In memory of Professor Jan Herget:

A Legacy of Passion and Dedication in Pulmonary Circulation Research

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This special issue was prepared in commemoration of the five-year death anniversary of a remarkable personality in pulmonary circulation research, and an outstanding physiology teacher and mentor, Professor Jan Herget, M.D. (March 16, 1945 Prague - March 14, 2019 Prague).

Jan Herget entered the international scientific scene with his methodological paper on how to catheterize the pulmonary artery in the intact rat [1]. This method is still the gold standard for assessing pulmonary circulation in this animal model. It heralded his lifelong interest, essentially a hobby, of "playing" in the lab with

how things could be done. Until his last days, he spent at least as much time in the lab as he did in his office.

He then contributed significantly to the discovery of various factors influencing the development of pulmonary hypertension, e.g. the effect of perinatal insults [2-6]. A number of his papers contributed to elucidating the role of oxygen radicals [7-13], mast cells [14-17], macrophages [18] and changes in the connective tissue of the pulmonary vascular wall [7,16,19-21], as well as the interaction of these factors, in the development of pulmonary hypertension. He also collaborated on the practical application of pulmonary circulation knowledge for the purposes of lung transplantation [22,23].

In 1984-1999, together with Prof. Jiří Widimský Sr. (1925-2020), Jan Herget was the main organizer of four international conferences "Pulmonary Circulation" in Prague. Especially those that took place during the communist regime in Czechoslovakia (1984 and 1988) were extremely difficult to organize, but they were also invaluable for the isolated Czechoslovak scientific community.

His lifelong scientific contributions to the field of pulmonary circulation were recognized in 2011 by the American Thoracic Society with the Robert F. Grover Award (Figure 1).

Jan Herget was a passionate teacher of both undergraduate medical students and PhD students (on average, he “produced” one PhD graduate every year). He cared a lot about his beloved Alma Mater, the 2nd Medical Faculty of Charles University in Prague. For 13 years, he served as its vice dean for research and international cooperation. He also participated in the establishment of the Grant Agency of the Czech Republic and thus the whole system of grant funding in the Czech Republic after the end of the communist regime in 1989. He was active on the Board of the European Society of Respiratory Physiology and Pathology in the first half of the 1990s (before its merger into the emerging European Respiratory Society). For decades, he was an active member of the editorial board of this journal.

But the most important thing that Prof. Herget had been always appreciated for by his subordinates, colleagues and students was his honest, unpretentious and humble commitment to science without any concern for his own promotion. His greatest concern was that his students would eventually outgrow him. He devoted a great deal to this priority, but he has set the bar high for his followers. He died full of ideas shortly after his beloved wife Hana.

The issue is composed of the works of scientists closely associated with Jan - his students, friends, and collaborators, both those at his home Department of Physiology at the 2nd Faculty of Medicine of Charles University in Prague - Motol, as well as the international ones.

In addition to the authors, other friends and colleagues of Jan Herget have generously contributed to this volume by helping with the review process. Of those, I would like, with their permission, to thank Andrea Olschewski, Kurt Stenmark, Ivan McMurtry, Ken Weir, Stephen Archer, and Hikmet Al-Hiti. Still other experts willingly participated in the review process even without their previous close personal experience with Jan Herget (e.g., Drs. Aaronson, Dřevínek, Melenovský, Kittnar, Wearing, Sedmera, Svatoň, Merkus).

After a personal remembrance of Jan's former PhD student Ivana Kawiková, the issue opens with a review article by Drs. Thenappan and Weir on the role of the gut microbiome in the mechanism of pulmonary hypertension [24], and a primary publication by Lorna Moore and colleagues on the effect of hypoxia on the fetus [25]. The choice of these seemingly disparate topics came about because Ken Weir and Lorna Moore were Herget's colleagues during his one-year fellowship funded by the B. Francis Parker Foundation at the *Cardiovascular and Pulmonary Research Laboratory* at the University of Colorado in 1984. This pair of papers is complemented by a review by Stephen Archer *et al.* [26] on the mechanisms of hypoxic pulmonary vasoconstriction (also co-authored by Ken Weir, once Stephen Archer’s mentor).

Next is a review by Ošťádal and colleagues on sex differences in cardiac tolerance to oxygen deprivation [27]. Prof. Ošťádal had been Jan Herget's friend and collaborator since their beginnings together in Prof. Otakar Poupa's laboratory in the 1960s. Although their professional paths soon diverged (Herget focused on pulmonary circulation and Ošťádal on myocardium), the collaboration continued (e.g. in the Center for Experimental Research of Cardiac and Vascular Diseases, jointly established by them in 1998) and showed that the mechanisms of ischemic cardioprotection (Ošťádal) and hypoxic pulmonary hypertension (Herget) have surprisingly much in common. Another strong research group at the Centre was led by Prof. Luděk Červenka, whose team is featured in this issue with a short communication on sex-linked differences in cardiac atrophy after heart transplantation [28]. These two articles illustrate that the Center explicitly focused on sex

differences long before they became mainstream in biomedical research. Prof. Herget contributed significantly to this trend [4,13].

Towards the end of the 1980s, Prof. Herget's group turned their attention to the lifelong consequences of perinatal insults on the pulmonary circulation [2-6]. They showed that, consistent with the then new Barker hypothesis [29], hypoxia acting at the time of birth, but not later in life, has a lifelong, irreversible effect on pulmonary vasoconstrictor reactivity, which likely contributes to the development of pulmonary hypertension in adulthood. This idea was taken up especially by the group of Prof. Jean-François Tolsa in Lausanne, focusing on the possible mechanisms of this effect [30-33]. This group's contribution in this issue [34] further develops this topic and also marks the 25th birthday of the Neonatal Research Laboratory at the University of Lausanne (October 1st, 1999).

Examples of the close interdisciplinary collaboration necessary for today's science are represented in this issue by the contributions of histologists Kamila Procházková & Jiří Uhlík [35] and biophysicist and cell biologist Lucie Bačáková (and her team) [36]. Herget's collaboration with both of these teams, including the prematurely deceased colleagues Jana Novotná (1954-2016), Václav Pelouch (1941-2010) and Luděk Vajner (1954-2018), was key to Herget's series of publications on the role of changes of extracellular matrix, particularly collagens, in the development of pulmonary hypertension [7,19-21]. These are summarized in a review by Bačáková *et al.* in this issue.

Herget's students are represented in this issue by the works of Drs Koubský [37] and Kawiková *et al.* [38]. Both articles well illustrate that guiding and mentoring PhD students in physiology and pathophysiology of pulmonary circulation can ultimately be a good preparation for successful work even in fields not necessarily directly related to the pulmonary vascular bed (pediatric cardiology for K. Koubský and even psychiatry in the case of I. Kawiková).

We have prepared this issue in the belief that individual personalities matter in science. May this commemoration of the legacy of the outstanding personality of Jan Herget contribute to a better understanding of the pathophysiology of his favorite pulmonary circulation and, in general, to the further advancement of his beloved science.

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Fig. 1. Jan Herget (right) and Robert F. "Bob" Grover during the American Thoracic Society Robert F. Grover Award ceremony in Denver, CO, on May 17, 2011.

References

1. Herget J, Paleček F. Pulmonary arterial blood pressure in closed chest rats. Changes after catecholamines, histamine and serotonin. Arch Int Pharmacodyn Ther 1972;198:107-117.

2. Herget J, Hampl V, Paleček F: Effect of perinatal hypoxia on hypoxic vascular reactivity in adult rats. In: *Interaction between Heart and Lung*. S Daum (eds), Thieme, Stuttgart, 1989, pp 63-66.
3. Hampl V, Herget J. Perinatal hypoxia increases hypoxic pulmonary vasoconstriction in adult rats recovering from chronic exposure to hypoxia. Am Rev Respir Dis 1990;142:619-624. https://doi.org/10.1164/ajrccm/142.3.619
4. Hampl V, Bíbová J, Ošťádalová I, Povýšilová V, Herget J. Gender differences in the long-term effects of perinatal hypoxia on the pulmonary circulation in rats. Am J Physiol Lung Cell Mol Physiol 2003;285:L386-L392. https://doi.org/10.1152/ajplung.00389.2002
5. Hampl V, Bíbová J, Herget J. Perinatal history of hypoxia leads to lower vascular pressures and hyporeactivity to angiotensin II in isolated lungs of adult rats. Physiol Res 2000;49:567-575.
6. Herget J, Hampl V, Povýšilová V, Slavík Z. Long-term effects of prenatal indomethacin administration on the pulmonary circulation in rats. Eur Respir J 1995;8:209-215. https://doi.org/10.1183/09031936.95.08020209
7. Herget J, Novotná J, Bíbová J, Povýšilová V, Vaňková M, Hampl V. Metalloproteinase inhibition by Batimastat attenuates pulmonary hypertension in chronically hypoxic rats. Am J Physiol Lung Cell Mol Physiol 2003;285:L199-L208. https://doi.org/10.1152/ajplung.00167.2002
8. Lachmanová V, Hniličková O, Povýšilová V, Hampl V, Herget J. N-acetylcysteine inhibits hypoxic pulmonary hypertension most effectively in the initial phase of chronic hypoxia. Life Sci 2005;77:175-182. https://doi.org/10.1016/j.lfs.2004.11.027
9. Hodyc D, Šnorek M, Brtnický T, Herget J. Superoxide dismutase mimetic tempol inhibits hypoxic pulmonary vasoconstriction in rats independently of nitric oxide production. Exp Physiol 2007;92:945-951. https://doi.org/10.1113/expphysiol.2007.037135
10. Chovanec M, Novotná J, Wilhelm J, Hampl V, Vízek M, Herget J. Hypercapnia attenuates hypoxic pulmonary hypertension by inhibiting lung radical injury. Physiol Res 2009;58:S79-S85. https://doi.org/10.33549/physiolres.931923
11. Hodyc D, Johnson E, Skoumalová A, Tkaczyk J, Maxová H, Vízek M, Herget J. Reactive oxygen species production in the early and later stage of chronic ventilatory hypoxia. Physiol Res 2012;61:145-151. https://doi.org/10.33549/physiolres.932206
12. Mizera R, Hodyc D, Herget J. ROS scavenger decreases basal perfusion pressure, vasoconstriction and NO synthase activity in pulmonary circulation during pulmonary microembolism. Physiol Res 2015;64:683-688. https://doi.org/10.33549/physiolres.932906
13. Mrázková H, Lischke R, Herget J. Influence of gender on ischemia-reperfusion injury in lungs in an animal model. Physiol Res 2016;65:953-958. https://doi.org/10.33549/physiolres.933273
14. Vajner L, Vytášek R, Lachmanová V, Uhlík J, Konrádová V, Novotná J, Hampl V *et al.* Acute and chronic hypoxia as well as 7-day recovery from chronic hypoxia affects the distribution of pulmonary mast cells and their MMP-13 expression in rats. Int J Exp Pathol 2006;87:383-391. https://doi.org/10.1111/j.1365-2613.2006.00493.x
15. Baňasová A, Maxová H, Hampl V, Vízek M, Povýšilová V, Novotná J, Vajnerová O *et al.* Prevention of mast cell degranulation by disodium cromoglycate attenuates the development of hypoxic pulmonary hypertension in rats exposed to chronic hypoxia. Respiration 2008;76:102-107. https://doi.org/10.1159/000121410
16. Maxová H, Novotná J, Vajner L, Tomášová H, Vytášek R, Vízek M, Bačáková L *et al.* In vitro hypoxia increases production of matrix metalloproteinases and tryptase in isolated rat lung mast cells. Physiol Res 2008;57:903-910. https://doi.org/10.33549/physiolres.931278
17. Maxova H, Vasilkova M, Novotna J, Vajnerova O, Bansova A, Vizek M, Herget J. Prevention of mast cell degranulation by disodium cromoglycate delayed the regression of hypoxic pulmonary hypertension in rats. Respiration 2010;80:335-339. https://doi.org/10.1159/000312403
18. Žaloudíková M, Vytášek R, Vajnerová O, Hniličková O, Vízek M, Hampl V, Herget J. Depletion of alveolar macrophages attenuates hypoxic pulmonary hypertension but not hypoxia-induced increase in serum concentration of MCP-1. Physiol Res 2016;65:763-768. https://doi.org/10.33549/physiolres.933187
19. Bacáková L, Wilhelm J, Herget J, Novotná J, Eckhart A. Oxidized collagen stimulates proliferation of vascular smooth muscle cells. Exp Mol Pathol 1997;64:185-194. https://doi.org/10.1006/exmp.1997.2219
20. Novotná J, Herget J. Exposure to chronic hypoxia induces qualitative changes of collagen in the walls of peripheral pulmonary arteries. Life Sci 1998;62:1-12. https://doi.org/10.1016/S0024-3205(97)01032-1

21. Novotná J, Bíbová J, Hampl V, Deyl Z, Herget J. Hyperoxia and recovery from hypoxia alter collagen in peripheral pulmonary arteries similarly. Physiol Res 2001;50:153-163. https://doi.org/10.33549/physiolres.930037
22. Hodyc D, Hniličková O, Hampl V, Herget J. Pre-arrest administration of the cell-permeable free radical scavenger tempol reduces warm ischemic damage of lung function in non-heart-beating donors. J Heart Lung Transplant 2008;27:890-897. https://doi.org/10.1016/j.healun.2008.05.019
23. Mrazkova H, Lischke R, Hodyc D, Herget J. The protective effect of hypercapnia on ischemia-reperfusion injury in lungs. Respir Physiol Neurobiol 2015;205:42-46. https://doi.org/10.1016/j.resp.2014.10.002
24. Thenappan T, Weir EK. Gut microbiome and pulmonary arterial hypertension - a novel and evolving paradigm. Physiol Res 2024;73, S477-S485. https://doi.org/10.33549/physiolres.935430
25. Moore LG, Julian CG, Lorca RA, Cioffi-Ragan D, Gumina D, Hobbins JC. Does hypoxia prompt fetal brain-sparing in the absence of fetal growth restriction? 2024;73, S487-S492. https://doi.org/10.33549/physiolres.935482
26. Archer SL, Snary KJD, Bentley RET, Alizadeh E, Weir K. Hypoxic pulmonary vasoconstriction: an important component of the homeostatic oxygen sensing system. Physiol Res 2024;73, S493-S510. https://doi.org/10.33549/physiolres.935431
27. Ostadal B, Drahota Z, Hlavackova M, Ostadal P. Sex differences in cardiac tolerance to oxygen deprivation - 40 years of cardiovascular research. Physiol Res 2024;73, S511-S525. https://doi.org/10.33549/physiolres.935429
28. Kolesár DM, Kujal P, Mrázová I, Pokorný M, Škaroupková P, Vaňourková Z, Sadowski J *et al.* Sex-linked differences in cardiac atrophy after heterotopic heart transplantation: no direct relation to the actions of sex steroid hormones. Physiol Res 2024;73, S527-S539. https://doi.org/10.33549/physiolres.935308
29. Barker DJ, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. Lancet 1986;327:1077-1081. https://doi.org/10.1016/S0140-6736(86)91340-1
30. Marino M, Beny JL, Peyter AC, Bychkov R, Diaceri G, Tolsa JF. Perinatal hypoxia triggers alterations in K+ channels of adult pulmonary artery smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 2007;293:L1171-L1182. https://doi.org/10.1152/ajplung.00126.2007
31. Peyter A-C, Muehlethaler V, Liaudet L, Marino M, Di Bernardo S, Diaceri G, Tolsa J-F. Muscarinic receptor M1 and phosphodiesterase 1 are key determinants in pulmonary vascular dysfunction following perinatal hypoxia in mice. Am J Physiol Lung Cell Mol Physiol 2008;295:L201-L213. https://doi.org/10.1152/ajplung.00264.2007
32. Marino M, Bény J-L, Peyter A-C, Diaceri G, Tolsa J-F. Perinatal hypoxia enhances cyclic adenosine monophosphate-mediated BKCa channel activation in adult murine pulmonary artery. J Cardiovasc Pharmacol 2011;57: https://doi.org/10.1097/FJC.0b013e3182016adf
33. Rexhaj E, Bloch J, Jayet PY, Rimoldi SF, Dessen P, Mathieu C, Tolsa JF *et al.* Fetal programming of pulmonary vascular dysfunction in mice: role of epigenetic mechanisms. Am J Physiol Heart CircPhysiol 2011;301:H247-H252. https://doi.org/10.1152/ajpheart.01309.2010
34. Peyter A-C, Muehlethaler V, Tolsa J-F. Long-term adverse effects of perinatal hypoxia on the adult pulmonary circulation vary between males and females in a murine model. Physiol Res 2024;73, S541-S556. https://doi.org/10.33549/physiolres.935481
35. Procházková K, Uhlík J. Influence of hypoxia on the airway epithelium. Physiol Res 2024;73, S557-S568. https://doi.org/10.33549/physiolres.935436
36. Bačáková L, Sedlář A, Musílková J, Eckhardt A, Žaloudíková M, Kolář F, Maxová H. Mechanisms controlling the behavior of vascular smooth muscle cells in hypoxic pulmonary hypertension: In memory of more than twenty years of cooperation with Prof. Jan Herget. Physiol Res 2024;73, S569-S596. https://doi.org/10.33549/physiolres.935394
37. Koubský K. Pediatric chronic heart failure: age-specific considerations of medical therapy. Physiol Res 2024;73, S597-S613. https://doi.org/10.33549/physiolres.935438
38. Kawikova I, Hakenova K, Lebedeva M, Kleteckova L, Jakob L, Spicka V, Wen L *et al.* Perinatal hypoxia and immune system activation in schizophrenia pathogenesis: Critical considerations during COVID-19 pandemic. Physiol Res 2024;73, S615-S639. https://doi.org/10.33549/physiolres.935501

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Jan Herget's impact on the field of pulmonary hypertension and beyond

In this Special issue of Physiological Research, we celebrate the life and work of Professor Jan Herget (1945-2019). He was a pathophysiologist focused on the responses of pulmonary circulation to hypoxia, which is highly relevant to common conditions, such as left heart failure or lung diseases. Jan, alias "Honza", pioneered experimental testing of the concept that events at early life may shape the course of a disease in adulthood. The topic has become intensively studied many years later, particularly concerning such complex organs as the brain or microbiota. In addition to being recognized as a significant player in his field, Honza was a genuinely enthusiastic teacher and supportive mentor. He also served for over two decades as the Head of the Department of Physiology at the 2nd Medical School of Karlova Universita in Prague and also as the Vice-Dean for Research and International Relations.

In 1968, after graduating from the Pediatric Medical School (today 2nd Medical School), Honza joined the laboratory of Professor Otakar Poupa to pursue his Ph.D. training at the Department of Pathological Physiology. However, as Allen Saunders said and John Lennon sang, "Life Is What Happens When You're Busy Making Other Plans", on August 21st, 1968, Czechoslovakia was occupied by the Soviet Union and its allies, and Jan's mentor left the country promptly, as did many others, and could not return until after 1989 when the communist regime collapsed. Another outstanding physiologist became the head of the Department, Prof. Jiří Křeček, but he was removed after a short time for political reasons. These blows must have been challenging for a young trainee, but Jan continued pursuing science and searching for his research identity.

At the end of 1970s, Honza spent almost a year in the laboratory of Professor Gwenda R. Barer at Sheffield University in the U.K. where he studied the role of hypoxia on pulmonary circulation. That hypoxemia causes vasoconstriction in lungs, in contrast to vasodilation in systemic circulation, was discovered in 1940's [1].

Jan Herget (right) with his colleague Ken Weir from the University of Minnesota (left) and their trainee, Vaclav Hampl (middle) at the Pulmonary Circulation Conference in Warsaw, Poland in June 2006.

Professor Barer contributed tremendously to understanding how hypoxia affects pulmonary arteries in acute and chronic settings [2]. Honza's stay in her lab was very productive and they became life-long friends. Professor Barer visited Prague on several occasions. She was like Agatha Christie's Miss Marple: mind sharp as a laser, kind and compassionate, and unassuming.

In 1982, Honza received the Parker B. Francis Fellowship that enabled him to spend one year in the National Jewish Center For Immunology and Respiratory Medicine in Denver, Colorado (today called National Jewish Health) with Robert Grover and John T. Reeves, two reknown high altitude physiologists and physicians. Whenever he spoke about Denver, his face lit up.

Shortly after returning to Prague, Honza became an Associate Professor and set up his independent lab focused on mechanisms of pulmonary hypertension. The assigned space was within a shabby underground of the old orphanage building at Ke Karlovu in Prague. A thin door separated the lab from the loud voices of construction workers and heaps of tools scattered on the corridor. Behind the doors, the world was neatly organized. The labyrinth of four interconnected rooms housed a large hypoxic chamber designed by Honza, a perfusion pump for studies on isolated lungs, a new marble stand for a beautiful, ancient mechanical balance, and some old wooden desks for two trainees (Vaclav Hampl and Ivana Kawikova) and a technician (Ruzena Ticha). The unforgettable aroma encompassed the old wall's sweat, odor from lab rat cages, coffee made in a beaker sitting above a Bunsen burner, and Honza's tobacco pipe. Behind the cellar windows was the world of communistic regime slowly crumbling away, while inside were done thought-provoking experiments on a remarkably skinny budget [3-8].

As a graduate and a faculty member of the Pediatric Medical School, Honza emphasized the need to view human health or diseases in the context of events during critical developmental stages. He followed steps of Professors Babak, Krecek, and others who founded the Pediatric Medical School in Prague with the notion that children are not small adults. The clinical observations were there, but there was no experimental evidence. Honza designed a landmark experiment where pregnant rats were placed into hypoxic chambers and were kept there until their offspring were ten days old. After placing the animals into the normoxic air, the animals recovered from hypoxia and had comparable pressure in pulmonary circulation as control mice unexposed to perinatal hypoxia. However, when these animals were re-exposed to acute hypoxia in adulthood, their responses were more severe than in animals born in normal air. The mechanism of this intriguing effect of early childhood events on the adaptation process in adulthood is not yet fully understood. Honza published 126 papers and his work helped clarify how chronic hypoxia influences pulmonary vascular remodeling and the role of various cellular and molecular factors in this process.

Honza's laboratory, or rather a den, was separated from the rest of the nearby Department of Pathophysiology and offered a refuge of openness, trust, and enthusiasm for research and teaching. Honza, his friend and colleague Martin Vizek, and several scientists from the Czechoslovak Academy of Sciences (such as Bohuslav Ostadal, known as "Boja," who served for five years as a Director of Institute of Physiology, Academy of Sciences of the Czech Republic) continued nourishing the legacy of systematic work, open-mind and tolerance fostered by Otakar Poupa (1916-1999), a Chair of the Department in 1961-1968. Professor Poupa was a physician-scientist, artist, philosopher, and a founding member of the interdisciplinary community of cardiology-oriented scientists, the so-called "Prague School [9]. His charismatic and insightful leadership influenced them, even though it was only early in his research career. In 1968, Professor Poupa contributed to formulating the manifesto "2000 Words," which expressed widespread frustration with the Communist regime and became a symbol of the reform movement Prague Spring 1968. The manifesto provoked a strong reaction from the establishment, and after the Warsaw Pact Army invaded Czechoslovakia, Professor Poupa and several other influential scientists emigrated in order avoid grave consequences (e.g., Karel Rakusan, an internationally respected physician-scientist who left Prague for Ottava, Canada). It was brave to leave everything behind and reinvent himself abroad in the sixth decade of his life, but Professor Poupa used to say that those who stayed behind and continued doing something meaningful were at least as courageous, if not more. Professor Poupa continued to follow events in the Czechoslovak research community from his new home in Goteborg, Sweden, but news about him was very sporadic and filtered by the Iron Curtain. He stood once on the border of Czechoslovakia, wanting to enter his homeland where his mother was passing away, but the entry was denied. We can only imagine how far Honza would fly under the mentorship of the man who established the field of comparative cardiology.

Then came 1989, and with it, the regime was changed. It was finally possible to freely cross the border,

to say what was on the mind, the military service was not obligatory anymore. The latest became critical for the Department of Pathophysiology because the army vacated barracks where the university's male students received military training. These buildings were near the main hospital campus of the Pediatric Medical School in Prague-Motol. Honza was critical in claiming these buildings for the medical school and creating an adjacent campus for pre-clinical fields. Who cared that the walls were as thin as paper? It was a significant step forward.

How did Honza affect other research areas? About a decade after the study on the effects of perinatal hypoxia [3-8], Paul Paterson published a paper demonstrating that exposure of dams to an infectious stimulus affects the behavior of their offspring in adulthood [10], which models phenotype of autism [11] and schizophrenia [12]. Altogether, these papers opened a new avenue to studies on neurodevelopmental disorders and may bring biological foundations to the socio-economic findings of the British birth cohort studies, which revealed the long-lasting impact of early childhood socio-economic conditions [13].

Honza's legacy continues also through his trainees. His mentoring was well-thought-through. He had well-defined goals and visualized a path towards reaching them. His expectations were realistic, and he provided as much support for his trainees as possible. There was always an air of informality, friendship, and generous space for self-realization. Honza was multifaceted, and when something was needed but was missing, he went on the limb and got it done. His trainees were around him and learned by osmosis. To name at least the trainees from the cellar on the street Ke Karlovu, Vaclav Hampl became an outstanding researcher in pulmonary circulation, and that per se was not a tiny deed at times when so many left science. He also became the youngest rector of Karlova Universita in its almost seven hundred-year history, served in this function for eight years, then went on to become Senator for another five years while never leaving the lab bench and medical students. He shaped graduate education at the University and supported research by facilitating multiple, long-lasting infrastructure projects funded by the European Union, e.g., BIOCEV. Meanwhile, Ivana Kawikova tackled some other unknowns related to metabolism in the immune system or the role of the immune system in neuropsychiatric diseases. Honza equipped her with interdisciplinary thinking [3,4], encouraged her to develop solid foundations in pathophysiology through teaching and instructed her on communicating complex problems to various audiences. This preparation became a real asset while building bridges between distant disciplines such as immunology and psychiatry. With Martin Vizek they also opened the path to Sweden for her and encouraged her to reach out to Otakar Poupa, who then quickly adopted her as his informal advisee and friend.

The careers of several other Honza's PhD students continued towards successful clinical work (Bíbová, Mizera, Ošťádal, Šnorek, Lachmanová, Šedivý,…), clinical-academic combination (Vytášek, Chovanec, Al-Hiti, Koubský,…) [14,15], or health care management (Hodyc, Šnorek).

If there is something like the river Styx, we can imagine elegant Professor Poupa and always energetic Honza gazing curiously into our world and cheering any success of anyone from their teams. They must have danced happily when they saw the recent opening of a modern Theoretical Institute building on the campus of 2nd Medical School of Charles University in Prague. All scientists are finally at the same place! Collaborating and teaching together! And somewhere, there is likely also Jan Evangelista Purkinje, a man who founded the first two independent institutes of physiology in the world– the first one in Wroclaw, Poland, and the other one in Prague. One hundred and seventy years later, Charles University has five medical faculties (each with a department of physiology and pathophysiology) and a Faculty of Science (also with a department of physiology), and the Czech Academy of Sciences has the Institute of Physiology. Honza was a remarkably functional part of this web, and it is an honor to raise a glass, blow a kiss in his direction and whisper: "Thank you!".

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References

1. von Euler U, Liljesttrand G. Observations on the pulmonary arterial blood pressure in the cat. Acta Physiologica Scandinavica 1946;12:301-312. https://doi.org/10.1111/j.1748-1716.1946.tb00389.x
2. Barer GR. Hypoxia and the pulmonary circulation: a brief review. Z Erkr Atmungsorgane 1989;173:109-115.
3. Herget J, Frydrychova M, Kawikova I, McMurtry IF. Thyroxine treatment increases the hypoxic pulmonary vasoconstriction in isolated lungs from thyroidectomized rats. Bull Eur Physiopathol Respir 1987;23:217-221.
4. Herget J, Kawikova I, Hampl V. Adrenalectomy in rats depresses hypoxic pulmonary vasoconstriction in vitro but does not attenuate the pulmonary hypertension of chronic hypoxia in vivo. Exp Clin Cardiol 1998;3:28-32.
5. Hampl V, Bibova J, Herget J. Perinatal history of hypoxia leads to lower vascular pressures and hyporeactivity to angiotensin II in isolated lungs of adult rats. Physiol Res 2000;49:567-575.
6. Hampl V, Bibova J, Ostadalova I, Povysilova V, Herget J. Gender differences in the long-term effects of perinatal hypoxia on pulmonary circulation in rats. Am J Physiol Lung Cell Mol Physiol 2003;285:L386-L392. https://doi.org/10.1152/ajplung.00389.2002
7. Hampl V, Herget J. Perinatal hypoxia increases hypoxic pulmonary vasoconstriction in adult rats recovering from chronic exposure to hypoxia. Am Rev Respir Dis 1990;142:619-624. https://doi.org/10.1164/ajrccm/142.3.619
8. Herget J, Hampl V, Povysilova V, Slavik Z. Long-term effects of prenatal indomethacin administration on the pulmonary circulation in rats. Eur Respir J 1995;8:209-215. https://doi.org/10.1183/09031936.95.08020209
9. Ostadal B, Kolar F. Sixty Years of Heart Research in the Institute of Physiology of the Czech Academy of Sciences. Physiol Res 2024;73,S35-S48; https://doi.org/10.33549/physiolres.935337
10. Shi L, Fatemi SH, Sidwell RW, Patterson PH. Maternal influenza infection causes marked behavioral and pharmacological changes in the offspring. J Neurosci 2003;23:297-302. https://doi.org/10.1523/JNEUROSCI.23-01-00297.2003
11. Patterson PH. Modeling autistic features in animals. Pediatr Res 2011;69:34R-40R. https://doi.org/10.1203/PDR.0b013e318212b80f
12. Meyer U, Feldon J, Schedlowski M, Yee BK. Towards an immuno-precipitated neurodevelopmental animal model of schizophrenia. Neurosci Biobehav Rev 2005;29:913-947. https://doi.org/10.1016/j.neubiorev.2004.10.012
13. Power C, Elliott J. Cohort profile: 1958 British birth cohort (National Child Development Study). Int J Epidemiol 2006;35:34-41. https://doi.org/10.1093/ije/dyi183
14. Koubský K. Pediatric chronic heart failure: age-specific considerations of medical therapy. Physiol Res 2024;73, S597-S613. https://doi.org/10.33549/physiolres.935438
15. Chovanec M, Ďurišová J, Vajnerová O, Baňasová A, Vízek M, Žaloudíková M, Uhlík J et al. Simple model of pulmonary hypertension secondary to left heart pressure overload induced by partial intravascular occlusion of the ascending aorta. Am J Physiol Lung Cell Mol Physiol 2024;327:L371-L381. https://doi.org/10.1152/ajplung.00243.2023

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INVITED REVIEW

Gut Microbiome and Pulmonary Arterial Hypertension – A Novel and Evolving Paradigm

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Summary

Pulmonary arterial hypertension is characterized by perivascular and systemic inflammation. The gut microbiome influences the host immune system. Here we review the emerging preclinical and clinical evidence that strongly suggests that alterations in the gut microbiome may either initiate or facilitate progression of established pulmonary arterial hypertension by modifying the systemic immune responses. We also briefly review the relationship between the gut microbiome and preeclampsia, a vascular disease also characterized by inflammation.

Key words

Dysbiosis • Right ventricle • Inflammation

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Introduction

Pulmonary arterial hypertension (PAH) is characterized by remodeling of the pulmonary vasculature causing reduced pulmonary arterial compliance (PAC) and increased pulmonary vascular resistance (PVR), ultimately resulting in right ventricular failure and death [1]. PAH remains as a fatal disease with a median survival of only 5-7 years and an in-hospital mortality of 6 % [2,3]. Current therapies are predominantly pulmonary vasodilators, which increase exercise capacity modestly and reduce hospital admission but are expensive and not curative [1]. Sotatercept, a novel fusion protein that traps activin and growth differentiation factors involved in the proliferation of pulmonary arterial smooth muscle cells in PAH, was recently approved by the Food and Drug Administration [4]. It is the only approved antiproliferative therapy for the treatment of PAH. Sotatercept also only improves exercise capacity and reduces time to clinical worsening. It is not curative and its effect on survival is unknown. Thus, there is an unmet need for better therapies for PAH to improve survival.

Inflammation and PAH

Inflammation plays a pivotal role in the pathogenesis of PAH [5]. PAC decreases early in the pathogenesis of PAH before there is an increase in the PVR [6]. This loss of vascular compliance correlates with extracellular matrix remodeling and fibrosis in the pulmonary vessels, which is linked to chronic perivascular inflammation and immune dysregulation [7]. Clinical and experimental evidence supports the role of inflammation in PAH [5]. Abnormalities in both adaptive and innate immunity occur in PAH. Clinically, PAH is associated with autoimmune diseases, including scleroderma and systemic lupus erythematosus, and infectious diseases, such as human immunodeficiency virus and schistosomiasis infection [1]. There is perivascular accumulation of inflammatory cells including macrophages, T cells, and B cells in the pulmonary arteries in PAH patients[5]. Increased serum cytokine levels in patients with PAH are associated with

increased mortality and reduced right ventricular function [8, 9]. Patients with PAH have increased serum autoantibody levels such as antinuclear antibodies [5]. The lower number of circulating Natural Killer cells is associated with poor survival in PAH patients, suggesting that they have a protective role [10].

Several preclinical studies prove a cause-and-effect relationship between inflammation and PAH. PAH can be induced experimentally in animals by exposure to various immune stimuli, including human immunodeficiency virus, schistosomiasis, and interleukin (IL)-6 overexpression [5]. Depletion of inflammatory macrophages in chronic hypoxic calves and rats prevents remodeling of the pulmonary vascular extracellular matrix and pulmonary hypertension (PH) [5]. Athymic rats deficient in T-cells and B-cells develop pulmonary vascular disease in response to Sugen and hypoxia [11]. Immune reconstitution of the athymic rats with regulatory T cells (Tregs) reduces the severity of PH caused by Sugen and hypoxia [11]. An imbalance of CD4 helper T-cell subsets occurs in PAH lungs. While there is an increase in TH1, TH2, and TH17 CD4 helper T-cells that induce inflammatory responses, Tregs, which have anti-inflammatory effects, are decreased [11]. Finally, autoantibodies from monocrotaline rats cause PH in naïve rats, supporting the importance of abnormal B-cells in the pathogenesis of PAH [5].

Gut microbiome and immune dysregulation

The gut microbiome consists of trillions of bacteria, viruses, and fungi. The individual gut microbiome composition and functionality are determined by geographic factors, drugs, diet, exercise, and genetics [12-15]. The gut microbiome regulates many physiological functions in the host including immune regulation, the intestinal mucosal barrier, energy homeostasis, xenobiotic metabolism, vitamin synthesis and degradation, and neurological development amongst others [16]. Detrimental changes in the intestinal microbial community structure and function is called dysbiosis, which can lead to immune dysregulation and chronic systemic inflammation [17]. The gut microbiome influences the host immune system through several different mechanisms. Increased bacterial translocation and release of endotoxins, due to dysbiosis, trigger activation of macrophages through upregulation of Toll-like receptor 4 (TLR4) and T cells including TH1, TH2, and TH17 CD4 helper T cells [18-21]. In addition, the gut microbiome can impact the host immune system through release of bacterial metabolites. Some of these metabolites such as short chain fatty acids (SCFA-acetate, butyrate, and propionate) have a beneficial, anti-inflammatory effect, including activation of Treg cells through G-protein-coupled receptors (GPCR), and through epigenetic modifications induced by inhibiting histone deacetylase [17,20,21]. Alternatively, some metabolites like Trimethylamine N-oxide (TMAO) can have a proinflammatory effect through activation of macrophages and T cells [22]. Thus, either an increase in the pathogenic bacteria, or a decrease in beneficial bacteria, in the gut can dysregulate the immune homeostasis, leading to chronic systemic inflammation. Gut dysbiosis is linked to the immunopathogenesis of numerous chronic inflammatory diseases including atherosclerotic vascular disease, obesity, diabetes mellitus (both type 1 and 2), depression, alcoholic and nonalcoholic steatohepatitis, multiple sclerosis, and chronic lung allograft rejection among others [17].

Gut microbiome and PAH (A Gut-Lung Axis)

There are several compelling and evolving observations that suggest that the inflammation in PAH is initiated by intestinal dysbiosis and altered circulating microbial products (Fig. 1).

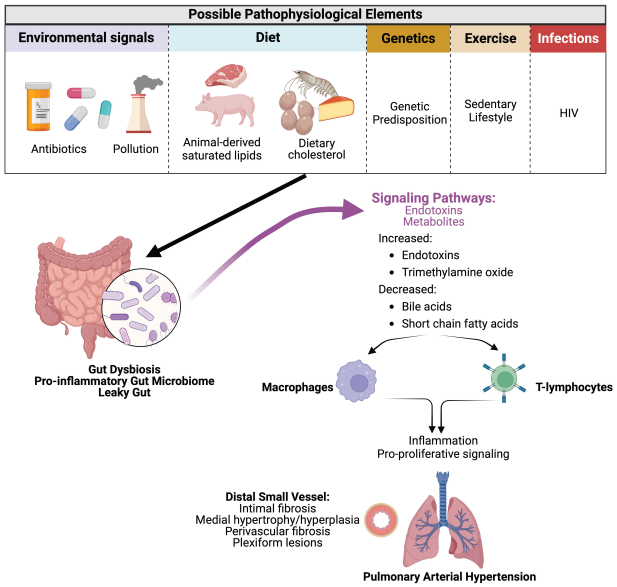

Fig. 1. Gut dysbiosis initiates or facilitates progression of pulmonary arterial hypertension by modifying systemic immune responses.

We and others have recently shown that PAH patients have a proinflammatory gut microbiome and altered circulating microbial metabolites. Compared to healthy controls, PAH patients had a less diverse and a more proinflammatory gut microbiome with reduced relative abundance of beneficial anti-inflammatory taxa and increased relative abundance of pro-inflammatory taxa, and lower plasma levels of anti-inflammatory microbial metabolites (SCFAs and secondary bile acids) and higher plasma levels of pro-inflammatory microbial metabolites such as TMAO [23,24]. Consistent with the circulating microbial metabolite levels, PAH patients have fewer copies of some of the gut microbial genes that produce anti-inflammatory microbial metabolites and a lower relative abundance of some of the species encoding these genes. PAH patients also have enrichment of species with microbial genes that encode the proinflammatory microbial metabolite trimethylamine [23,24]. Moreover, serum TMAO levels in PAH patients are associated with disease severity and poor prognosis [25,26].

Bacteriophages or phages are viruses that infect bacteria. By infecting the gut bacteria, phages can increase or decrease the abundance of the host bacteria or alter the functionality of the host bacteria without a change in their relative abundance. Thus, phages can indirectly be linked to chronic systemic diseases through their interaction with the host gut bacteria. In addition to abnormal gut bacteria, PAH patients also have altered gut bacteriophages with enrichment of *Enterococcal*, and relative depletion of *Lactococcal* phages compared to healthy subjects [24]. The increase in *Enterococcus* phage in PAH is associated with increased relative abundance of the pro-inflammatory *Enterococcus* taxa in PAH patients [24].

While human association data does not prove a direct cause-and-effect relationship between PAH and gut dysbiosis, preclinical data suggest a mechanistic role for gut dysbiosis in PAH. Preclinical animal models of PH exhibit gut dysbiosis. Wistar rats treated with Sugen and chronic hypoxia to cause PH demonstrate gut dysbiosis on taxonomy-based analysis with increased Firmicutes and decreased Bacteroidota [27]. The normal Firmicutes/Bacteroidetes (F/B) ratio helps to maintain intestinal homeostasis. Thus, altered F/B ratio, both increased and decreased, is considered as a marker of gut dysbiosis. In this animal model, serum acetate (an anti-inflammatory SCFA) levels are lower. Interestingly, gut microbiota modification with antibiotic treatment significantly suppresses the vascular remodeling, right ventricular hypertrophy, and increase in the right ventricular systolic pressure in Sugen/hypoxia rats, suggesting a causative role for gut dysbiosis in the pathogenesis of PAH [28].

Similarly, alteration of the circulating microbial metabolites either prevents or regresses pulmonary vascular remodeling in preclinical animal models. In an hypoxic rat model of PH, administration of butyrate, an anti-inflammatory SCFA predominantly derived from the gut microbiome, prevents and regresses hypoxia-induced pulmonary vascular remodeling, increase in right ventricular systolic pressure, and right ventricular hypertrophy [29]. Mechanistically, butyrate reduced infiltration of alveolar and interstitial macrophages in the lungs [29]. Parallel to this, reduction in circulating levels of TMAO, a proinflammatory bacterial metabolite, reduces right ventricular hypertrophy and pulmonary vascular remodeling in the monocrotaline-induced rat model of PH and the hypoxia-induced mouse model of PH through decreasing macrophage production of cytokines and chemokines [30].

There are also several compelling circumferential observations that suggest that the immune dysregulation and perivascular inflammation in PAH is initiated by intestinal dysbiosis and a greater burden of the circulating microbiome and microbial products. First, the pulmonary vascular macrophages encounter a greater burden of microbial products from the gut in experimental PH and in patients with liver disease and portopulmonary hypertension [31]. Serum endotoxin levels are elevated in both experimental PH and human PAH. Monocrotaline-induced PH rats have increased gut permeability, increased circulating levels of endotoxin in the portal vein, and increased circulating levels of soluble CD14, a marker of macrophage activation, in the systemic venous blood [32]. The increase in serum endotoxin levels is associated with upregulation of TLR4, the main receptor for endotoxin, in pulmonary arterial smooth muscle.

Common bile duct ligation (CBDL) in a rat recapitulates human pulmonary vascular disease related to cirrhosis [31]. Our prior work described an increased medial thickness and loss of lumen in the resistance pulmonary arteries in CBDL rats compared with sham animals [31]. In this experimental model, circulating endotoxin levels are elevated, which in turn recruit and activate pulmonary intravascular macrophages. Endotoxin released from the gut bypasses the liver

through the portosystemic shunts resulting from cirrhosis and portal hypertension, avoiding hepatic uptake and inactivation, passes through the right heart, and enters the pulmonary vasculature. In the lungs the endotoxin activates macrophages, leading to pulmonary arteriovenous malformations, capillary dilatation, and proliferative arteriopathy of the distal pulmonary arteries. Treatment of CBDL rats with the antibiotic norfloxacin decreases pulmonary intravascular macrophage accumulation and reduces pulmonary vascular remodeling [33]. Likewise, depletion of the pulmonary intravascular macrophages in the CBDL rats prevents, as well as reverses, the pulmonary vascular changes [31].

This pathological mechanism has also been described in PH associated with congenital porto-systemic shunts in the Abernathy malformation. Patients with the Abernethy malformation often develop pulmonary vascular disease, including portopulmonary hypertension and hepatopulmonary syndrome [34]. Increased systemic endotoxin levels are described in PAH associated with the Abernethy malformation [35]. Importantly, correction of the portosystemic shunt in patients with Abernethy malformation reverses PAH, suggesting a causative role for the gut-lung axis in the pathogenesis of PAH [35]. Likewise, patients with cirrhosis, who undergo a trans-jugular intrahepatic portosystemic shunt, are more likely to develop portopulmonary hypertension [36]. Patients with idiopathic PAH and heritable PAH have increased serum levels of endotoxin and soluble CD14 compared with healthy controls [32]. In patients with PAH, the increase in serum CD14 levels parallels the increase in serum endotoxin levels. Furthermore, patients with PAH have increased blood TLR4 expression compared with healthy controls [37]. Mutations in the bone morphogenic protein receptor II (BMPR II) signaling pathway underlie 80 % of heritable PAH but disease penetrance is only 20 %, suggesting a requirement for additional triggers [38]. Chronic administration of lipopolysaccharide causes PH in Bmpr2+/- mice but not in littermate controls, signifying that endotoxin-induced inflammation can be an important cofactor for disease penetrance [39]. Interestingly, a recent bidirectional Mendelian randomization study demonstrates a causal relationship between nine specific gut bacterial taxa and PAH [40].

Taken together, all these observations, strongly suggest that gut dysbiosis either initiates PAH or facilitates progression of already established PAH by modifying systemic immune responses (Fig. 1).

Gut dysbiosis and PAH – cause or consequence?

Chronic right heart failure (RHF) from PAH is seen in conjunction with increased intestinal congestion, reduced bowel perfusion, increased intestinal permeability and gut dysbiosis. If RHF can cause gut dysbiosis, then markers of gut dysbiosis should correlate with poor right ventricular function. The Shannon diversity index is a measure of the number of species living in a habitat (richness) and their relative abundance (evenness). There is no relationship between Shannon diversity index and various measures of right ventricular function [23]. Conversely, the Shannon diversity index correlates only with the measures of pulmonary vascular disease [23]. More importantly, modification of the gut microbiome with antibiotics or alteration of the circulating microbial metabolites in preclinical models, can prevent or regress pulmonary vascular modeling [28,29,41]. Alteration of the gut microbiome with intermittent fasting improves right ventricular function in the monocrotaline rat model of PH [42]. These clinical and preclinical data support the thinking that gut dysbiosis in PAH is less likely to be due to right ventricular failure.

Targeting gut dysbiosis to treat PAH

There is persuasive basic and clinical science evidence in favor of modulating the gut microbiome to treat PAH. Modulation of the gut microbiome may not only reduce pulmonary vascular remodeling but may also improve right ventricular function. There are several ways to regulate the gut microbiome. Fecal microbiota transplant (FMT), which involves transfer of fecal microbiota from an healthy individual into the recipient patient, has proven to be a clinically effective approach to treat patients with recurrent *Clostridioides difficile* infection [43]. FMT is also currently being evaluated as a treatment approach for patients with chronic inflammatory conditions including ulcerative colitis [44] and malignant melanoma that is resistant to immune-therapy [45,46]. Unlike treatment of recurrent *Clostridioides difficile* infection, which responds to a single treatment of fecal microbiota transplantation, to change the established dysbiotic intestinal microbiota community structure and functionality in a chronic disease, multiple sequential administrations of microbiota or preconditioning with

antibiotics will be required. This approach is called microbiota transplant therapy. Alternatively, prebiotics (oral fermentable fibers) increase serum levels of anti-inflammatory SCFAs, such as butyrate. In a recent pilot clinical trial, fermentable fiber supplementation reduced systemic blood pressure [47]. Finally, increasing intake of the anti-inflammatory bacterial metabolite butyrate (postbiotics) is an alternate option to target the gut microbiome-lung axis in PAH.

Preeclampsia and gut microbiome

Preeclampsia is a life-threatening pregnancy disorder characterized by systemic hypertension and proteinuria after 20 weeks of gestation [48]. Preeclampsia shares many factors in common with PAH. Both vascular disorders initiate with vascular endothelial cell dys-function followed by vascular stiffness and elevated vascular pressures [48]. The etiology of preeclampsia is not completely understood. Early onset preeclampsia has been linked to placental ischemia from inadequate angiogenesis leading to oxidative stress, chronic inflammation, maternal vascular endothelial dysfunction, systemic vascular stiffness, and hyper-tension [49]. Increased levels of serum lipopolysaccharide released from bacteria has been speculated to play a role in the development of preeclampsia [50]. Like PAH, interestingly, gut dysbiosis has been ass-ociated with preeclampsia. Compared to healthy individuals in early, middle, and late pregnancy, those with preeclampsia in late pregnancy have a higher relative abundance of pathogenic bacteria, *Clostridioides perfringens* and *Solobacterium moorei* and a lower relative abundance of beneficial anti-inflammatory bacteria, *Coprococcus catus* [51]. Similarly, the relative abundance of the butyrate-producing anti-inflammatory genus *Odoribacter* is inversely related to systemic blood pressure in obese pregnant patients [52]. In a two-sample Mendelian randomization study, the anti-inflammatory probiotic taxa, *Bifido-bacterium* had a protective role against development of preeclampsia [53].

Conclusion

Evolving preclinical and clinical evidence demonstrates that gut dysbiosis and altered circulating microbial metabolites are drivers of perivascular inflammation in PAH, either initiating or accelerating already established pulmonary vascular remodeling in PAH. Hence, modulating the gut microbiome is a promising novel treatment paradigm in the management of PAH.

Conflict of Interest

There is no conflict of interest.

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References

1. Thenappan T, Ormiston ML, Ryan JJ, Archer SL. Pulmonary arterial hypertension: pathogenesis and clinical management. BMJ 2018;360:j5492. https://doi.org/10.1136/bmj.j5492
2. Chang KY, Duval S, Badesch DB, Bull TM, Chakinala MM, De Marco T, Frantz RP, Hemnes A, Mathai SC, Rosenzweig EB, Ryan JJ, Thenappan T, et al. Mortality in pulmonary arterial hypertension in the modern era: early insights from the pulmonary hypertension association registry. J Am Heart Assoc 2022;11:e024969. https://doi.org/10.1164/ajrccm-conference.2021.203.1_MeetingAbstracts.A1181
3. Anand V, Roy SS, Archer SL, Weir EK, Garg SK, Duval S, Thenappan T. Trends and outcomes of pulmonary arterial hypertension-related hospitalizations in the United States: Analysis of the nationwide inpatient sample database from 2001 through 2012. JAMA Cardiol 2016;1:1021-1029. https://doi.org/10.1001/jamacardio.2016.3591
4. Hoeper MM, Badesch DB, Ghofrani HA, Gibbs JSR, Gomberg-Maitland M, McLaughlin VV, Preston IR, Souza R, Waxman AB, Grünig E, Kopeć G, Meyer G, Olsson KM, Rosenkranz S, Xu Y, Miller B, Fowler M, Butler J, Koglin J, de Oliveira Pena J, Humbert M, Investigators ST. Phase 3 Trial of Sotatercept for Treatment of Pulmonary Arterial Hypertension. N Engl J Med 2023;388:1478-1490. https://doi.org/10.1056/NEJMoa2213558
5. Rabinovitch M, Guignabert C, Humbert M, Nicolls MR. Inflammation and immunity in the pathogenesis of pulmonary arterial hypertension. Circ Res 2014;115:165-175. https://doi.org/10.1161/CIRCRESAHA.113.301141

6. Thenappan T, Prins KW, Pritzker MR, Scandurra J, Volmers K, Weir EK. The critical role of pulmonary arterial compliance in pulmonary hypertension. Ann Am Thorac Soc 2016;13:276-284. https://doi.org/10.1513/AnnalsATS.201509-599FR
7. Thenappan T, Chan SY, Weir EK. Role of extracellular matrix in the pathogenesis of pulmonary arterial hypertension. Am J Physiol Heart Circ Physiol 2018;315:H1322-H1331. https://doi.org/10.1152/ajpheart.00136.2018
8. Soon E, Holmes AM, Treacy CM, Doughty NJ, Southgate L, Machado RD, Trembath RC, Jennings S, Barker L, Nicklin P, Walker C, Budd DC, Pepke-Zaba J, Morrell NW. Elevated levels of inflammatory cytokines predict survival in idiopathic and familial pulmonary arterial hypertension. Circulation 2010;122:920-927. https://doi.org/10.1161/CIRCULATIONAHA.109.933762
9. Prins KW, Archer SL, Pritzker M, Rose L, Weir EK, Sharma A, Thenappan T. Interleukin-6 is independently associated with right ventricular function in pulmonary arterial hypertension. J Heart Lung Transplant 2018;37:376-384. https://doi.org/10.1016/j.healun.2017.08.011
10. Ormiston ML, Chang C, Long LL, Soon E, Jones D, Machado R, Treacy C, Toshner MR, Campbell K, et al. Impaired natural killer cell phenotype and function in idiopathic and heritable pulmonary arterial hypertension. Circulation 2012;126:1099-1109. https://doi.org/10.1161/CIRCULATIONAHA.112.110619
11. Tamosiuniene R, Tian W, Dhillon G, Wang L, Sung YK, Gera L, Patterson AJ, Agrawal R, Rabinovitch M, et al. Regulatory T cells limit vascular endothelial injury and prevent pulmonary hypertension. Circ Res 2011;109:867-879. https://doi.org/10.1161/CIRCRESAHA.110.236927
12. Singh RK, Chang HW, Yan D, Lee KM, Ucmak D, Wong K, Abrouk M, Farahnik B, Nakamura M, Zhu TH, Bhutani T, Liao W. Influence of diet on the gut microbiome and implications for human health. J Transl Med 2017;15:73. https://doi.org/10.1186/s12967-017-1175-y
13. Liu Z, Liu HY, Zhou H, Zhan Q, Lai W, Zeng Q, Ren H, Xu D. Moderate-intensity exercise affects gut microbiome composition and influences cardiac function in myocardial infarction mice. Front Microbiol 2017;8:1687. https://doi.org/10.3389/fmicb.2017.01687
14. Hall AB, Tolonen AC, Xavier RJ. Human genetic variation and the gut microbiome in disease. Nat Rev Genet 2017;18:690-699. https://doi.org/10.1038/nrg.2017.63
15. Bretler T, Weisberg H, Koren O, Neuman H. The effects of antipsychotic medications on microbiome and weight gain in children and adolescents. BMC Med 2019;17:112. https://doi.org/10.1186/s12916-019-1346-1
16. Cresci GA, Bawden E. Gut microbiome: What we do and don't know. Nutr Clin Pract 2015;30:734-746. https://doi.org/10.1177/0884533615609899
17. Lynch SV, Pedersen O. The human intestinal microbiome in health and disease. New England J Med 2016;375:2369-2379. https://doi.org/10.1056/NEJMra1600266
18. Mokkala K, Roytio H, Munukka E, Pietila S, Ekblad U, Ronnemaa T, Eerola E, Laiho A, Laitinen K. Gut microbiota richness and composition and dietary intake of overweight pregnant women are related to serum zonulin concentration, a marker for intestinal permeability. J Nutr 2016;146:1694-1700. https://doi.org/10.3945/jn.116.235358
19. Castro A, Bemer V, Nobrega A, Coutinho A, Truffa-Bachi P. Administration to mouse of endotoxin from gram-negative bacteria leads to activation and apoptosis of T lymphocytes. Eur J Immunol 1998;28:488-495. https://doi.org/10.1002/(SICI)1521-4141(199802)28:02<488::AID-IMMU488>3.0.CO;2-R
20. Tan J, McKenzie C, Potamitis M, Thorburn AN, Mackay CR, Macia L. The role of short-chain fatty acids in health and disease. Adv Immunol 2014;121:91-119. https://doi.org/10.1016/B978-0-12-800100-4.00003-9
21. Yuille S, Reichardt N, Panda S, Dunbar H, Mulder IE. Human gut bacteria as potent class I histone deacetylase inhibitors in vitro through production of butyric acid and valeric acid. PLoS One 2018;13:e0201073. https://doi.org/10.1371/journal.pone.0201073
22. Tan Y, Sheng Z, Zhou P, Liu C, Zhao H, Song L, Li J, Zhou J, Chen Y, Wang L, Qian H, Sun Z, Qiao S, Xu B, Gao R, Yan H. Plasma trimethylamine N-oxide as a novel biomarker for plaque rupture in patients with ST-segment-elevation myocardial infarction. Circ Cardiovasc Interv 2019;12:e007281. https://doi.org/10.1161/CIRCINTERVENTIONS.118.007281

23. Moutsoglou DM, Tatah J, Prisco SZ, Prins KW, Staley C, Lopez S, Blake M, Teigen L, Kazmirczak F, Weir EK, Kabage AJ, Guan W, Khoruts A, Thenappan T. Pulmonary Arterial hypertension patients have a proinflammatory gut microbiome and altered circulating microbial metabolites. Am J Respir Crit Care Med 2023;207:740-756. https://doi.org/10.1164/rccm.202203-0490OC
24. Kim S, Rigatto K, Gazzana MB, Knorst MM, Richards EM, Pepine CJ, Raizada MK. Altered gut microbiome profile in patients with pulmonary arterial hypertension. Hypertension 2020;75:1063-1071. https://doi.org/10.1161/HYPERTENSIONAHA.119.14294
25. Yang Y, Zeng Q, Gao J, Yang B, Zhou J, Li K, Li L, Wang A, Li X, Liu Z, Luo Q, Zhao Z, Liu B, Xue J, Jiang X, Konerman MC, Zheng L, Xiong C. High-circulating gut microbiota-dependent metabolite trimethylamine N-oxide is associated with poor prognosis in pulmonary arterial hypertension. Eur Heart J Open 2022;2:oeac021. https://doi.org/10.1093/ehjopen/oeac021
26. Yang Y, Yang B, Li X, Xue L, Liu B, Liang Y, Zhao Z, Luo Q, Liu Z, Zeng Q, Xiong C. Higher circulating Trimethylamine N-oxide levels are associated with worse severity and prognosis in pulmonary hypertension: a cohort study. Respir Res 2022;23:344. https://doi.org/10.1186/s12931-022-02282-5
27. Callejo M, Mondejar-Parreno G, Barreira B, Izquierdo-Garcia JL, Morales-Cano D, Esquivel-Ruiz S, Moreno L, Cogolludo A, Duarte J, Perez-Vizcaino F. Pulmonary arterial hypertension affects the rat gut microbiome. Sci Rep 2018;8:9681. https://doi.org/10.1038/s41598-018-27682-w
28. Sanada TJ, Hosomi K, Shoji H, Park J, Naito A, Ikubo Y, Yanagisawa A, Kobayashi T, Miwa H, Suda R, Sakao S, Mizuguchi K, Kunisawa J, Tanabe N, Tatsumi K. Gut microbiota modification suppresses the development of pulmonary arterial hypertension in an SU5416/hypoxia rat model. Pulm Circ 2020;10:2045894020929147. https://doi.org/10.1177/2045894020929147
29. Karoor V, Strassheim D, Sullivan T, Verin A, Umapathy NS, Dempsey EC, Frank DN, Stenmark KR, Gerasimovskaya E. The short-chain fatty acid butyrate attenuates pulmonary vascular remodeling and inflammation in hypoxia-induced pulmonary hypertension. Int J Mol Sci 2021;22,9916. https://doi.org/10.3390/ijms22189916
30. Huang Y, Lin F, Tang R, Bao C, Zhou Q, Ye K, Shen Y, Liu C, Hong C, Yang K, Tang H, Wang J, Lu W, Wang T. Gut Microbial Metabolite Trimethylamine. Am J Respir Cell Mol Biol 2022;66:452-460. https://doi.org/10.1165/rcmb.2021-0414OC
31. Thenappan T, Goel A, Marsboom G, Fang YH, Toth PT, Zhang HJ, Kajimoto H, Hong Z, Paul J, Wietholt C, Pogoriler J, Piao L, Rehman J, Archer SL. A central role for CD68(+) macrophages in hepatopulmonary syndrome. Reversal by macrophage depletion. Am J Respir Crit Care Med 2011;183:1080-1091. https://doi.org/10.1164/rccm.201008-1303OC
32. Ranchoux B, Bigorgne A, Hautefort A, Girerd B, Sitbon O, Montani D, Humbert M, Tcherakian C, Perros F. Gut-Lung Connection in Pulmonary Arterial Hypertension. Am J Respir Cell Mol Biol 2017;56:402-405. https://doi.org/10.1165/rcmb.2015-0404LE
33. Rabiller A, Nunes H, Lebrec D, Tazi KA, Wartski M, Dulmet E, Libert JM, Mougeot C, Moreau R, Mazmanian M, Humbert M, Herve P. Prevention of gram-negative translocation reduces the severity of hepatopulmonary syndrome. Am J Respir Crit Care Med 2002;166:514-517. https://doi.org/10.1164/rccm.200201-027OC
34. Lin KY, Chen H, Yu L. Pulmonary arterial hypertension caused by congenital extrahepatic portocaval shunt: a case report. BMC Cardiovasc Disord 2019;19:141. https://doi.org/10.1186/s12872-019-1124-1
35. Iida T, Ogura Y, Doi H, Yagi S, Kanazawa H, Imai H, Sakamoto S, Okamoto S, Uemoto S. Successful treatment of pulmonary hypertension secondary to congenital extrahepatic portocaval shunts (Abernethy type 2) by living donor liver transplantation after surgical shunt ligation. Transpl Int 2010;23:105-109. https://doi.org/10.1111/j.1432-2277.2009.00964.x
36. Tatah JH, Weir EK, Prins KW, Thenappan T. A case report of portopulmonary hypertension precipitated by transjugular intrahepatic portosystemic shunt. Chest 2021;159:e193-e196. https://doi.org/10.1016/j.chest.2020.11.014

37. Chesne J, Danger R, Botturi K, Reynaud-Gaubert M, Mussot S, Stern M, Danner-Boucher I, Mornex JF, Pison C, Dromer C, Kessler R, Dahan M, Brugiere O, Le Pavec J, Perros F, Humbert M, Gomez C, Brouard S, Magnan A, Consortium C. Systematic analysis of blood cell transcriptome in end-stage chronic respiratory diseases. PLoS One 2014;9:e109291. https://doi.org/10.1371/journal.pone.0109291
38. Morrell NW, Aldred MA, Chung WK, Elliott CG, Nichols WC, Soubrier F, Trembath RC, Loyd JE. Genetics and genomics of pulmonary arterial hypertension. Eur Respir J 2019;53, 1801899. https://doi.org/10.1183/13993003.01899-2018
39. Soon E, Crosby A, Southwood M, Yang P, Tajsic T, Toshner M, Appleby S, Shanahan CM, Bloch KD, Pepke-Zaba J, Upton P, Morrell NW. Bone morphogenetic protein receptor type II deficiency and increased inflammatory cytokine production. A gateway to pulmonary arterial hypertension. Am J Respir Crit Care Med 2015;192:859-872. https://doi.org/10.1164/rccm.201408-1509OC
40. Zhang C, Xi Y, Zhang Y, He P, Su X, Li Y, Zhang M, Liu H, Yu X, Shi Y. Causal effects between gut microbiota and pulmonary arterial hypertension: A bidirectional Mendelian randomization study. Heart Lung 2024;64:189-197. https://doi.org/10.1016/j.hrtlng.2024.01.002
41. Huang Y, Lin F, Tang R, Bao C, Zhou Q, Ye K, Shen Y, Liu C, Hong C, Yang K, Tang H, Wang J, Lu W, Wang T. Gut microbial metabolite trimethylamine N-oxide aggravates pulmonary hypertension. Am J Respir Cell Mol Biol 2022. https://doi.org/10.1165/rcmb.2021-0414OC
42. Prisco SZ, Eklund M, Moutsoglou DM, Prisco AR, Khoruts A, Weir EK, Thenappan T, Prins KW. Intermittent fasting enhances right ventricular function in preclinical pulmonary arterial hypertension. J Am Heart Assoc 2021;10:e022722. https://doi.org/10.1161/JAHA.121.022722
43. McDonald LC, Gerding DN, Johnson S, Bakken JS, Carroll KC, Coffin SE, Dubberke ER, Garey KW, Gould CV, Kelly C, Loo V, Shaklee Sammons J, Sandora TJ, Wilcox MH. Clinical practice guidelines for clostridium difficile infection in adults and children: 2017 update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). Clin Infect Dis 2018;66:e1-e48. https://doi.org/10.1093/cid/cix1085
44. Quagliariello A, Del Chierico F, Reddel S, Russo A, Onetti Muda A, D'Argenio P, Angelino G, Romeo EF, Dall'Oglio L, De Angelis P, Putignani L, All The Other Fmt Opbg Committee Collaborators. Fecal microbiota transplant in two ulcerative colitis pediatric cases: gut microbiota and clinical course correlations. Microorganisms 2020;8. https://doi.org/10.3390/microorganisms8101486
45. Davar D, Dzutsev AK, McCulloch JA, Rodrigues RR, Chauvin JM, Morrison RM, Deblasio RN, et al. Fecal microbiota transplant overcomes resistance to anti-PD-1 therapy in melanoma patients. Science 2021;371:595-602. https://doi.org/10.1126/science.abf3363
46. Baruch EN, Youngster I, Ben-Betzalel G, Ortenberg R, Lahat A, Katz L, Adler K, et al. Fecal microbiota transplant promotes response in immunotherapy-refractory melanoma patients. Science 2021;371:602-609. https://doi.org/10.1126/science.abb5920
47. Jama HA, Rhys-Jones D, Nakai M, Yao CK, Climie RE, Sata Y, Anderson D, et al. Prebiotic intervention with HAMSAB in untreated essential hypertensive patients assessed in a phase II randomized trial. Nat Cardiovasc Res 2023;2:35-43. https://doi.org/10.1038/s44161-022-00197-4
48. Valensise H, Vasapollo B, Gagliardi G, Novelli GP. Early and late preeclampsia: two different maternal hemodynamic states in the latent phase of the disease. Hypertension 2008;52:873-880. https://doi.org/10.1161/HYPERTENSIONAHA.108.117358
49. Rana S, Lemoine E, Granger JP, Karumanchi SA. Preeclampsia: pathophysiology, challenges, and perspectives. Circ Res 2019;124:1094-1112. https://doi.org/10.1161/CIRCRESAHA.118.313276
50. Kell DB, Kenny LC. A Dormant microbial component in the development of preeclampsia. Front Med (Lausanne) 2016;3:60. https://doi.org/10.3389/fmed.2016.00060
51. Liu J, Yang H, Yin Z, Jiang X, Zhong H, Qiu D, Zhu F, Li R. Remodeling of the gut microbiota and structural shifts in Preeclampsia patients in South China. Eur J Clin Microbiol Infect Dis 2017;36:713-719. https://doi.org/10.1007/s10096-016-2853-z

52. Gomez-Arango LF, Barrett HL, McIntyre HD, Callaway LK, Morrison M, Dekker Nitert M, Group ST. Increased systolic and diastolic blood pressure is associated with altered gut microbiota composition and butyrate production in early pregnancy. Hypertension 2016;68: 974-981. https://doi.org/10.1161/HYPERTENSIONAHA.116.07910
53. Li P, Wang H, Guo L, Gou X, Chen G, Lin D, Fan D, Guo X, Liu Z. Association between gut microbiota and preeclampsia-eclampsia: a two-sample Mendelian randomization study. BMC Med 2022;20:443. https://doi.org/10.1186/s12916-022-02657-x

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SHORT COMMUNICATION

Does Hypoxia Prompt Fetal Brain-Sparing in the Absence of Fetal Growth Restriction?

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Summary

The fetus develops normally in a hypoxic environment but exaggerated hypoxia late in pregnancy is a worrisome sign often observed in hypertensive disorders of pregnancy, placental insufficiency, or fetal growth restriction (FGR). Serial fetal biometry and the cerebroplacental ratio (CPR, calculated as the middle cerebral artery [MCA] / the umbilical artery [UmbA] pulsatility indices [PI]), are commonly used to indicate fetal "brain sparing" resulting from exaggerated fetal hypoxia. But unclear is the extent to which a low CPR indicates pathology or is a physiological response for maintaining cerebral blood flow. We studied 31 appropriate for gestational age (AGA) pregnancies at low (LA, 1670 m) or high (HA, 2879 m) altitude, given the chronic hypoxia imposed by HA residence, and 54 LA women with a clinical diagnosis of FGR. At week 34, the MCA PI was lower in the LA-FGR than the LA-AGA group but lower still in the HA-AGA compared to either LA groups due to a trend toward higher end-diastolic velocity (EDV). We concluded that the lower MCA PI was likely due to greater cerebral vasodilation in the HA-AGA group and an indication of physiological versus pathological fetal hypoxia. Future reporting of serial MCA and UmbA values and their determinants along with the CPR could improve our ability to distinguish between physiological and pathological fetal brain sparing.

Keywords

Birth weight • Cerebroplacental ratio • Fetal physiology • HDP • High altitude

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Hypoxia plays a central role during prenatal life. During 1st-trimester embryogenesis and placental development, fetal epithelial-derived trophoblast cells invade maternal uterine tissue and plug the maternal spiral end-arterioles, making the amniotic cavity extremely hypoxic [1]. The very low resultant tissue pO_2 (<20 mmHg) is thought favorable for preventing reactive oxygen species production and damage to developing tissues [2]. Trophoblast plugs are removed after the completion of placentation late in the 1st trimester, enabling a pronounced rise in uterine artery (UtA) blood flow and oxygen, as well as other nutrient, delivery to the developing fetus.

Chronic hypoxia later in pregnancy is a worrisome sign commonly observed in hypertensive disorders of pregnancy, placental abruption or other signs of placental insufficiency, or fetal growth restriction (FGR). These conditions are not only associated with increased perinatal morbidity and mortality but also greater susceptibility to cardiovascular and other diseases later in life for both mother and child. Therefore, indices of fetal hypoxia are an important means for identifying pregnancies at increased risk. Such indices include elevated resistance indices obtained by Doppler ultrasound for the UtA, umbilical (UmbA), middle cerebral arteries (MCA), and the ductus venosus; serial fetal biometry measures indicative of slowed or restricted growth; and reduced birth weight after adjusting for gestational age and sex [3]. Of these, birth weight is the least sensitive since it alone cannot discriminate between

babies who are constitutionally small *versus* those who are growth restricted, nor can it identify those who are constitutionally small but have been subject to fetal hypoxia. While birth weight percentiles continue to be used, especially since they are only such measure available in many parts of the world, the use of serial fetal-biometry and vascular resistance measures are preferable as they can be monitored over time and therefore provide a timelier means for clinical management.

The commonly used vascular resistance indices are the systolic to diastolic ratio (S/D, calculated as the peak systolic [PSV] to end-diastolic velocity [EDV]), the pulsatility index (PI, calculated as the [PSV-EDV] divided by the mean flow velocity throughout the cardiac cycle or TAM), and the resistive index (RI, calculated as PSV-EDV divided by the PSV). These indices describe the velocities or mean velocities observed, using Doppler ultrasound, of the middle cerebral artery which serves as a major source of blood flow to the brain and of the umbilical artery which describes the velocity profile of blood returning to the placenta when propelled by the fetal heart. These indices play key roles in the surveillance, diagnosis, and clinical management of suspected FGR and are particularly useful for distinguishing early (< 32 weeks) *versus* late (≥ 32 weeks) suspected FGR [3]. Early FGR cases generally show increased UtA and UmbA PI due to placental insufficiency, whereas late-gestation FGR are more often marked by redistribution of cerebral blood flow to favor the cerebral circulation or "brain sparing" and calculated as the MCA PI/UmbA PI [3]. Given that the estimated fetal weight (EFW) may be insufficient for diagnosing FGR, a low cerebroplacental ratio (CPR) is commonly used as an index of brain sparing due to exaggerated fetal hypoxia.

The multiple physiological factors contributing to brain sparing have been extensively reviewed elsewhere [4]. The question being addressed in this short communication is whether a low CPR necessarily indicates pathology or whether it may also be a physiological response by which the fetus maintains cerebral blood flow under conditions of chronic maternal hypoxia. Studies at high altitude (HA, conventionally defined as > 2500 m or 8250 ft as that is where maternal arterial oxygen saturation [SaO_2] measurably declines), provide a means to answer this question since pregnant women at such altitudes necessarily experience chronic hypoxia. However, even though average birth weight falls ~100 gm/1000 m elevation gain and FGR is approximately 3-fold more common at HA [5], some HA babies are appropriate for gestational age and sex (AGA). Therefore, we address the question of whether hypoxia prompts fetal brain-sparing in the absence of FGR by comparing CPR values in clinically diagnosed FGR pregnancies at low altitude (LA, <1670 m) to those observed in healthy, LA- and HA-AGA pregnancies. Additionally, since the CPR is a ratio of two ratios and therefore affected by several factors, chiefly the MCA and the UmbA PSV and EDV, we ask which of these CPR determinants differed in the three groups.

Our study measured CPR and its key determinants in 31 healthy women experiencing uncomplicated AGA pregnancies at LA (Denver, Colorado [1670 m]), 27 AGA pregnancies at HA (Summit and adjacent Colorado counties [2879 m]), and 54 LA (Denver) women with a clinical diagnosis of FGR based on repeated EFW values below the 10^{th} percentile for gestational age and sex. All studies were performed in accordance with the 2000 Declaration of Helsinki, with approval from the Colorado Multiple Institutional Human Subjects Review Committee, and all Doppler ultrasound exams were conducted by the same ultrasonographers. The LA- and HA-AGA pregnancies were studied at pregnancy weeks 20 and 34 (20.2 ± 0.8 and 20.8 ± 1.0, 34.7 ± 1.4 and 34.3 ± 1.2 at LA and HA, respectively), whereas data from LA-FGR women were only available at week 34 (34.0 ± 2.0). Information collected in all three groups included maternal demographics (age, pre-pregnancy body mass index [BMI]), obstetric history (gravidity, parity), fetal biometry, CPR (averaged from both UmbA and MCA), and delivery characteristics (vaginal or Cesarean section delivery, gestational age at birth, birth weight, infant sex and ponderal index). Additionally, maternal hemoglobin (Hb), SaO_2, average right and left UtA diameter, time-averaged mean blood flow velocity (TAM) for calculating total UtA blood, and the average PI were measured at pregnancy weeks 20 and 34 in LA- and HA-AGA groups as previously described [6]. The same variables except for UtA blood flow, UmbA PSV and EDV were recorded in LA-FGR women at week 34. Further details concerning these LA- or HA-AGA and LA-FGR groups are available elsewhere [7,8]. Comparisons between two groups were conducted using student's t-tests and among the three groups by one-way analysis of variance with post-hoc comparisons to identify the specific groups whose values differed. Statistical significance was assessed as a p value < 0.05 and trends noted when the $0.05 < p < 0.10$. All comparisons were conducted using Graph Pad Prism version 10.2.3.

Table 1. Subject characteristics

Variable	LA-AGA	HA-AGA	LA-FGR	P value
n	31	27	54	
Maternal characteristics				
Age, yr	32.0 ± 4.7[a]	32.0 ± 4.3[a]	28.1 ± 4.9[b]	<0.0001
European ancestry, %	74 [56, 86]	100 [88, 100]	80 [68, 89] [b]	<0.01
Parity, no.	1.6 ± 0.6	1.5 ± 0.7	1.8 ± 1.0	NS
Pre-pregnant BMI, kg/m^2	23.2 ± 2.8	23.5 ± 3.2	24.4 ± 4.6	NS
SaO_2, wk 34, %	97.4 ± 1.3	94.7 ± 1.3	--	<0.0001
Hb, wk 34, gm/dL	13.0 ± 2.6	13.5 ± 1.5	--	NS
Wk 20 UtA diam, cm	0.31 ± 0.08	0.29 ± 0.07	--	NS (0.08)
TAM, cm/s	35 ± 12	34 ± 13	--	NS
blood flow, ml/min	356 ± 166	264± 102	--	<0.05
PI	0.82± 0.17	0.84 ± 0.29		NS
Wk 34 UtA diam, cm	0.34 ± 0.08	0.32 ± 0.08	--	NS
TAM, cm/s	42 ± 11	47 ± 15	--	NS
blood flow, ml/min	429 ± 175	469± 243	--	NS
PI	0.66± 0.18	0.65 ± 0.24	0.72 ± 0.21	NS
C-section, %	16 [7, 33]	36 [21, 54]	35 [22, 50]	NS
Fetal characteristics, wk 20				
MCA PSV, cm/s	26.5 ± 4.0	25.4 ± 4.4	--	NS
EDV, cm/s	5.8 ± 0.7	6.4 ± 1.6	--	NS
PI	1.60 ± 0.37	1.38 ± 0.12	--	<0.01
UmbA PSV, cm/s	35.1 ± 5.9	33.2 ± 4.9	--	NS
EDV, cm/s	9.1 ± 3.6	9.6 ± 2.8	--	NS
PI	1.36 ± 0.18	1.25 ± 0.16	--	<0.05
CPR	1.18 ± 0.25	1.12 ± 0.17	--	NS
EFW, gm	351 ± 58	356 ± 56	--	NS
Fetal characteristics, wk 34				
CPR	2.08 ± 0.47	1.80 ± 0.53	1.94 ± 0.53	NS
CPR < 1.08 (no.)	0 [0, 12]	6 [1, 27]	6 [2, 15]	NS
EFW, gm	2444 ± 301[a]	2412 ± 2653[a]	1878 ± 222[b]	<0.0001
Infant characteristics				
Birth weight, g	3460 ± 436[a]	3141 ± 328[b]	2442 ± 410[c]	<0.0001
Male sex, %	55 [38, 70]	54 [36, 70]	33 [22, 46]	NS (0.07)
Ponderal index, kg/cm^3	2.68 + 0.27[a]	2.54 + 0.26[a]	2.40 + 0.25[b]	<0.001
Gest. age at birth, wk	39.7 ± 1.1[a]	39.1 ± 1.5[a]	37.6 ± 1.4[b]	<0.0001
Preterm, %	3 [0, 16]	14 [6, 31]	15 [7, 28]	NS
<10th percentile, %	0 [0.9, 1.0]	11 [4, 27]	52 [38, 67]	<0.0001
<5th percentile, %	--	3 [0.2, 18]	22 [12, 38]	<0.01

Abbreviations: AGA, appropriate-for-gestational-age and sex; BMI body mass index; CPR, cerebral placental ratio; C-section, Cesarean section; EDV, end-diastolic velocity; EFW, estimated fetal weight; Hb, hemoglobin; HA, high altitude; LA, low altitude; MCA, middle cerebral artery; no, number; PSV, peak systolic velocity; PI, pulsatility index; SaO_2, arterial oxygen saturation; TAM, time-averaged mean; UtA, uterine artery; Wk, week. Mean ± standard deviation or 95% confidence intervals in brackets. Different superscripts indicates the specific groups whose values differed using post-hoc tests for results obtained using one-way analyses of variance.

The LA-FGR women were younger and more often self-identified as being of non-European ancestry than LA- or HA-AGA women but similar in parity, gravidity (data not shown), and pre-pregnant BMI (Table 1). Compared to LA-AGA pregnancies, the HA-AGA had lower SaO_2, higher Hb, and lower UtA blood flow at week 20 due to a trend toward smaller vessel diameter, but UtA blood flow parameters were similar at week 34. UtA PI was similar in LA-AGA and HA-AGA women at week 20 and in all three groups at week 34. The frequency of Cesarean-section delivery was also similar in all three groups.

With respect to fetal characteristics, those in the HA-compared to the LA-AGA group had lower week 20 MCA and UmbA PI values, but changes were proportionate such that the CPR values did not differ (Table 1). At week 34, the UmbA PSV, EDV, and PI values were similar among all three groups but there were considerable differences in the MCA PSV, EDV, and PI values (Fig. 1). Specifically, the LA-FGR group tended to have lower MCA PSV and EDV than the LA-AGA, a trend toward lower values than the HA-AGA, and higher MCA PI than the HA-AGA but no differences in the CPR or the percent with CPR values below the cutoff value of 1.08 (Table 1) [9]. Of note, the MCA PI values in the HA-AGA fetuses were lower than those seen in either the LA-AGA or LA-FGR groups (Fig. 1 and Table 1).

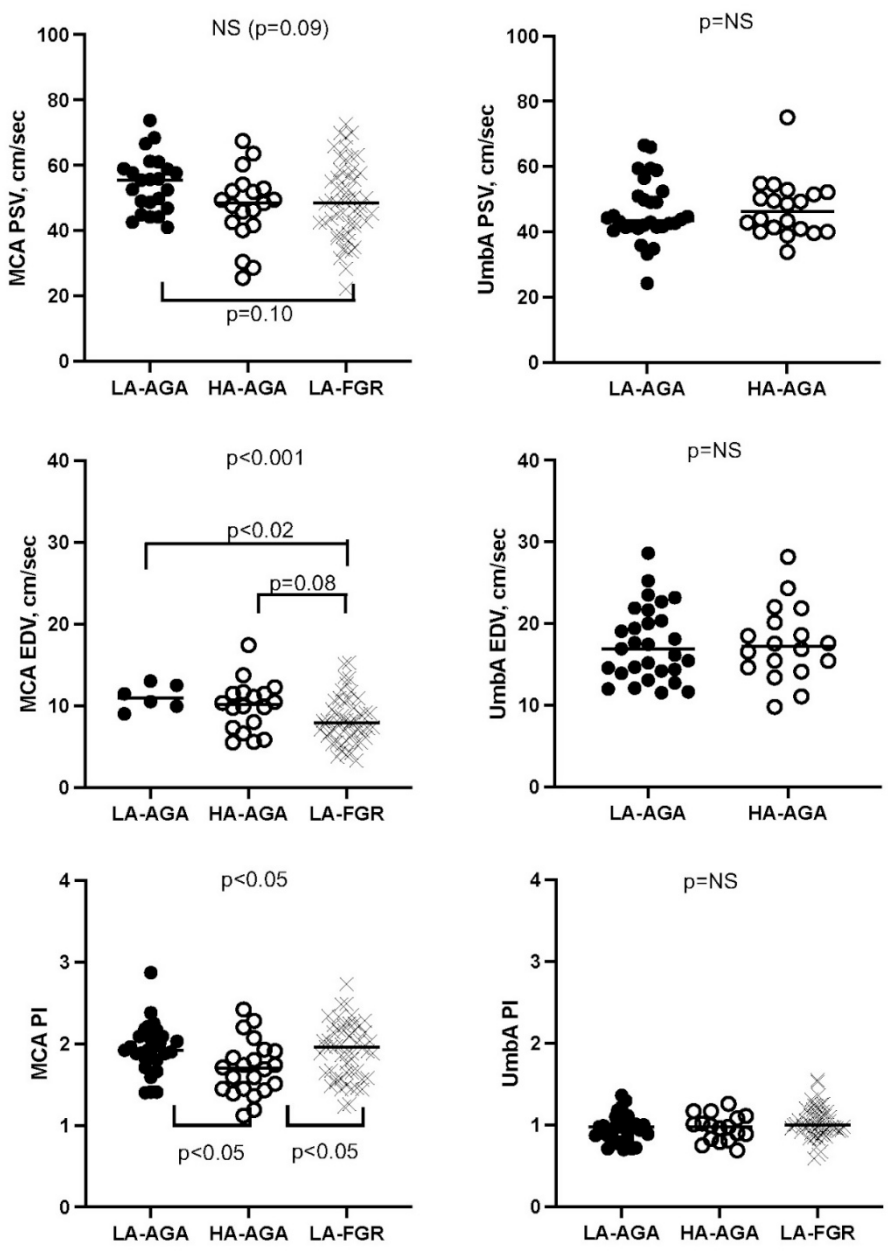

Fig. 1. Lefthand panels: Pregnant women were studied at week 34 who resided either at low (LA, <1670 m, n=31), high altitude (HA, 2879m, n=27) with fetal weights that were appropriate for gesta-tional age and sex (AGA) and in 55 LA women with a clinical diagnosis of fetal growth restriction (FGR). Fetuses in the LA-FGR tended to have lower middle cerebral artery (MCA) peak systolic velocity (PSV) and lower end-diastolic velocity (EDV) than the LA-AGA group, as well as a trend toward lower EDV compared to HA-AGA babies. The MCA pulsa-tility index (PI) was lower in the HA-AGA fetuses than in either the LA-AGA or -FGR groups. Righthand panels: The umbilical artery (UmbA) PSV and EDV values were similar in the LA- or HA-AGA groups at week 34, as were the PI values in all three groups. Filled circles designate LA-AGA, open circles HA-AGA, and x's LA-FGR groups.

EFW was similar in the LA- and HA-AGA groups at pregnancy weeks 20 and 34 but, as expected, both groups had higher values at week 34 than those observed in the LA-FGR cases (Table 1). Birth weights and ponderal indices were highest in the LA-AGA, moderately reduced in HA-AGA, and lowest in LA-FGR groups, with corresponding differences seen in the frequencies of birth weights below the 10^{th} or 5^{th} percentiles. There was a trend toward fewer male LA-FGR babies but no differences in gestational age at delivery or the percentage of preterm births (Table 1).

We interpreted these study findings as showing that differences in fetal growth evident at week 34 or at birth were not accompanied by alterations in the UmbA's PI or its PSV or EDV components, but were paralleled by changes in the MCA PSV, EDV and PI. Moreover, these MCA parameters appeared to differ in physiological *versus* pathological reductions in fetal growth; specifically, whereas the MCA PI was the same in the LA-FGR and LA-AGA pregnancies, the HA-AGA had a lower MCA PI due to a trend toward higher EDV. While it must be acknowledged that neither PSV, EDV nor PI truly measure vascular resistance given that by definition vascular resistance is blood pressure divided by blood flow, the lower PI and trend toward higher EDV in HA-AGA group suggested that the lower MCA PI resulted from greater cerebral vasodilation.

Our understanding of how a fetus can maintain normal growth under conditions of chronic hypoxia is limited by the technical difficulties encountered for directly measuring fetal cardiovascular function *in vivo* as well as to the fact that chronic hypoxia is usually accompanied by some other pregnancy complication such as preeclampsia or placental insufficiency [4]. Given such limitations, we considered that a comparison of the non-invasive fetal vascular resistance parameters obtained by Doppler ultrasound in clinically-diagnosed FGR *versus* AGA yet chronically hypoxic babies would be useful for differentiating between fetal brain-sparing indicative of adaptive or physiological responses to hypoxia *versus* that seen in pathological conditions associated with stillbirth and perinatal loss [10]. HA studies have the unique advantage of being able to examine the mechanisms by which the fetus responds to chronic hypoxia in the absence of overt pathology. Studies at HA provide several lenses through which physiological *versus* pathological fetal responses to chronic hypoxia can be distinguished since HA birth weights vary due to 1) the increased frequency of FGR but also the existence of AGA fetuses and also to 2) the operation of natural selection that has resulted in genetic protection from altitude-associated FGR in multigenerational HA populations (Tibetans and Andeans) *versus* HA newcomers [11-14]. The present study conducted in Colorado LA and HA residents suggested that a strategy distinguishing physiological from pathological fetal hypoxia is a reduction in the MCA PI due, in part, to higher EDV relative to the PSV that, in turn, helps defend cerebral blood flow. Speculatively, this preference on the part of the fetus for sending more blood to the brain as opposed to the placenta suggests a possible role for hypoxic vasoconstriction. Future studies are required to determine, however, such a mechanism as well as investigations in multigenerational HA populations to determine if lower MCA PI values and other indices of physiological brain sparing are present and, if so, whether genetic factors are involved.

The strengths of our study stemmed from the availability of subjects residing at either LA or HA within sufficient proximity that Doppler ultrasound studies could be performed by the same ultrasonographers at each location. While our study was not designed to evaluate the mechanisms by which the HA fetuses sustained a lower MCA PI and possibly greater cerebral blood flow, our observation of lower PI in the MCA and UmbA at week 20 indicated that altitudinal differences were already present by mid-gestation. Since cord blood Hb values were similar in the LA- and HA-AGA groups (15.3 ± 1.8 *versus* 15.5 ± 2.3 g/dL, respectively; p=NS) and there were no relationships between Hb and the MCA or UmbA vascular resistance parameters, the lower PIs at HA did not appear to be due to altitudinal differences in blood viscosity. But our study also had several limitations. One was that 3^{rd} trimester measurements were only available at week 34 for the LA-FGR group, so early FGR or that present by week 32 could not be studied. Another was that not all the babies with a clinical diagnosis of FGR met Delphi criteria [15] since only 52 % weighed less than the 10^{th} percentile at birth.

We concluded that the brain sparing seen in the high-altitude AGA fetuses indicated that indeed, fetal brain-sparing can occur in the absence of FGR. Therefore, while convenient, reliance on a low CPR for diagnosing FGR could be misleading insofar as it might not detect more subtle changes in the MCA PSV or EDV flow velocity parameters. Given the difficulties inherent

in making direct measurements of fetal MCA or UmbA vascular resistance, we suggest that reporting serial MCA and UmbA PSV, EDV, and PI along with the CPR measurements in future studies could improve our ability to distinguish between physiological and pathological fetal brain sparing.

Conflict of Interest

There is no conflict of interest.

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References

1. Jauniaux E, Gulbis B, and G.J. Burton, Physiological implications of the materno-fetal oxygen gradient in human early pregnancy. Reprod Biomed Online 2003;7:250-253. https://doi.org/10.1016/S1472-6483(10)61760-9
2. Burton GJ. Oxygen, the Janus gas; its effects on human placental development and function. J Anat 2009;215:27-35. https://doi.org/10.1111/j.1469-7580.2008.00978.x
3. Lees CC, Romero R, Stampalija T, Dall'Asta A, DeVore GA, Prefumo F, Frusca T, et al. Clinical Opinion: The diagnosis and management of suspected fetal growth restriction: an evidence-based approach. Am J Obstet Gynecol 2022;226:366-378. https://doi.org/10.1016/j.ajog.2021.11.1357
4. Giussani DA. The fetal brain sparing response to hypoxia: physiological mechanisms. J Physiol 2016;594:1215-1230. https://doi.org/10.1113/JP271099
5. Jensen GM, Moore LG. The effect of high altitude and other risk factors on birthweight: independent or interactive effects? Am J Public Health 1997;87:1003-1007. https://doi.org/10.2105/AJPH.87.6.1003
6. Palmer SK, Zamudio S, Coffin C, Parker S, Stamm E, Moore LG. Quantitative estimation of human uterine artery blood flow and pelvic blood flow redistribution in pregnancy. Obstet Gynecol 1992;80:1000-1006.
7. Chassen SS, Zemski-Berry K, Raymond-Whish S, Driver C, Hobbins JC, Powell TL. Altered cord blood lipid concentrations correlate with birth weight and doppler velocimetry of fetal vessels in human fetal growth restriction pregnancies. Cells 2022;11,3110. https://doi.org/10.3390/cells11193110
8. Moore LG, Lorca RA, Gumina DL, Wesolowski SR, Reisz JA, Cioffi-Ragan D, Houck JA, et al. Maternal AMPK pathway activation with uterine artery blood flow and fetal growth maintenance during hypoxia. Am J Physiol: Heart and Circ Physiol 2024;327:H778-H792. https://doi.org/10.1152/ajpheart.00193.2024
9. Dogru S, Akkus F, AcarA. Cerebroplacental ratio and perinatal outcomes in mild-to-moderate idiopathic polyhydramnios cases. Int J Gynaecol Obstet 2024;324. https://doi.org/10.1002/ijgo.15556
10. Khalil, A. and B. Thilaganathan, Role of uteroplacental and fetal Doppler in identifying fetal growth restriction at term. Best Pract Res Clin Obstet Gynaecol 2017;38:38-47. https://doi.org/10.1016/j.bpobgyn.2016.09.003
11. Moore LG, Young D, McCullough RE, Droma T, Zamudio S. Tibetan protection from intrauterine growth restriction (IUGR) and reproductive loss at high altitude. Am J Hum Biol 2001;13:635-644. https://doi.org/10.1002/ajhb.1102
12. Julian CG, Vargas E, Armaza JF, Wilson MJ, Niermeyer S, Moore LG. High-altitude ancestry protects against hypoxia-associated reductions in fetal growth. Arch Dis Child Fetal Neonatal Ed 2007;92:F372-F377. https://doi.org/10.1136/adc.2006.109579
13. Soria R, Julian CG, Vargas E, Moore LG, Giussani DA. Graduated effects of high-altitude hypoxia and highland ancestry on birth size. Pediatr Res 2013;74:633-638. https://doi.org/10.1038/pr.2013.150
14. Dolma P, Angchuk PT, Jain V, Dadhwal V, Kular D, Williams DJ, Montgomery HE, Hillman SL. High-altitude population neonatal and maternal phenotypes associated with birthweight protection. Pediatr Res 2022;91:137-142. https://doi.org/10.1038/s41390-021-01593-5
15. Gordijn SJ, Beune IM, Thilaganathan B, Papageorghiou A, Baschat AA, Baker PN, Silver RM, Wynia K, Ganzevoort W. Consensus definition of fetal growth restriction: a Delphi procedure. Ultrasound Obstet Gynecol 2016;48:333-339. https://doi.org/10.1002/uog.15884

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REVIEW

Hypoxic Pulmonary Vasoconstriction: An Important Component of the Homeostatic Oxygen Sensing System

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Summary

Hypoxic pulmonary vasoconstriction (HPV) rapidly and reversibly matches lung ventilation (V) and perfusion (Q), optimizing oxygen uptake and systemic oxygen delivery. HPV occurs in small pulmonary arteries (PA), which uniquely constrict to hypoxia. Although HPV is modulated by the endothelium the core mechanism of HPV resides in PA smooth muscle cells (PASMC). The PASMC's mitochondrial oxygen sensor lies within the electron transport chain (ETC) and includes NDUFS2 in ETC Complex-I. PASMC mitochondria respond to hypoxia by varying production of reactive oxygen species (ROS) and hydrogen peroxide in proportion to alveolar oxygen tension. Hypoxic ROS inhibition results in a state of reduction which triggers a redox-mediated inhibition of oxygen-sensitive, voltage-gated, potassium channels, including Kv1.5 and Kv2.1. Kv channel inhibition depolarizes the PASMC, opening of large-conductance calcium channels (Ca_L), elevating cytosolic calcium and activating the contractile apparatus. HPV is strongest in small PAs where sensors (hypoxia-responsive mitochondria) and effectors (oxygen-sensitive K^+ channels) are enriched. Oxygenation at birth reverses fetal HPV, contributing to the rapid neonatal drop in pulmonary vascular resistance (PVR). A similar mitochondrial-K^+ channel sensor-effector mechanism exists in the ductus arteriosus (DA), however in DASMC it is oxygen-induced increases in mitochondrial ROS that inhibit DASMC K^+ channels, causing DA constriction. Atelectasis and pneumonia elicit HPV, which optimises V/Q matching, increasing systemic oxygenation. Whilst HPV in response to localized hypoxia in a single lung lobe does not increase PA pressure; global airway hypoxia, as occurs with altitude or sleep apnea, causes pulmonary hypertension. HPV can be inhibited by drugs, including calcium channel blockers, or used to maintain a dry operative field during single lung anesthesia for lung surgery. HPV does not normally cause lung edema but excessive, heterogenous HPV contributes to high altitude pulmonary edema. HPV is suppressed in COVID-19 pneumonia by a SARS-CoV-2 mitochondriopathy. HPV is a component of the body's homeostatic oxygen sensing system.

Keywords

Ductus arteriosus • Redox • NDUFS2 • Oxygen sensitive potassium • Channels • High altitude pulmonary edema (HAPE) • Mitochondrial electron transport chain • COVID-19 pneumonia • Atelectasis

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Introduction

Hypoxic pulmonary vasoconstriction (HPV) was reported first in 1894 by Bradford and Dean [1], and subsequently more precisely described by von Euler and Liljestrand [2]. In the Bradford and Dean paper hypoxic stimulation was achieved by transient asphyxia (a form of global airway hypoxia), whereas in the more definitive work of von Euler and Liljestrand the lung was ventilated with hypoxic gas mixtures (Fig. 1). These authors

concluded *"The experiments seem to warrant the conclusion, that the regulation of the pulmonary blood flow is mainly mediated by a local action of the blood and alveolar gases leading to an adequate distribution of the blood through the various parts of the lungs according to the effeciency [sic] of aeration.*" [2].

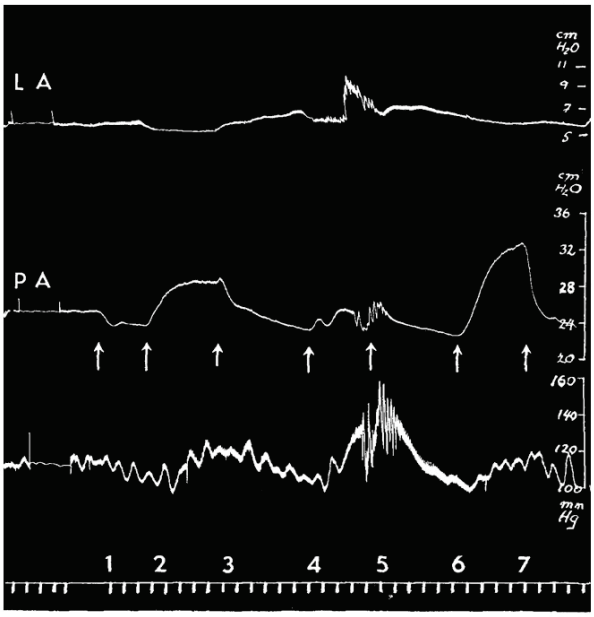

Fig. 1. Original description of HPV by von Euler and Liljestrand: Experiments were performed in a cat under chloralose anesthesia. The uppermost trace shows pressure in left auricle, the middle trace pulmonary arterial pressure, and the lower trace shows systemic blood pressure. The cat received artificial respiration and the thorax was open. Time scale is in units of 30 seconds.
Note point 6, which reflects hypoxic ventilation. HPV is rapid in onset, fully reversible, and not associated with significant changes in systemic blood pressure.
1. Oxygen (room air)
2. 6.5 % CO_2 in oxygen
3. Oxygen (room air)
4. 18.7 % CO_2 in room air
5. Oxygen (room air)
6. 10.5 % oxygen in nitrogen (hypoxia)
7. Oxygen (room air).
Reproduced with permission from [2].

HPV has long intrigued physicians and physiologists perhaps because it is involved in so many physiologic and pathologic events in life, including the transition of the fetal circulation at birth (withdrawal of HPV), the regulation of ventilation/perfusion matching to optimize systemic oxygenation in normal lungs, counteracting intrapulmonary shunting of blood in pneumonia and atelectasis, and mediating the pulmonary hypertensive response to ascent to altitude. When excessive and heterogenous, HPV also contributes to high altitude pulmonary edema (HAPE). Loss of HPV, whether caused by drugs that are pulmonary vasodilators or due to infectious agents, as occurs with COVID-19 and coronavirus infection [3], can exacerbate systemic hypoxia by causing V/Q mismatch and intrapulmonary shunting (perfusion of unventilated lung segments).

At the heart of HPV is the unique ability of small pulmonary arteries (PAs) (<400 μm diameter) to constrict to hypoxia. Virtually all systemic arteries (and large PAs) relax upon exposure to hypoxia [4-6]. We have made the case that intrapulmonary arteries (PA)s are part of the body's homeostatic oxygen sensing system (HOSS), a system which includes the carotid body, ductus arteriosus, and fetal placental arteries [7]. These specialized HOSS tissues each initiate changes in vascular tone and/or neural output that serve to optimize systemic oxygen delivery. For example, at altitude the type 1 cells within the carotid body trigger hyperventilation to increase oxygen uptake from rarefied air. Likewise, at birth the ductus arteriosus SMC constrict, achieving functional DA closure which diverts blood from the right ventricle (RV) into the pulmonary circulation of the new ventilated lung. Simultaneously, with the first breath, the neonatal PASMC relax as the lung's fetal hypoxic milieu is replaced with the oxygen-rich neonatal environment. The withdrawal of HPV relaxes the pulmonary circulation, preparing it to receive the RV output at low PA pressure. One could argue that systemic arteries, by dilating to hypoxia, also contribute to optimizing systemic oxygen delivery in states of hypoxia and thus may too be considered part of the HOSS. The tissues of the HOSS (at least the type 1 cell carotid body, DASMC, PASMC) all use mitochondria as oxygen sensors and ion channels, notably potassium and calcium channels, as effector mechanisms [7].

It should be noted that the HOSS, as originally described [7], uses conserved mitochondrial-ROS-ion channel signalling pathways in specialized tissues to rapidly (in seconds) elicit adaptive responses to counteract hypoxia. However, there are equally important response to hypoxia that occur more slowly in response to hypoxia in most tissues. These transcriptional responses are often initiated by redox triggers (including ROS) but are mediated by transcriptional regulators, such as hypoxia inducible factors like HIF-1α [8] and prolyl hydroxylases. These transcriptional hypoxic responses onset more slowly and are sustained throughout exposure to hypoxia. The HIF-1α pathways interact with the mechanism of HPV. For example, patrial deficiency of

HIF-1α in mice impairs acute HPV (specifically impairing hypoxia-induced depolarization and K^+ current inhibition in PASMC from HIF-1α heterozygous knockout mice) and inhibits chronic hypoxic pulmonary hypertension [9]. Moreover, the acute mitochondrial oxygen sensing pathway is involved in regulation of HIF-1α. For example, in pulmonary arterial hypertension there is normoxic activation of HIF-1α due to epigenetic silencing of mitochondrial superoxide dismutase 2, which reduces production of mitochondrial-derived hydrogen peroxide [10]. Normoxic activation of HIF-1α, whether spontaneous or achieved by cobalt exposure, rapidly activates dynamin related protein 1 (Drp1) in human PASMC which triggers mitotic mitochondrial fission, changes mitochondrial redox control [11] and promotes the hyperproliferation of PASMC. Thus, there is interaction between acute mitochondrial-redox mechanisms of oxygen sensing in the HOSS and longer-term transcriptional responses to hypoxia both in HOSS tissues and less specialized tissues.

HPV has certain hallmarks which any proposed mechanism must address. HPV is rapid in onset, beginning within seconds of exposure to even mild airway hypoxia [12,13]. Moreover, although HPV can be sustained for pronged periods [14], it is rapidly reversible when airway hypoxia is relieved. HPV is also dependent, in large part, on influx of extracellular calcium [15]. However, there is also evidence for a role for hypoxia-induced release of intracellular calcium and activation of rho kinase in triggering HPV, see review [16]. Although many endothelium-derived vasodilators (notably nitric oxide (NO), prostaglandins) and vasoconstrictors (notably endothelin) attenuate and enhance HPV, respectively, HPV can be elicited in PA rings denuded of endothelium or even in isolated PASMC [17]. Nonetheless, the magnitude of HPV is clearly augmented by inhibition of nitric oxide synthase [18,19] or cyclooxygenase [20].

The cellular electrophysiology of HPV

In 1992 a partnership between the Weir-Archer lab and the laboratory of Professor Joe Hume made the first demonstration that hypoxia inhibited the outward K^+ currents of canine PASMC, leading to membrane depolarization. This hypoxic inhibition of I_K was unique to PASMC and did not occur in canine renal arterial SMCs [21]. In PASMC voltage-gated potassium channels (Kv) maintain a resting membrane potential of ~ -60 mV. The membrane potential is set by a tonic efflux of positively charged potassium ions down a concentration gradient of ~ 140 mM (intracellular) to 5 mM (extracellular). The resulting negative membrane potential decreases the open state probability of voltage-gated, L-type calcium channels (Ca_L). With hypoxic PASMC depolarization Ca_L have more and longer openings and extracellular Ca^{2+} enters the PASMC, traveling down a 20,000:1 extracellular:intracellular Ca^{2+} gradient, reviewed in [22]. The resulting increase in cytosolic calcium triggers vasoconstriction, which is subsequently reinforced by Rho-kinase-mediated phosphorylation of myosin light chain, which mediates calcium sensitization contributing to a sustained phase of pulmonary arterial vasoconstriction [15,21]. Based on this cellular electrophysiology, performed using the whole-cell patch clamp technique, it is not surprising that the pulmonary circulation in isolated perfused rat lungs constrict in response to the Kv channel inhibitor, 4-aminopyridine [23]. Moreover, HPV is inhibited by Ca_L inhibitors, like nifedipine and verapamil, while it is enhanced by CaL agonists like BAYK8644 [15,24].

Molecular electrophysiology of HPV

After identification of the role of K^+ channels in HPV a search began to obtain molecular precision in specifying which of the many candidate Kv channels (or other channels, like TASK channels) was involved. Ultimately a key role for Shaker channels, notably Kv1.5, and Kv2.1 [25,26], was identified. Subsequently we showed that a Kv1.5 knockout mouse had reduced HPV [27]. However, there is evidence for involvement of other classes of K^+ and Ca^{2+} channels in the mechanism of HPV [28], including two-pore acid sensitive K^+ channels (TASK) [29,30]. TASK-1 channels are active at very negative membrane potentials and contribute to regulation of the resting membrane potential in PASMC and may also modulate the sensitivity of membrane potential to acid-base disturbances [31]. However, TASK-1 is not essential for HPV in murine PAs, since HPV persists in TASK-1 knockout mice and in the presence of a chemical TASK-1 inhibitor (A293) [32]. Nonetheless, mutations in the gene encoding TASK, *KCNK3*, predispose to WHO Group 1 pulmonary hypertension (pulmonary arterial hypertension, PAH) in transgenic rats [33] and humans [34].

The mitochondrial electron transport chain (ETC) is an O_2-sensor in DA and adult PA SMC [35,36].

One of the earliest papers to identify a potential role for the mitochondrial ETC in HPV was by Rounds and McMurtry [37]. They found that five substances that blocked different steps of oxidative ATP production (10 mM azide, 1 mM cyanide, 1 mM dinitrophenol, 5 or 10 µM antimycin A, or 0.5 µM rotenone) trigger pulmonary vasoconstriction, which like HPV, was inhibited by the calcium channel blocker, verapamil [37]. Subsequently we showed rotenone and antimycin did indeed mimic hypoxia but do not achieve this by causing bioenergetic depletion, rather they inhibit normoxic ROS production, thereby regulating redox-sensitive potassium channels in a manner mimicking authentic hypoxia [38,39].

The ETC conducts electrons from donors derived from Krebs' cycle (NADH and $FADH_2$), down an electrochemical gradient to molecular O_2 (Fig. 2). These donors transfer electrons to Fe-S clusters, within the quinone chambers (Q-site) of Complex I and III. One such Fe-S center, NADH dehydrogenase [ubiquinone] iron-sulfur protein 2 (NDUFS2) in Complex I, is a source of oxygen-sensitive, mitochondrial-derived ROS (Fig. 2) and is implicated in O_2-sensing in adult PASMC and carotid body type-1 cells [17,40]. During electron transfer ~1-3 % of electrons leak from Q-sites, such as NDUFS2 in Complex I and the Rieske Fe-S center in Complex III [41,42]. More contemporary measurements of electron leakage from the ETC suggest that electron leak comprises < 0.5 % of total electron flux under normal conditions [43-45].

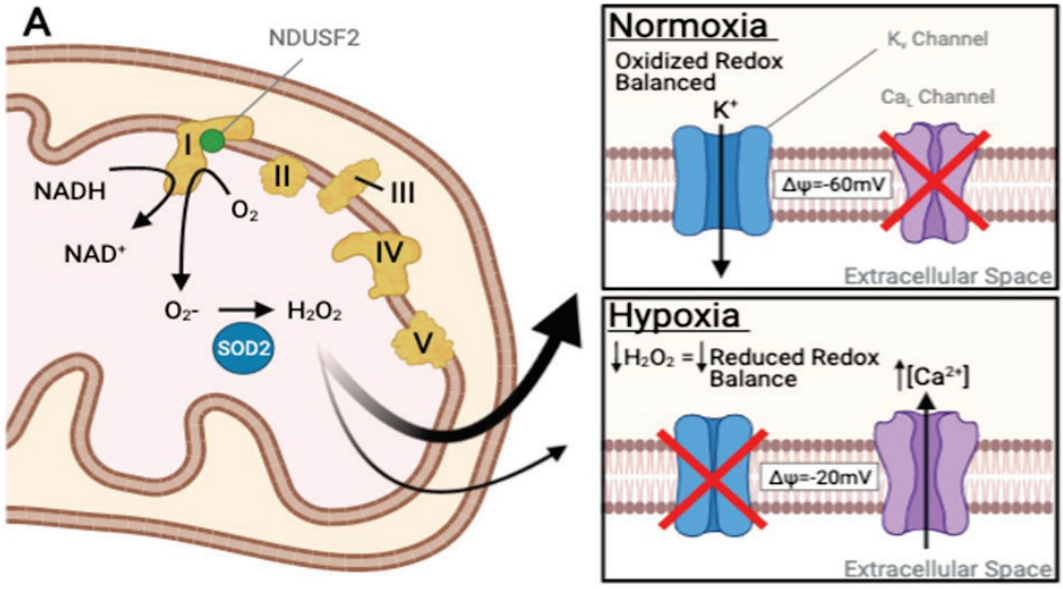

Fig. 2. Mitochondrial O_2-Sensing in PASMC: In adult, normoxic, PASMC mitochondria generate superoxide from NDUFS2 in ETC Complex I. This is dismutated to hydrogen peroxide by superoxide dismutase 2 (SOD2). Hydrogen peroxide diffuses to plasmalemmal Kv channels which it oxidizes and opens. Inset on **right**: In normoxia, the resulting hyperpolarization of the SMC membrane potential decreases the opening of large conductance, voltage-gated calcium channels (Ca_L), lowering $[Ca^{2+}]_i$ and relaxing tone. In hypoxia the pathway reverses, causing HPV. It is unknown if this pathway is conserved in fetal PASMC.

Uncoupled electron leak generates superoxide anion ($O_2^{\bullet-}$). $O_2^{\bullet-}$ is rapidly changed by mitochondrial superoxide dismutase 2 (SOD2) into H_2O_2, which serves as a diffusible, redox signaling molecule, connecting ETC function to cellular electrophysiology (Fig. 2). Mitochondrial ROS control vascular tone by regulating the gating kinetics of redox-sensitive ion channels, such as the voltage-gated potassium channel, Kv1.5, and enzymes, such as rho kinase [25,36,46-49]. In adult PA and fetal DA, inhibitors of ETC Complex I (rotenone) or Complex III (antimycin A) mimic hypoxia's opposing effects on ROS production and vascular tone [36,39,41,50-52]. Like hypoxia, inhibitors of ETC Complex I (rotenone) and -III (antimycin A) inhibit K^+ currents and cause PA constriction [39]. The parallel effects of hypoxia and ETC inhibitors on subcellular signaling (K+ channel opening in DASMC versus inhibition in PASMC) and vascular tone (DA vasodilatation versus PA vasoconstriction) suggest a role for mitochondrial *spatial heterogeneity* in

the opposing PA and DA O_2-responses. The effects of hypoxia and ETC inhibitors are mimicked by reducing agents (e.g., duroquinone). Conversely, oxidants (e.g., diamide) mimic oxygen and increase I_K while causing pulmonary vasodilatation [46].

Molecular Identification of the O_2 sensor in HPV: Identification of NDUFS2 as a mitochondrial O_2 sensor in adult PASMC

In 2019 we used a targeted proteomic strategy in mitochondria derived from adult rat PAs vs renal arteries (RAs), which dilate to hypoxia, to show that NDUFS2 is essential for acute O_2-sensing [17]. We isolated mitochondria from PA vs RA SMC and performed ETC complex pull-down by immunocapture to resolve the 45 ETC Complex I proteins. NDUFS2 expression was greater in PASMC vs RASMC. Small inhibitory RNA targeting NDUFS2 (siNDUFS2), selectively inhibited normoxic ROS production and suppressed HPV without altering responses to other vasoconstrictors or the function of RASMC. We also showed that isolated mitochondria derived from normoxic lungs were producing hydrogen peroxide during normoxia and the media surrounding these mitochondria inhibited HPV, when infused into an isolated lung bioassay model. We also found that redox-sensitive Ndufs2 cysteine residues within the lung were reduced during hypoxia and showed that both hypoxia and reducing agents caused functional inhibition of ETC-I. Thus, we concluded that NDUFS2 is an O_2-sensor in ETC Complex I of adult PASMC and regulates the downstream effector mechanism by generating ROS which are converted to hydrogen peroxide [17]. Although mitochondria were not initially believed to be oxygen sensors in the carotid body, this position has been revised. López-Barneo *et al.* in 2015 demonstrated that knockdown of a component of ETC Complex I, Ndufs2, selectively eliminated O_2 sensing in the mouse carotid body [40], much as occurs in rats in which Ndufs2 in the lung is depleted *in vivo* by siRNA, in which HPV is selectively inhibited [17].

Regional heterogeneity in the expression of O_2 sensitive K+ channels

The pulmonary circulation displays heterogeneity in the distribution of ion channels along its length. The proximal PASMC (derived from conduit arteries that display no vasoconstriction or weak vasoconstriction in response to hypoxia) are enriched in large conductance, calcium-sensitive, K^+ channels (BK_{Ca}) whereas the resistance PASMC, the site of HPV, are enriched in Kv channels. Moreover, Kv1.5 and Kv2.1, two key O_2-sensitive channels, account for much of the O_2-sensitive current in resistance-level PASMC [25]. HPV occurs in this Kv-enriched resistance PA portion of the pulmonary vasculature, in part, because resistance PASMCs preferentially express these O_2-sensitive Kv-channels [53]. This work was subsequently confirmed and extended by the group of Dr. Jason Yuan, who also noted the electrophysiologic heterogeneity of PASMC, which they showed partially relates not only to differential expression and function of Kv channels but also to regional differences in intrinsic mechanisms involved in regulating cytosolic calcium [54].

Mitochondrial diversity in pulmonary versus systemic arteries

We have studied the role of mitochondrial heterogeneity in the opposing O_2-responses in adult PA versus systemic arteries. The composition of mitochondrial ETC megacomplexes I and III differs between adult PA (which constrict in hypoxia) and adult renal arteries (RA), which relax in hypoxia) [5]. In renal artery SMC (RASMC), hypoxia increases mitochondrial ROS and Kv current, leading to RA relaxation; whilst the opposite occurs in PAs [5]. Complex I inhibitors also elicit opposing ROS responses and hemodynamic changes in PA vs RA (constriction vs relaxation) [5], illustrating that acute changes in ETC complex function can generate opposing ROS signals and vasomotor responses in adult arteries. Compared to RASMC, adult PASMC mitochondria also have lower O_2-consumption rates (OCR), a more depolarized mitochondrial membrane potential (ΔYm), and higher rates of normoxic mitochondrial ROS production. These functional differences in mitochondria are associated with lower expression of several ETC Complex I and Complex III subunits and higher expression of the H_2O_2-generating enzyme, mitochondrial SOD2 [5] in PASMC vs. RASMC. This spatial ETC heterogeneity confers upon adult PASMC a unique ability to vary ROS in proportion to PO_2, which is key to O_2-sensing. We propose that heterogeneity in ETC Complex structure and function underlies opposing O_2-sensing in adult PA vs RA [5].

Controversies in the mechanism of HPV

The role of mitochondria as oxygen-sensors is generally accepted, as is the hypothesis that ETC-derived ROS regulate the downstream ion channel effector

pathway (the gating of K^+ and Ca_L channels) that elicit vasoconstriction [38,39,55]. This fits nicely with the original Redox hypothesis of HPV published by Archer, Will, and Weir in 1986 [56]. However, disagreement persists as to whether, in adult PAs, physiologic levels of hypoxia (anoxia is not a stimulus for HPV) increase or decrease ROS/H_2O_2 levels [28,57-61]. Some groups, notably the Schumacker group at Northwestern University, Chicago, have shown that hypoxia increases mitochondrial ROS production, which they attribute to an hypoxic inhibition of the distal ETC, particularly at ETC Complex III [59,60]. They propose that inhibition of the distal ETC by hypoxia causes a net reduction of sites within the proximal ETC. The addition of electrons due to distal ETC inhibition and retrograde electron flux serves to increase electron leak from the proximal ETC and react with O_2 to form ROS. In contrast, our group, consistent with findings by the Wolin group [55,62-64], finds that PASMC ROS production increases in direct proportion to PO_2 and decreases with hypoxia. Production of ROS (including the major signalling molecule, hydrogen peroxide) falls in hypoxia due a reduced rate of electron flux (and a related decrease in electron leak) caused by decreased availability of molecular oxygen, the ETC's terminal electron acceptor [7]. It appears the hypoxic fall in ROS is associated with a reduction in oxygen consumption rate (OCR). We recently showed for example that siNDUFS2, which eliminates HPV and reduces ROS, reduces OCR in PASMC [17]. We also believe that reduced availability of O_2 as an acceptor of the leaked electrons may contribute to reduced ROS in hypoxia, although our data suggest this reflects inhibition of antegrade electron flow, rather than enhanced retrograde electron flow.

This direct relationship between PO_2 and ROS production is evident in other tissues in the HOSS, such as the ductus arteriosus (DA) in which ROS in DASMC increase with rising PO_2 at birth [52,65]. A direct relationship of ROS and PO_2 is also seen in non-HOSS tissues, like the heart. When the heart is studied in a Langendorff model and ROS are measured from the cardiac surface using enhanced chemiluminescence, ROS levels fall from a normoxic baseline during global cardiac ischemia and then increase far above baseline with reoxygenation, accounting for the reperfusion phase of myocardial ischemia-reperfusion injury [66]. This oxygen-induced rise in ROS in cardiac ischemia-reperfusion injury is largely due to mitochondrial fission mediated by dynamin related protein 1 (Drp1) in cardiac myocytes [67]. Although we find a direct relationship between PO_2 and ROS production, Lopez-Barneo's laboratory found the opposite relationship in type 1 cells of the carotid body in a study where they identified the role of Ndufs2 in response to hypoxia in these cells [40].

The basis for the discrepancies between those that find hypoxia to be an oxidized state with high ROS [59,60] versus those that report hypoxia is a reduced state with low ROS [7,61] remains unclear. However, we have identified and reported several methodological discrepancies amongst groups related to the use (or lack of use) of: (i) freshly isolated PASMC from resistance arteries and isolated perfused lungs (ii) focus on physiologic hypoxia (versus anoxia), (iii) attention to/reporting of pH and PCO_2, and (iv) performing dynamic and accurate ROS measurement in subcellular compartments [68]. The proposal that hypoxia is a chemical state of reduction (not oxidation), is supported by the observations that the opposing effects of hypoxia on PA versus systemic vascular tone (constriction versus dilation) and the opposing effects of hypoxia on cellular electrophysiology in PASMC versus systemic arterial SMC are mimicked by reducing agents (such as dithiothreitol), and not reproduced by exogenous administration of ROS and oxidants [5,50]. Any proposed mechanism of HPV should be judged by the degree to which it accounts for the physiological core properties of HPV, notably the response onset should be rapid (as HPV onsets within a few breaths of hypoxia) [13], reversible (like HPV), and should not induce edema [68]. A unifying theory for oxygen sensing should also (in our view) *"account for the opposing effects of hypoxia on tone in the pulmonary circulation (constriction) versus the ductus arteriosus and systemic vasculature (vasodilatation)"* [68].

We acknowledge that the use of ETC inhibitors like rotenone and antimycin A to study redox signaling is suboptimal as the effects of these agents may be confounded by the possibility that they inhibit mitochondrial metabolism. Therefore, Brand *et al.* developed electron leak suppressors [43], S1QEL and S3QEL. S1QEL prevents electron leak and superoxide production (both in the forward and reverse direction of electron flux) from site I_Q in ETC Complex 1 [45]. We recently used S1QEL to study oxygen sensing in the DASMC and showed that S1QEL, but not S3QEL, reversed oxygen-induced constriction, but not phenylephrine constriction, in rabbit DA rings. S1QEL did not inhibit mitochondrial metabolism or ETC Complex I activity [69]. Moreover, in human DASMC, S1QEL and rotenone inhibited oxygen-induced increases in cytosolic calcium, a surrogate for DA constriction [69].

In future studies of oxygen sensing the use of these electron leak suppressors should be considered.

Oxygen sensing in the ductus arteriosus and fetal PA

The DA is a vital fetal vessel that shunts placentally oxygenated blood from the PA to the aorta, bypassing the unventilated fetal lung. The simultaneous, opposing, O_2-responses of the fetal PA and DA at birth achieve two goals required for transition from placentally oxygenated fetus to air-breathing neonate: 1) perfusion of newly ventilated lungs and 2) elimination of right to left shunting. This *miracle of birth* occurs flawlessly in term infants but often fails in prematurity, promoting congenital heart diseases, such as patent ductus arteriosus and persistent pulmonary hypertension of the newborn (PPHN). The PA and DA have different embryologic origins, mesodermal lung buds vs left 6th branchial arch, respectively, but we do not fully understand the molecular basis for their opposing O_2-responses. Although functional DA closure is modulated by the endothelium (reinforced by withdrawal of vasodilatory prostaglandin E[70] and increases in the vasoconstrictor, endothelin-1 [71]), human DAs constrict to O_2 in the absence of endothelium [72] and despite blockade of the endothelin pathway [48]. The core of the DA's O_2-sensor mechanism resides in DASMC [48,72].

In unpublished, preliminary data we examined the opposing effects of oxygen on intracellular calcium (a surrogate for vascular tone) in fetal DA vs PA SMC harvested from term rabbits measured using confocal imaging and the calcium indicator, Cal-520AM. These DASMC and PASMC were isolated from the same rabbit kits and grown under identical hypoxic conditions, (PO_2~40 mmHg), to mimic the fetal oxygen condition) (Fig. 3). DASMC respond to normoxia with a rise in $[Ca^{2+}]_i$, which triggers contraction, whilst PASMC have a fall in $[Ca^{2+}]_i$, favouring relaxation (Fig. 3). It is noteworthy, although only a preliminary finding, that elimination of NDUFS2 (achieved using siRNA) inhibits the effects of oxygen in fetal DASMC without altering the effects on fetal PASMC, perhaps indicating different mitochondrial sensors within these two types of SMC (Fig. 3).

This opposing response to oxygen is nicely imaged in a preliminary microCT study of fetal rabbits, which demonstrates that within 15 minutes of birth (and breathing oxygen) the DA is functionally occluded by vasoconstriction while the adjacent pulmonary circulation is vasodilated (Fig. 4). We speculate that this spatial heterogeneity in O_2-responses in adjacent fetal arteries, which is intrinsic to their respective SMCs, reflects mitochondrial heterogeneity. The term *"mitochondrial spatial heterogeneity"* refers to differences in quantity and/or structure of mitochondrial proteins between fetal PA and DA. The fact that ETC inhibitors and redox agents mimic hypoxia and exert opposing effects on both ROS production and vascular tone in the DA and PA, further supports the contention that mitochondrial spatial heterogeneity underlies opposing PA vs DA responses.

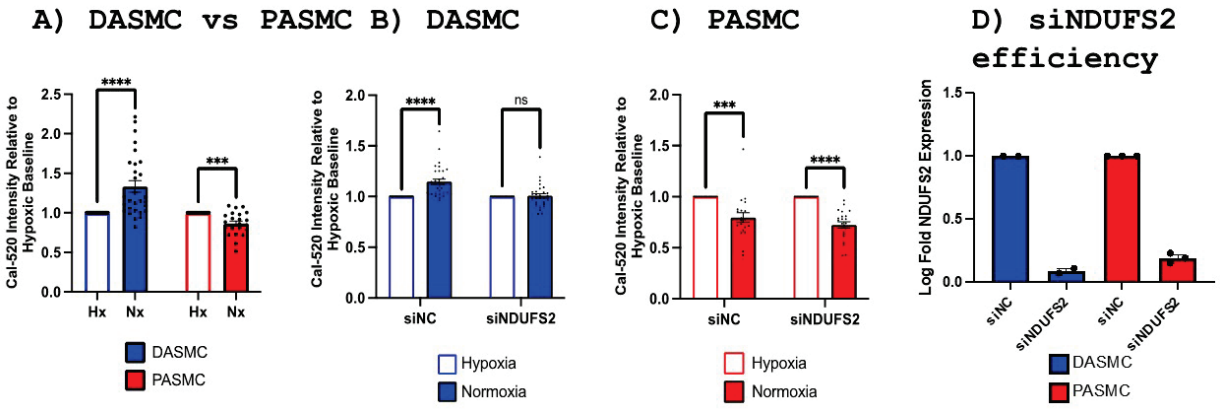

Fig. 3. Oxygen increases $[Ca^{2+}]_i$ DASMC and lowers $[Ca^{2+}]_i$ in PASMC in term, fetal rabbits. **A)** Oxygen increases cytosolic calcium in fetal rabbit DASMC and reduces cytosolic calcium in fetal rabbit PASMC, isolated from the same kit. **B)** Oxygen-induced increases in cytosolic calcium in fetal DASMC are inhibited by small inhibitory RNA (siRNA) targeting NDUFS2. **C)** Oxygen-induced reductions in cytosolic calcium in fetal PASMC are not affected by siNDUFS2. **D)** siNDUFS2 was equally effective in reducing NDUFS2 mRNA expression in both cell types.
**, *** $p<0.01$ and <0.001, respectively. These preliminary data support a role for NDUFS2 as a DA O_2-sensor but also suggests that fetal PASMC may have an alternate sensor for normoxia-induced PA vasodilation. The two blue bars in panel B do not significantly differ from each other statistically; further supporting the interpretation of this preliminary study as showing siNDUFS2 does not alter the oxygen induced fall in PASMC calcium. n=1 DASMC and 1 PASMC cell culture from 1 rabbit kit; data points are individual cells.

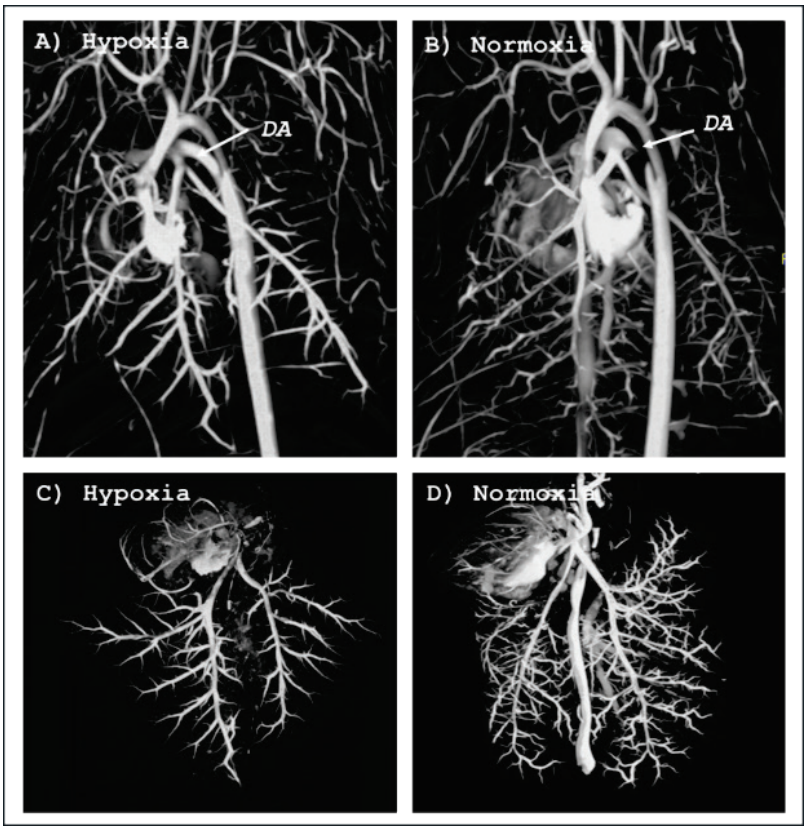

Fig. 4. Contrast-enhanced microcomputed tomography (micro-CT) demonstrates the opposing effects of oxygen on the DA *vs* the pulmonary circulation in newborn, term rabbits. **A-B** *In situ* maximum intensity projection (MIP) micro-CT images taken after perfusion of fetal rabbit kits with an intravascular contrast agent (a mixture of 25 % barium sulfate and 3 % gelatin), via injection of the agent into the beating left ventricle. In panel **A**, acquired during hypoxia, the DA is widely patent (connecting the PA to the aorta). The fetal DA is <0.5 mm in diameter. In panel **B,** acquired within 20 minutes of breathing 100 % O_2, the DA is constricted and functionally closed. **C-D** *Ex vivo* MIP micro-CT images after dissection of heart and lungs from the same (**C**) hypoxic and (**D**) normoxic rabbit kits, indicating the perfusion of the newly ventilated lung vasculature and pulmonary circulation. Note the vasodi-latation of the pulmonary circulation in normoxia. Micro-CT scans were conducted using VECTor^{4}CT (MILabs B.V., Utrecht, Netherlands) preclinical tri-modality ima-ging platform.

In contrast, downstream portions of the O_2-sensing pathways are similar in PA and DA. For example, Kv channel inhibitors, like 4-aminopyridine, cause concordant vasoconstriction in both PA and DA [5,36], whilst Ca_L inhibitors cause concordant vasodilatation [73,74]. Moreover, PA [25] and DA [36] utilize similar ion channels for O_2-responses, including Kv1.5 and Kv2.1.

HPV in lung diseases

Pneumonia, atelectasis HPV optimizes systemic PO_2 in people with atelectasis, pneumonia, COPD, and asthma by optimizing V/Q mismatch and reducing intrapulmonary shunting (Table 1, reproduced with modification from [68]). In these conditions, HPV decreases perfusion of the hypoxic lung segments, shunting blood to better oxygenated lung segments. HPV rapidly reverses upon resolution of pneumonia or once atelectasis resolves (as occurs upon removal of a mucous plug or with lung expansion by incentive spirometry). The intensity of HPV varies between individuals and is reduced in certain diseases. HPV can also be reduced or augmented by medications (Table 1). Calcium channel blockers, like nifedipine or verapamil, reduce HPV [75]. This can result in hypoxemia due to increased intrapulmonary shunting. For example, patients with WHO Group 3 pulmonary hypertension due to COPD, when given nifedipine (20 mg) suffered a fall in arterial PO_2 (52 to 47 mmHg) related to impaired V/Q matching [75]. In contrast, inhaled NO, though a potent pulmonary vasodilator, does not reduce, and may even enhance V/Q matching, because this gas only enters well-ventilated lung segments (where HPV is not active). In contrast, intravenous vasodilators usually decrease V/Q matching by relaxing PAs that perfuse poorly ventilated lung segments [76]. In an *ex vivo* perfused, rabbit lung model, Walmrath *et al.* showed that while intravenous PGI_2 reduced mean PA pressure, it also increased shunt fraction (to ~60 %). In this same study, inhaled NO caused a similar beneficial decrease in mPAP but did not impair V/Q matching (i.e., the shunt fraction remained ~25 %) [76].

Impaired HPV in COVID-19 pneumonia

Our group showed that infection with a murine coronavirus, MHV-1, cause a murine pneumonia which mimics SARS-CoV-2 pneumonia in patients [3]. The suppression of HPV and systemic hypoxemia in this mouse model can be partially reversed by administration of the Ca_L opener, BAYK8644 (Fig. 5). HPV is also suppressed in patients with COVID-19 pneumonia [77].

Table 1. The role of HPV in respiratory diseases

Disease	Role of HPV	Treatment	References
High altitude pulmonary edema (HAPE)	Exaggerated HPV; over perfusion of non-constricted pulmonary vasculature	The calcium channel blocker nifedipine reduces the incidence of HAPE from 63 to 10 %	[78,79,92,93]
Asthma	HPV maintains V/Q balance during acute bronchoconstriction	Intravenous isoprenaline decreases mPAP in an isolated perfused rat lung model of asthma (by 3-5 mmHg)	[92,94-96]
Chronic obstructive pulmonary disease (COPD)	HPV is reduced, however the residual HPV response improves V/Q balance and systemic oxygenation	In COPD patients Almitrine (100mg/day), a drug that enhances HPV, increases PaO_2 from 52 to 59 mmHg. In COPD patient with pulmonary hypertension and respiratory failure prostacyclin IV is a nonselective vasodilator that causes mild hypoxemia.	[92,97-99] [100]
Pneumonia	Diversion of blood flow away from the diseased lobe(s) of lung optimizes systemic PO_2	Pneumonia impairs HPV in experimental models. In COPD patients with hypoxic exacerbations, intravenous prostaglandins increase systemic hypoxemia, consistent with the adverse effects of suppressing HPV	[92,97,100-102]
Acute Lung Injury (ALI)/Acute Respiratory Distress Syndrome (ARDS)	HPV is reduced in ALI however the residual HPV improves V/Q balance and reduces shunting	In an ovine model of ARDS inhibition of inducible NOS restores HPV, increases PaO_2/FiO_2, and decreases shunt fraction	[92,97,103,104]
COVID-19 pneumonia	HPV is suppressed in an MHV-1 murine model of COVID-19 pneumonia HPV is also suppressed in patients with COVID-19 pneumonia [77]	BAYK8644 (an opener of Ca_L channels) enhances HPV	[3]

Modified and reproduced from [68]

We showed that the SARS-CoV-2 virus (and its proteins) directly attack the mitochondria in human and rodent PASMC, leading to impaired oxygen sensing and reduced HPV [3]. Indeed, this effect is seen with other coronaviruses too, including HCoV-OC43 and MHV-1. SARS-CoV-2 infections downregulate expression of ETC Complex I and ATP synthase genes and upregulate apoptosis-inducing genes. In additional to the adverse transcriptional effects of coronaviruses on mitochondria we demonstrated direct protein-protein interactions between viral proteins and key components of the mitochondrial ETC that are relevant to oxygen sensing. For example, in normal human PASMC, lentiviral transduction with SARS-CoV-2's M or Nsp9 proteins inhibits HPV [3]. This is SARS-CoV-2 mitochondriopathy is directly relevant to HPV in vivo. For example, in a murine model of COVID-19 pneumonia, created by infection of mice with the murine coronavirus MHV-1, HPV is suppressed and a ventilation-perfusion mismatch lowers systemic oxygenation, much as occurs in patients with COVID-19 pneumonia [77]. BAY K8644, a calcium channel agonist, increased HPV and improved systemic oxygenation in this murine COVID pneumonia model. This SARS-CoV-2 mitochondriopathy also causes mitochondrial apoptosis in airway epithelial cells, contributing to diffuse alveolar damage in COVID-19 pneumonia (illustrated in Fig. 6) [3].

Excessive HPV in High Altitude Pulmonary Edema (HAPE)

While HPV is largely beneficial, excessive and spatially heterogenous HPV can be harmful, as is the case

in HAPE [78,79] (Table 1). Rapid ascent to high altitude without acclimation triggers a non-cardiogenic pulmonary edema that manifests as cough, dyspnea, and reduced exercise performance, within 2-5 days of ascent to altitudes exceeding 2500 m [80]. In people with HAPE, excessive HPV stresses and distends the more proximal arterial walls ultimately rupturing the basement membrane and disrupting the alveolar-capillary barrier [81]. Hypoxia also inhibits alveolar fluid clearance within the lung by inhibiting sodium exchangers, thereby decreasing sodium transport and reducing the lung's ability to reabsorb fluid [81]. Evidence that excessive HPV promotes HAPE comes from a study of simulated rapid ascent to altitude. Dehnert et al found that individuals with the strongest HPV response are most at risk of HAPE. They exposed 421 healthy Caucasians to hypoxia (simulating their elevation to 4500 m over 24 hours). 13 % of subjects with exaggerated HPV (systolic PA pressure in hypoxia 51±6 mmHg on echo Doppler, n=4/39) developed HAPE within 48 hours. Subjects with milder HPV (systolic PA pressure at altitude of 33±5 mmHg) did not develop HAPE [82]. Likewise, in a rabbit model of ascent to altitude, suppression of HPV (using acetazolamide) reduced HAPE [83]. HAPE is also reversible with supplemental oxygen and rapid descent from altitude [84] and in the short term can be treated or prevented by inhibitors of HPV, such as nifedipine or sildenafil, while descent is in progress [85].

Pulmonary hypertension

There is an intersection between pulmonary arterial hypertension (WHO Group 1 PH) and HPV. In some regards, the pulmonary circulation in people or animals with Group 1 PH behaves as if there were a failure of oxygen sensing, meaning that despite normal FiO_2 pathways are activated as if the lung were hypoxic. We refer to this normoxic elevation of pulmonary vascular tone, with downregulation of oxygen-sensitive Kv channels and normoxic activation of HIF-1α, as a state of *pseudohypoxia*, reviewed in [86]. A human condition exemplifying the role of dysregulated oxygen sensing in pulmonary hypertension was brought to our attention by our late colleague, Dr John Newman, a pioneering expert in high altitude pulmonary

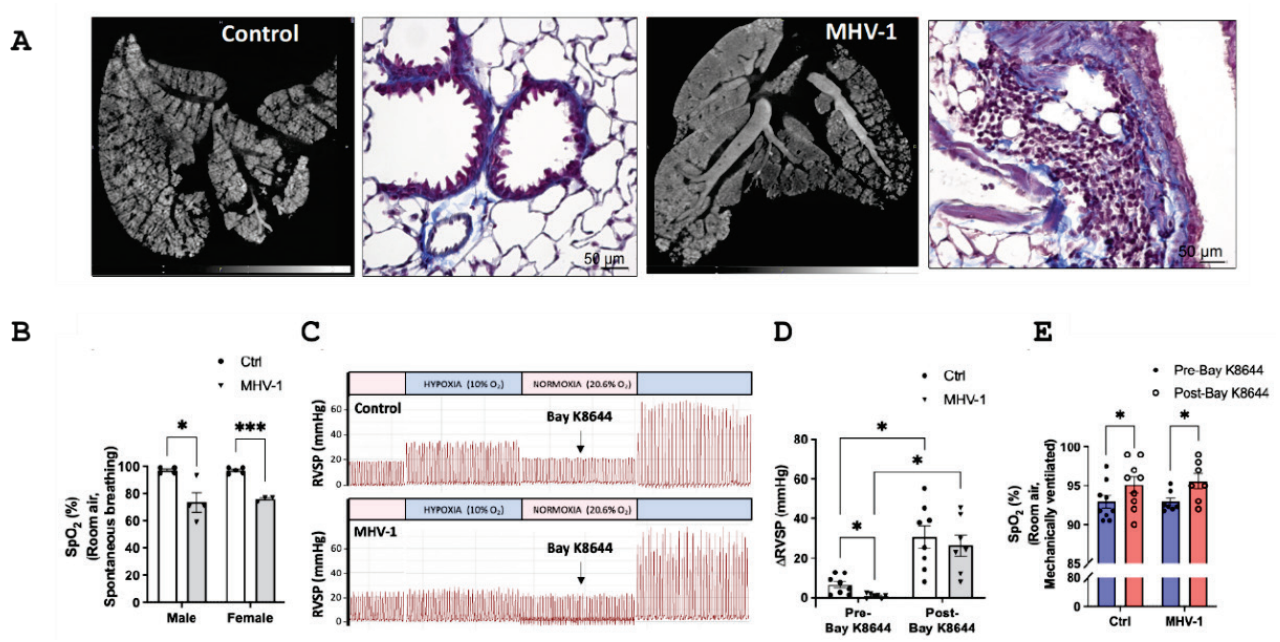

Fig. 5. Suppression of hypoxic pulmonary vasoconstriction in a murine coronavirus model of COVID-19 pneumonia. Loss of HPV may contribute to hypoxemia in COVID-19 pneumonia. Here the effects of coronavirus infection on HPV are studied in a murine model created by MHV-1 infection at days 4–6. (n=4 for male Ctrl, n=4 for male MHV-1, n=5 for female Ctrl, n=3 for female MHV-1). Micro-CT images acquired using a VECTor^{4}CT scanner. The CT scans show lung consolidation in MHV-1 infected mice. The histology insets (on right) show inflammatory infiltrates in the MHV-1 infected lungs. Reduction in oxygen saturation occurs in both male and female mice with MHV-1 pneumonia. **C)** Representative RV pressure traces and mean data show that HPV is suppressed in MHV-1 mice. There was no significant increase in RV systolic pressure with hypoxia in MHV-1 infected mice, in contrast to the robust rise in RVSP (HPV) in uninfected mice. **D)** Intraperitoneal treatment with the CaL agonist BAYK8644 significantly augmented HPV (defined as the increase in RVSP in response to hypoxia) in both control and infected mice. (n=8 for Ctrl, n=7 for MHV-1). **E)** Bay K8644 (1mg/kg IP) increases O_2 saturation. (n=9 for Ctrl, n=7 for MHV-1). The higher O_2 saturations in this panel (vs panel **B**) reflect the fact these mice were being mechanically ventilated with room air (versus spontaneous breathing of room air in panel **A**). **P*<0.05, ***P*<0.01, ****P*<0.001. Reproduced with permission from [3].

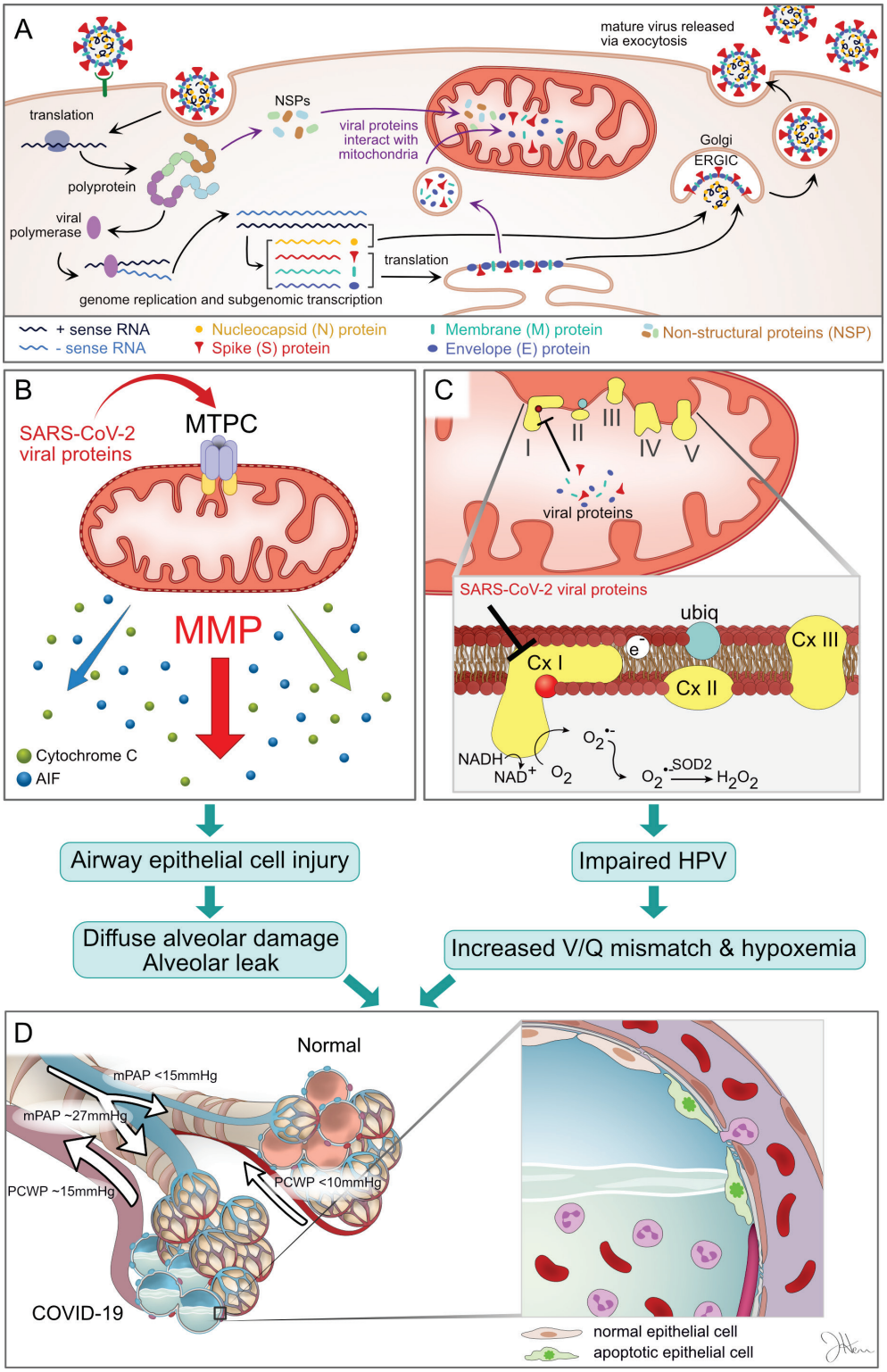

Fig. 6. Proposed mechanism by which SARS-CoV-2 mitochondriopathy contributes to hypoxemia in COVID-19 pneumonia. SARS-CoV-2 mitochondriopathy causes diffuse alveolar damage (DAD) and hypoxemia in part by inducing apoptosis (left) in airway epithelial cells and in part by suppressing HPV in PASMC (right)**. A)** Viral replication floods the cell with viral proteins. **B)** In airway epithelial cells some viral proteins target the mitochondria transition pore complex (MTPC) proteins which permeabilizes the membrane (MMP) and leads to leak of apoptosis mediators, like AIF and cytochrome *c*, resulting in apoptosis. **C)** In PASMC viral proteins interact with components of ETC Complex I, the site of O_2-sensing, and impair HPV. **D)** The consequences of this mitochondriopathy include impaired HPV, which inappropriately floods capillaries in infected segments with blood, exacerbating capillary leak by promoting V/Q mismatch. Excessive apoptosis damages the alveoli and contributes to DAD. The loss of HPV combined with AEC apoptosis exacerbate systemic hypoxemia in coronavirus pneumonia syndromes, including COVID-19 pneumonia. In addition, there is profound mitochondrial fission and bioenergetic impairment which contribute to the lung injury (not shown in this figure). Reproduced with permission from [3].

hypertension and respirologist who worked at Vanderbilt University [87]. After hearing of the impaired oxygen sensing and spontaneous pulmonary hypertension in fawn-hooded rats (FHR), which relates to their normoxic activation of HIF-1α [88], Dr. Newman acquainted our group with Chuvash disease. People with Chuvash disease, named for a mid-Volga River region in Russia, have enhanced HPV, polycythemia and pulmonary hypertension, despite normal inspired oxygen levels [89]. Chuvash disease results from a missense mutation within the von Hippel-Lindau (VHL) gene which impairs VHL's ability to interact with the α-subunits of hypoxia inducible factors (HIF-1α and HIF-2α). Normally, HIF is hydroxylated by prolyl hydroxylases, marking these proteins for ubiquit-ination by VHL, which ultimately leads to their proteasomal degradation. The Chuvash VHL mutation impairs this degradation pathway resulting in normoxic HIF stabilization and paradoxical normoxic transcription of HIF-regulated genes during normoxia, including erythropoietin [89-91]. Thus, patients with Chuvash PH are afflicted by a failure of oxygen sensing and aspects of this are seen in in patinets with WHO Group 1 PH.

FHR, like Chuvash patients, develop mild polycythemia with age and manifest normoxic activation of HIF-1α. However, in FHR the syndrome does not relate to a VHL mutation; rather the state of pseudohypoxia reflects epigenetic silencing of mitochondrial SOD2 by DNA methyltransferase [10]. Interestingly in FHR, as in residents of high altitude, the signal transduction of acute HPV is downregulated, meaning that despite adverse pulmonary vascular remodeling and pulmonary hypertension the acute response to hypoxia (the magnitude of HPV) is depressed in FHR, compared to control rats.

It is a pleasure to contribute this review of the mechanisms of oxygen sensing to an issue of the journal published in honor of Dr. Jan Herget. Jan had an intense interest in the effects of hypoxia on the pulmonary vasculature, both acute and chronic. Over 30 years, between 1985 and 2015, he was an author on 21 papers dealing with the pathophysiologic mechanisms involved, in both perinatal and adult animals. In most he was the first or senior author. The mechanisms he studied involved the study of potassium channels, reactive oxygen species and nitric oxide. In addition to his research, Jan organized a series of international pulmonary hypertension meetings in Prague. These took place both before and after the fall of the Berlin wall and were the premiere venue in which to present and discuss pulmonary hypertension research. He was always amazingly hospitable to clinicians and scientists from all over the world.

Conclusion

The homeostatic oxygen sensing system plays a vital role in adapting humans, and most mammalian species, to reduced oxygen availability (hypoxia) which they experience in utero, at altitude and during disease. The oxygen sensing mechanisms found in PASMC, DASMC and type 1 cells of the carotid body are largely based in the mitochondrial electron transport chain and are mediated by ETC subunits, including NDUFS2. Changes in PO_2 rapidly alter production of mitochondrial reactive oxygen species, including hydrogen peroxide, which in turn regulate the opening of redox-sensitive potassium channels. The K+ channels regulate membrane potential and thereby control calcium influx through voltage-gated calcium channels. In PASMC hypoxia induces a fall in ROS production which inhibits Kv1.5 and other Kv channels, thereby activating Ca_L channels and initiating vasoconstriction. There is a diversity in the types of mitochondria found in the PASMC versus systemic arterial SMC such that PASMC respond uniquely to hypoxia. Likewise, there is regional heterogeneity in the expression and activity of oxygen-sensitive K+ channels along the length of the pulmonary circulation, such that they are enriched in small resistance PAs, the site of HPV. HPV remains as fascinating a subject of study in 2024 as it was in 1894.

Conflict of Interest

There is no conflict of interest.

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References

1. Bradford JR, Dean HP. The Pulmonary Circulation. J Physiol 1894;16:34-158 25. https://doi.org/10.1113/jphysiol.1894.sp000493
2. Euler USv, Liljestrand G. Observations on the Pulmonary Arterial Blood Pressure in the Cat. Acta Physiologica Scandinavica 1946;12:301-20. https://doi.org/10.1111/j.1748-1716.1946.tb00389.x
3. Archer SL, Dasgupta A, Chen KH, Wu D, Baid K, Mamatis JE, Gonzalez V *et al.* SARS-CoV-2 mitochondriopathy in COVID-19 pneumonia exacerbates hypoxemia. Redox Biol 2022;58:102508. https://doi.org/10.1016/j.redox.2022.102508
4. Madden JA, Vadula MS, Kurup VP. Effects of hypoxia and other vasoactive agents on pulmonary and cerebral artery smooth muscle cells. Am J Physiol 1992;263:L384-93. https://doi.org/10.1152/ajplung.1992.263.3.L384
5. Michelakis ED, Hampl V, Nsair A, Wu X, Harry G, Haromy A, Gurtu R *et al.* Diversity in mitochondrial function explains differences in vascular oxygen sensing. Circ Res 2002;90:1307-15. https://doi.org/10.1161/01.RES.0000024689.07590.C2
6. Yuan XJ, Tod ML, Rubin LJ, Blaustein MP. Contrasting effects of hypoxia on tension in rat pulmonary and mesenteric arteries. Am J Physiol 1990;259:H281-9. https://doi.org/10.1152/ajpheart.1990.259.2.H281
7. Weir EK, Lopez-Barneo J, Buckler KJ, Archer SL. Acute oxygen-sensing mechanisms. N Engl J Med 2005;353:2042-55. https://doi.org/10.1056/NEJMra050002
8. Semenza GL. A compendium of proteins that interact with HIF-1αlpha. Exp Cell Res 2017;356:128-35. https://doi.org/10.1016/j.yexcr.2017.03.041
9. Shimoda LA, Manalo DJ, Sham JS, Semenza GL, Sylvester JT. Partial HIF-1αlpha deficiency impairs pulmonary arterial myocyte electrophysiological responses to hypoxia. Am J Physiol Lung Cell Mol Physiol 2001;281:L202-8. https://doi.org/10.1152/ajplung.2001.281.1.L202
10. Archer SL, Marsboom G, Kim GH, Zhang HJ, Toth PT, Svensson EC, Dyck JR *et al.* Epigenetic attenuation of mitochondrial superoxide dismutase 2 in pulmonary arterial hypertension: a basis for excessive cell proliferation and a new therapeutic target. Circulation 2010;121:2661-71. https://doi.org/10.1161/CIRCULATIONAHA.109.916098
11. Marsboom G, Toth PT, Ryan JJ, Hong Z, Wu X, Fang YH, Thenappan T *et al.* Dynamin-related protein 1-mediated mitochondrial mitotic fission permits hyperproliferation of vascular smooth muscle cells and offers a novel therapeutic target in pulmonary hypertension. Circ Res 2012;110:1484-97. https://doi.org/10.1161/CIRCRESAHA.111.263848
12. Baraka AS, Taha SK, Yaacoub CI. Alarming hypoxemia during one-lung ventilation in a patient with respiratory bronchiolitis-associated interstitial lung disease. Can J Anaesth 2003;50:411-4. https://doi.org/10.1007/BF03021041
13. Jensen KS, Micco AJ, Czartolomna J, Latham L, Voelkel NF. Rapid onset of hypoxic vasoconstriction in isolated lungs. J Appl Physiol (1985) 1992;72:2018-23. https://doi.org/10.1152/jappl.1992.72.5.2018
14. Grant JL, Naylor RW, Crandell WB. Bronchial adenoma resection with relief of hypoxic pulmonary vasoconstriction. Chest 1980;77:446-9. https://doi.org/10.1378/chest.77.3.446
15. McMurtry IF, Davidson AB, Reeves JT, Grover RF. Inhibition of hypoxic pulmonary vasoconstriction by calcium antagonists in isolated rat lungs. Circ Res 1976;38:99-104. https://doi.org/10.1161/01.RES.38.2.99
16. Aaronson PI, Robertson TP, Knock GA, Becker S, Lewis TH, Snetkov V, Ward JP. Hypoxic pulmonary vasoconstriction: mechanisms and controversies. J Physiol 2006;570:53-8. https://doi.org/10.1113/jphysiol.2005.098855
17. Dunham-Snary KJ, Wu D, Potus F, Sykes EA, Mewburn JD, Charles RL, Eaton P *et al.* Ndufs2, a core subunit of mitochondrial complex i, is essential for acute oxygen-sensing and hypoxic pulmonary vasoconstriction. Circ Res 2019;124:1727-1746. https://doi.org/10.1161/CIRCRESAHA.118.314284
18. Archer SL, Tolins JP, Raij L, Weir EK. Hypoxic pulmonary vasoconstriction is enhanced by inhibition of the synthesis of an endothelium derived relaxing factor. Biochem Biophys Res Commun 1989;164:1198-1205. https://doi.org/10.1016/0006-291X(89)91796-8

19. Hampl V, Archer SL, Nelson DP, Weir EK. Chronic EDRF inhibition and hypoxia: effects on pulmonary circulation and systemic blood pressure. J Appl Physiol (1985) 1993;75:1748-1757. https://doi.org/10.1152/jappl.1993.75.4.1748
20. Leeman M, de Beyl VZ, Biarent D, Maggiorini M, Melot C, Naeije R. Inhibition of cyclooxygenase and nitric oxide synthase in hypoxic vasoconstriction and oleic acid-induced lung injury. Am J Respir Crit Care Med 1999;159:1383-90. https://doi.org/10.1164/ajrccm.159.5.9807114
21. Post JM, Hume JR, Archer SL, Weir EK. Direct role for potassium channel inhibition in hypoxic pulmonary vasoconstriction. Am J Physiol 1992;262:C882-C890. https://doi.org/10.1152/ajpcell.1992.262.4.C882
22. Weir EK, Archer SL. The mechanism of acute hypoxic pulmonary vasoconstriction: the tale of two channels. FASEB J 1995;9:183-189. https://doi.org/10.1096/fasebj.9.2.7781921
23. Hasunuma K, Rodman DM, McMurtry IF. Effects of K+ channel blockers on vascular tone in the perfused rat lung. Am Rev Respir Dis 1991;144:884-887. https://doi.org/10.1164/ajrccm/144.4.884
24. McMurtry IF. BAY K 8644 potentiates and A23187 inhibits hypoxic vasoconstriction in rat lungs. Am J Physiol 1985;249:H741-H746. https://doi.org/10.1152/ajpheart.1985.249.4.H741
25. Archer SL, Souil E, Dinh-Xuan AT, Schremmer B, Mercier JC, El Yaagoubi A, Nguyen-Huu L *et al.* Molecular identification of the role of voltage-gated K+ channels, Kv1.5 and Kv2.1, in hypoxic pulmonary vasoconstriction and control of resting membrane potential in rat pulmonary artery myocytes. J Clin Invest 1998;101:2319-2330. https://doi.org/10.1172/JCI333
26. Firth AL, Remillard CV, Platoshyn O, Fantozzi I, Ko EA, Yuan JX. Functional ion channels in human pulmonary artery smooth muscle cells: Voltage-dependent cation channels. Pulm Circ 2011;1:48-71. https://doi.org/10.4103/2045-8932.78103
27. Archer SL, London B, Hampl V, Wu X, Nsair A, Puttagunta L, Hashimoto K *et al.* Impairment of hypoxic pulmonary vasoconstriction in mice lacking the voltage-gated potassium channel Kv1.5. FASEB J 2001;15:1801-1803. https://doi.org/10.1096/fj.00-0649fje
28. Ward JP. Point: Hypoxic pulmonary vasoconstriction is mediated by increased production of reactive oxygen species. J Appl Physiol (1985) 2006;101:993-5; discussion 9. https://doi.org/10.1152/japplphysiol.00480.2006
29. Duprat F, Guillemare E, Romey G, Fink M, Lesage F, Lazdunski M, Honore E. Susceptibility of cloned K+ channels to reactive oxygen species. Proc Natl Acad Sci U S A 1995;92:11796-11800. https://doi.org/10.1073/pnas.92.25.11796
30. Gardener MJ, Johnson IT, Burnham MP, Edwards G, Heagerty AM, Weston AH. Functional evidence of a role for two-pore domain potassium channels in rat mesenteric and pulmonary arteries. Br J Pharmacol 2004;142:192-202. https://doi.org/10.1038/sj.bjp.0705691
31. Gurney AM, Osipenko ON, MacMillan D, McFarlane KM, Tate RJ, Kempsill FE. Two-pore domain K channel, TASK-1, in pulmonary artery smooth muscle cells. Circ Res 2003;93:957-964. https://doi.org/10.1161/01.RES.0000099883.68414.61
32. Murtaza G, Mermer P, Goldenberg A, Pfeil U, Paddenberg R, Weissmann N, Lochnit G *et al.* TASK-1 potassium channel is not critically involved in mediating hypoxic pulmonary vasoconstriction of murine intra-pulmonary arteries. PLoS One 2017;12:e0174071. https://doi.org/10.1371/journal.pone.0174071
33. Lambert M, Capuano V, Boet A, Tesson L, Bertero T, Nakhleh MK, Remy S *et al.* Characterization of Kcnk3-Mutated Rat, a Novel Model of Pulmonary Hypertension. Circ Res 2019;125:678-695. https://doi.org/10.1161/CIRCRESAHA.119.314793
34. Ma L, Roman-Campos D, Austin ED, Eyries M, Sampson KS, Soubrier F, Germain M *et al.* A novel channelopathy in pulmonary arterial hypertension. N Engl J Med 2013;369:351-361. https://doi.org/10.1056/NEJMoa1211097
35. Hong Z, Kutty S, Toth PT, Marsboom G, Hammel JM, Chamberlain C, Ryan JJ *et al.* Role of dynamin-related protein 1 (Drp1)-mediated mitochondrial fission in oxygen sensing and constriction of the ductus arteriosus. Circ Res 2013;112:802-815. https://doi.org/10.1161/CIRCRESAHA.111.300285
36. Michelakis ED, Rebeyka I, Wu X, Nsair A, Thebaud B, Hashimoto K, Dyck JR *et al.* O2 sensing in the human ductus arteriosus: regulation of voltage-gated K+ channels in smooth muscle cells by a mitochondrial redox sensor. Circ Res 2002;91:478-486. https://doi.org/10.1161/01.RES.0000035057.63303.D1

37. Rounds S, McMurtry IF. Inhibitors of oxidative ATP production cause transient vasoconstriction and block subsequent pressor responses in rat lungs. Circ Res 1981;48:393-400. https://doi.org/10.1161/01.RES.48.3.393
38. Archer SL, Nelson DP, Weir EK. Simultaneous measurement of O_2 radicals and pulmonary vascular reactivity in rat lung. J Appl Physiol (1985) 1989;67:1903-1911. https://doi.org/10.1152/jappl.1989.67.5.1903
39. Archer SL, Huang J, Henry T, Peterson D, Weir EK. A redox-based O2 sensor in rat pulmonary vasculature. Circ Res 1993;73:1100-1112. https://doi.org/10.1161/01.RES.73.6.1100
40. Fernandez-Aguera MC, Gao L, Gonzalez-Rodriguez P, Pintado CO, Arias-Mayenco I, Garcia-Flores P, Garcia-Perganeda A *et al.* Oxygen sensing by arterial chemoreceptors depends on mitochondrial complex I signaling. Cell Metab 2015;22:825-837. https://doi.org/10.1016/j.cmet.2015.09.004
41. Archer SL, Nelson DP, Weir EK. Detection of activated O_2 species in vitro and in rat lungs by chemiluminescence. J Appl Physiol (1985) 1989;67:1912-1921. https://doi.org/10.1152/jappl.1989.67.5.1912
42. Jastroch M, Divakaruni AS, Mookerjee S, Treberg JR, Brand MD. Mitochondrial proton and electron leaks. Essays Biochem 2010;47:53-67. https://doi.org/10.1042/bse0470053
43. Brand MD, Goncalves RL, Orr AL, Vargas L, Gerencser AA, Borch Jensen M, Wang YT *et al.* Suppressors of Superoxide-H(2)O(2) Production at Site I(Q) of Mitochondrial Complex I Protect against Stem Cell Hyperplasia and Ischemia-Reperfusion Injury. Cell Metab 2016;24:582-592. https://doi.org/10.1016/j.cmet.2016.08.012
44. Fang J, Wong HS, Brand MD. Production of superoxide and hydrogen peroxide in the mitochondrial matrix is dominated by site IQ of complex I in diverse cell lines. Redox Biol 2020;37:101722. https://doi.org/10.1016/j.redox.2020.101722
45. Gibbs ET, Lerner CA, Watson MA, Wong HS, Gerencser AA, Brand MD. Site IQ in mitochondrial complex I generates S1QEL-sensitive superoxide/hydrogen peroxide in both the reverse and forward reactions. Biochem J 2023;480:363-384. https://doi.org/10.1042/BCJ20220611
46. Reeve HL, Weir EK, Nelson DP, Peterson DA, Archer SL. Opposing effects of oxidants and antioxidants on K+ channel activity and tone in rat vascular tissue. Exp Physiol 1995;80:825-834. https://doi.org/10.1113/expphysiol.1995.sp003890
47. Archer SL, Huang JM, Reeve HL, Hampl V, Tolarova S, Michelakis E, Weir EK. Differential distribution of electrophysiologically distinct myocytes in conduit and resistance arteries determines their response to nitric oxide and hypoxia. Circ Res 1996;78:431-442. https://doi.org/10.1161/01.RES.78.3.431
48. Michelakis E, Rebeyka I, Bateson J, Olley P, Puttagunta L, Archer S. Voltage-gated potassium channels in human ductus arteriosus. Lancet 2000;356:134-137. https://doi.org/10.1016/S0140-6736(00)02452-1
49. Thebaud B, Michelakis ED, Wu XC, Moudgil R, Kuzyk M, Dyck JR, Harry G *et al.* Oxygen-sensitive Kv channel gene transfer confers oxygen responsiveness to preterm rabbit and remodeled human ductus arteriosus: implications for infants with patent ductus arteriosus. Circulation 2004;110:1372-1379. https://doi.org/10.1161/01.CIR.0000141292.28616.65
50. Reeve HL, Michelakis E, Nelson DP, Weir EK, Archer SL. Alterations in a redox oxygen sensing mechanism in chronic hypoxia. J Appl Physiol (1985) 2001;90:2249-2256. https://doi.org/10.1152/jappl.2001.90.6.2249
51. Reeve HL, Tolarova S, Nelson DP, Archer S, Weir EK. Redox control of oxygen sensing in the rabbit ductus arteriosus. J Physiol 2001;533:253-61. https://doi.org/10.1111/j.1469-7793.2001.0253b.x
52. Archer SL, Wu XC, Thebaud B, Moudgil R, Hashimoto K, Michelakis ED. O2 sensing in the human ductus arteriosus: redox-sensitive K+ channels are regulated by mitochondria-derived hydrogen peroxide. Biol Chem 2004;385:205-216. https://doi.org/10.1515/BC.2004.014
53. Archer SL, Wu XC, Thebaud B, Nsair A, Bonnet S, Tyrrell B, McMurtry MS *et al.* Preferential expression and function of voltage-gated, O2-sensitive K+ channels in resistance pulmonary arteries explains regional heterogeneity in hypoxic pulmonary vasoconstriction: ionic diversity in smooth muscle cells. Circ Res 2004;95:308-318. https://doi.org/10.1161/01.RES.0000137173.42723.fb
54. Platoshyn O, Yu Y, Ko EA, Remillard CV, Yuan JX. Heterogeneity of hypoxia-mediated decrease in I(K(V)) and increase in $[Ca^{2+}]$(cyt) in pulmonary artery smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 2007;293:L402-416. https://doi.org/10.1152/ajplung.00391.2006

55. Paky A, Michael JR, Burke-Wolin TM, Wolin MS, Gurtner GH. Endogenous production of superoxide by rabbit lungs: effects of hypoxia or metabolic inhibitors. J Appl Physiol (1985) 1993;74:2868-2874. https://doi.org/10.1152/jappl.1993.74.6.2868
56. Archer SL, Will JA, Weir EK. Redox status in the control of pulmonary vascular tone. Herz 1986;11:127-41.
57. Chandel NS, McClintock DS, Feliciano CE, Wood TM, Melendez JA, Rodriguez AM, Schumacker PT. Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1alpha during hypoxia: a mechanism of O2 sensing. J Biol Chem 2000;275:25130-8. https://doi.org/10.1074/jbc.M001914200
58. Guzy RD, Hoyos B, Robin E, Chen H, Liu L, Mansfield KD, Simon MC *et al.* Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing. Cell Metab 2005;1:401-408. https://doi.org/10.1016/j.cmet.2005.05.001
59. Waypa GB, Chandel NS, Schumacker PT. Model for hypoxic pulmonary vasoconstriction involving mitochondrial oxygen sensing. Circ Res 2001;88:1259-1266. https://doi.org/10.1161/hh1201.091960
60. Waypa GB, Marks JD, Mack MM, Boriboun C, Mungai PT, Schumacker PT. Mitochondrial reactive oxygen species trigger calcium increases during hypoxia in pulmonary arterial myocytes. Circ Res 2002;91:719-726. https://doi.org/10.1161/01.RES.0000036751.04896.F1
61. Weir EK, Archer SL. Counterpoint: Hypoxic pulmonary vasoconstriction is not mediated by increased production of reactive oxygen species. J Appl Physiol (1985) 2006;101:995-8; discussion 8. https://doi.org/10.1152/japplphysiol.00480a.2006
62. Ahmad M, Kelly MR, Zhao X, Kandhi S, Wolin MS. Roles for Nox4 in the contractile response of bovine pulmonary arteries to hypoxia. Am J Physiol Heart Circ Physiol 2010;298:H1879-88. https://doi.org/10.1152/ajpheart.01228.2009
63. Wolin MS, Alruwaili N, Kandhi S. Studies on Hypoxic Pulmonary Vasoconstriction Detect a Novel Role for the Mitochondrial Complex I Subunit Ndufs2 in Controlling Peroxide Generation for Oxygen-Sensing. Circ Res 2019;124:1683-5. https://doi.org/10.1161/CIRCRESAHA.119.315137
64. Wolin MS, Gupte SA, Neo BH, Gao Q, Ahmad M. Oxidant-redox regulation of pulmonary vascular responses to hypoxia and nitric oxide-cGMP signaling. Cardiol Rev 2010;18:89-93. https://doi.org/10.1097/CRD.0b013e3181c9f088
65. Thebaud B, Wu XC, Kajimoto H, Bonnet S, Hashimoto K, Michelakis ED, Archer SL. Developmental absence of the O2 sensitivity of L-type calcium channels in preterm ductus arteriosus smooth muscle cells impairs O2 constriction contributing to patent ductus arteriosus. Pediatr Res 2008;63:176-81. https://doi.org/10.1203/PDR.0b013e31815ed059
66. Henry TD, Archer SL, Nelson D, Weir EK, From AH. Postischemic oxygen radical production varies with duration of ischemia. Am J Physiol 1993;264:H1478-84. https://doi.org/10.1152/ajpheart.1993.264.5.H1478
67. Piao L, Fang YH, Fisher M, Hamanaka RB, Ousta A, Wu R, Mutlu GM *et al.* Dynamin-related protein 1 is a critical regulator of mitochondrial calcium homeostasis during myocardial ischemia/reperfusion injury. FASEB J 2024;38:e23379. https://doi.org/10.1096/fj.202301040RR
68. Dunham-Snary KJ, Wu D, Sykes EA, Thakrar A, Parlow LR, Mewburn JD, Parlow JL *et al.* Hypoxic Pulmonary Vasoconstriction: From Molecular Mechanisms to Medicine. Chest 2017;151:181-92. https://doi.org/10.1016/j.chest.2016.09.001
69. Read AD, Bentley RET, Martin AY, Mewburn JD, Alizadeh E, Wu D, Lima PDA *et al.* Electron Leak From the Mitochondrial Electron Transport Chain Complex I at Site I(Q) Is Crucial for Oxygen Sensing in Rabbit and Human Ductus Arteriosus. J Am Heart Assoc 2023;12:e029131. https://doi.org/10.1161/JAHA.122.029131
70. Coceani F, Olley PM. The response of the ductus arteriosus to prostaglandins. Can J Physiol Pharmacol 1973;51:220-5. https://doi.org/10.1139/y73-031
71. Coceani F, Kelsey L, Seidlitz E. Evidence for an effector role of endothelin in closure of the ductus arteriosus at birth. Can J Physiol Pharmacol 1992;70:1061-4. https://doi.org/10.1139/y92-146
72. Tristani-Firouzi M, Reeve HL, Tolarova S, Weir EK, Archer SL. Oxygen-induced constriction of rabbit ductus arteriosus occurs via inhibition of a 4-aminopyridine-, voltage-sensitive potassium channel. J Clin Invest 1996;98:1959-65. https://doi.org/10.1172/JCI118999

73. Nakanishi T, Gu H, Hagiwara N, Momma K. Mechanisms of oxygen-induced contraction of ductus arteriosus isolated from the fetal rabbit. Circ Res 1993;72:1218-28. https://doi.org/10.1161/01.RES.72.6.1218
74. Archer SL, Yankovich RD, Chesler E, Weir EK. Comparative effects of nisoldipine, nifedipine and bepridil on experimental pulmonary hypertension. J Pharmacol Exp Ther 1985;233:12-7.
75. Melot C, Hallemans R, Naeije R, Mols P, Lejeune P. Deleterious effect of nifedipine on pulmonary gas exchange in chronic obstructive pulmonary disease. Am Rev Respir Dis 1984;130:612-6.
76. Walmrath D, Schermuly R, Pilch J, Grimminger F, Seeger W. Effects of inhaled versus intravenous vasodilators in experimental pulmonary hypertension. Eur Respir J 1997;10:1084-92. https://doi.org/10.1183/09031936.97.10051084
77. Caravita S, Baratto C, Di Marco F, Calabrese A, Balestrieri G, Russo F, Faini A *et al.* Haemodynamic characteristics of COVID-19 patients with acute respiratory distress syndrome requiring mechanical ventilation. An invasive assessment using right heart catheterization. Eur J Heart Fail 2020;22:2228-37. https://doi.org/10.1002/ejhf.2058
78. Hackett PH, Creagh CE, Grover RF, Honigman B, Houston CS, Reeves JT, Sophocles AM *et al.* High-altitude pulmonary edema in persons without the right pulmonary artery. N Engl J Med 1980;302:1070-3. https://doi.org/10.1056/NEJM198005083021907
79. Hultgren HN. High-altitude pulmonary edema: current concepts. Annu Rev Med 1996;47:267-84. https://doi.org/10.1146/annurev.med.47.1.267
80. Bhagi S, Srivastava S, Singh SB. High-altitude pulmonary edema: review. J Occup Health 2014;56:235-43. https://doi.org/10.1539/joh.13-0256-RA
81. Bartsch P, Mairbaurl H, Swenson ER, Maggiorini M. High altitude pulmonary oedema. Swiss Med Wkly 2003;133:377-84. https://doi.org/10.4414/smw.2003.09657
82. Dehnert C, Mereles D, Greiner S, Albers D, Scheurlen F, Zugel S, Bohm T *et al.* Exaggerated hypoxic pulmonary vasoconstriction without susceptibility to high altitude pulmonary edema. High Alt Med Biol 2015;16:11-7. https://doi.org/10.1089/ham.2014.1117¨
83. Deem S, Hedges RG, Kerr ME, Swenson ER. Acetazolamide reduces hypoxic pulmonary vasoconstriction in isolated perfused rabbit lungs. Respir Physiol 2000;123:109-19. https://doi.org/10.1016/S0034-5687(00)00148-1
84. Maggiorini M. High altitude-induced pulmonary oedema. Cardiovasc Res 2006;72:41-50. https://doi.org/10.1016/j.cardiores.2006.07.004
85. Oelz O, Maggiorini M, Ritter M, Waber U, Jenni R, Vock P, Bartsch P. Nifedipine for high altitude pulmonary oedema. Lancet 1989;2:1241-4. https://doi.org/10.1016/S0140-6736(89)91851-5
86. Archer SL, Gomberg-Maitland M, Maitland ML, Rich S, Garcia JG, Weir EK. Mitochondrial metabolism, redox signaling, and fusion: a mitochondria-ROS-HIF-1αlpha-Kv1.5 O2-sensing pathway at the intersection of pulmonary hypertension and cancer. Am J Physiol Heart Circ Physiol 2008;294:H570-8. https://doi.org/10.1152/ajpheart.01324.2007
87. Humphrey N. VUMC mourns loss of renowned pulmonary medicine physician-scientist John H. Newman. VUMC News, 2024.
88. Bonnet S, Michelakis ED, Porter CJ, Andrade-Navarro MA, Thebaud B, Bonnet S, Haromy A *et al.* An abnormal mitochondrial-hypoxia inducible factor-1alpha-Kv channel pathway disrupts oxygen sensing and triggers pulmonary arterial hypertension in fawn hooded rats: similarities to human pulmonary arterial hypertension. Circulation 2006;113:2630-41. https://doi.org/10.1161/CIRCULATIONAHA.105.609008
89. Ang SO, Chen H, Hirota K, Gordeuk VR, Jelinek J, Guan Y, Liu E *et al.* Disruption of oxygen homeostasis underlies congenital Chuvash polycythemia. Nat Genet 2002;32:614-21. https://doi.org/10.1038/ng1019
90. Hickey MM, Richardson T, Wang T, Mosqueira M, Arguiri E, Yu H, Yu QC *et al.* The von Hippel-Lindau Chuvash mutation promotes pulmonary hypertension and fibrosis in mice. J Clin Invest 2010;120:827-39. https://doi.org/10.1172/JCI36362
91. Smith TG, Brooks JT, Balanos GM, Lappin TR, Layton DM, Leedham DL, Liu C *et al.* Mutation of von Hippel-Lindau tumour suppressor and human cardiopulmonary physiology. PLoS Med 2006;3:e290. https://doi.org/10.1371/journal.pmed.0030290

92. Lumb AB, Slinger P. Hypoxic pulmonary vasoconstriction: physiology and anesthetic implications. Anesthesiology 2015;122:932-46. https://doi.org/10.1097/ALN.0000000000000569
93. Bartsch P, Maggiorini M, Ritter M, Noti C, Vock P, Oelz O. Prevention of high-altitude pulmonary edema by nifedipine. N Engl J Med 1991;325:1284-9. https://doi.org/10.1056/NEJM199110313251805
94. Rodriguez-Roisin R, Ballester E, Roca J, Torres A, Wagner PD. Mechanisms of hypoxemia in patients with status asthmaticus requiring mechanical ventilation. Am Rev Respir Dis 1989;139:732-9. https://doi.org/10.1164/ajrccm/139.3.732
95. Piercy V, Smith H, Arch JR. Effects of isoprenaline, adrenaline and selective alpha 1- and alpha 2- adrenoceptor stimulation on hypoxic pulmonary vasoconstriction in rat isolated perfused lungs. Pulm Pharmacol 1990;3:59-63. https://doi.org/10.1016/0952-0600(90)90033-F
96. Knudson RJ, Constantine HP. An effect of isoproterenol on ventilation-perfusion in asthmatic versus normal subjects. J Appl Physiol 1967;22:402-6. https://doi.org/10.1152/jappl.1967.22.3.402
97. Sylvester JT, Shimoda LA, Aaronson PI, Ward JP. Hypoxic pulmonary vasoconstriction. Physiol Rev 2012;92:367-520. https://doi.org/10.1152/physrev.00041.2010
98. Weitzenblum E, Kessler R, Oswald M, Fraisse P. Medical treatment of pulmonary hypertension in chronic lung disease. Eur Respir J 1994;7:148-52. https://doi.org/10.1183/09031936.94.07010148
99. Melot C, Naeije R, Rothschild T, Mertens P, Mols P, Hallemans R. Improvement in ventilation-perfusion matching by almitrine in COPD. Chest 1983;83:528-533. https://doi.org/10.1378/chest.83.3.528
100. Archer SL, Mike D, Crow J, Long W, Weir EK. A placebo-controlled trial of prostacyclin in acute respiratory failure in COPD. Chest 1996;109:750-755. https://doi.org/10.1378/chest.109.3.750
101. Light RB. Indomethacin and acetylsalicylic acid reduce intrapulmonary shunt in experimental pneumococcal pneumonia. Am Rev Respir Dis 1986;134:520-525.
102. McCormack DG, Paterson NA. Loss of hypoxic pulmonary vasoconstriction in chronic pneumonia is not mediated by nitric oxide. Am J Physiol 1993;265:H1523-H1528. https://doi.org/10.1152/ajpheart.1993.265.5.H1523
103. Dantzker DR, Brook CJ, Dehart P, Lynch JP, Weg JG. Ventilation-perfusion distributions in the adult respiratory distress syndrome. Am Rev Respir Dis 1979;120:1039-1052.
104. Enkhbaatar P, Murakami K, Shimoda K, Mizutani A, Traber L, Phillips GB, Parkinson JF *et al.* The inducible nitric oxide synthase inhibitor BBS-2 prevents acute lung injury in sheep after burn and smoke inhalation injury. Am J Respir Crit Care Med 2003;167:1021-1026. https://doi.org/10.1164/rccm.200209-1031PP

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REVIEW

Sex Differences in Cardiac Tolerance to Oxygen Deprivation – 40 Years of Cardiovascular Research

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Summary

Experimental and clinical studies have clearly demonstrated significant sex differences in myocardial structure and function, both under physiological and pathological conditions. The best example are significant sex differences in the cardiac tolerance to ischemia/reperfusion injury: pre-menopausal adult female hearts are more resistant as compared to the male myocardium. The importance of these findings is supported by the fact that the number of studies dealing with this issue increased significantly in recent years. Detailed molecular and cellular mechanisms responsible for sex differences are yet to be elucidated; however, it has been stressed that the differences cannot be explained only by the effect of estrogens. In recent years, a promising new hypothesis has been developed, suggesting that mitochondria may play a significant role in the sex differences in cardiac tolerance to oxygen deprivation. However, one is clear already today: sex differences are so important that they should be taken into consideration in the clinical practice for the selection of the optimal diagnostic and therapeutic strategy in the treatment of ischemic heart disease. The present review attempts to summarize the progress in cardiovascular research on sex-related differences in cardiac tolerance to oxygen deprivation during the last 40 years, i.e. from the first experimental observation. Particular attention was paid to the sex-related differences of the normal heart, sex-dependent tolerance to ischemia-reperfusion injury, the role of hormones and, finally, to the possible role of cardiac mitochondria in the mechanism of sex-dependent differences in cardiac tolerance to ischemia/reperfusion injury.

Key words

Female heart • Cardiac hypoxic tolerance • Ischemia-reperfusion injury • Sex differences

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Introduction

The most frequent (and hence the most widely studied) cardiovascular diseases of modern times undoubtedly include hypoxic states. They originate as a result of disproportion between the amount of oxygen supplied to the cardiac cell and the amount actually required by the cell. Degree of hypoxic injury depends not only on the intensity and duration of hypoxic stimuli but also on cardiac tolerance to oxygen deprivation. 40 years ago, in the study comparing cardiopulmonary responses of male and female rats to intermittent high-altitude hypoxia, we have observed significant sex differences in cardiac resistance to acute anoxia *in vitro* [1]. The myocardium of control adult female rats was significantly more resistant to oxygen deprivation as compared with males of the same age (Fig. 1). Adaptation to chronic hypoxia significantly increased resistance in both sexes, yet the sex difference was maintained. Unfortunately, our scientific interest was at that time concentrated on the protective mechanisms of cardiac adaptation to chronic hypoxia and the possible sex-dependent differences remained out of our research program. To our surprise, starting ten years later we have seen repeatedly information published in the high-quality journals that our paper from 1984 first described sex differences of myocardial resistance in female and male rats exposed to acute hypoxia [2-4]. Mistrust to this statement led us to the search for objective information:

and really, according to data from Web of Science, the number of studies investigating sex-related differences in the cardiovascular system was negligible still in 1989 [5] (Fig. 2). However, the number of clinical and experimental studies has grown exponentially over the past 30 years. This trend is obviously the result of several facts: the number of examples of different behaviour of the male and female heart under physiological and pathological conditions is steadily increasing and there are controversial reports on the beneficial and adverse effects of hormonal replacement therapy (HRT) in women during menopause. Moreover, the increasing interest undoubtedly reflects the importance of this topic and the urgent need to explain underlying mechanisms for better understanding sex determinants of outcomes and to minimize bias in the management and treatment of ischemic heart disease (IHD) in women.

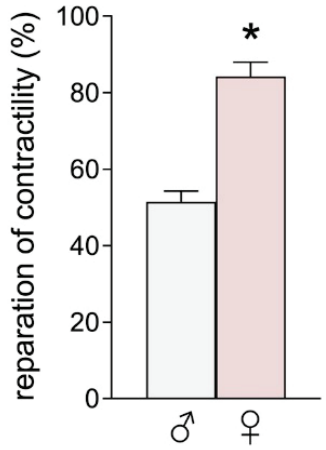

Fig. 1. Sex differences in the cardiac tolerance to acute oxygen deprivation in rats (expressed as % of the reparation of contractility of the isolated right ventricle after acute anoxia). *p<0.01; data from [1].

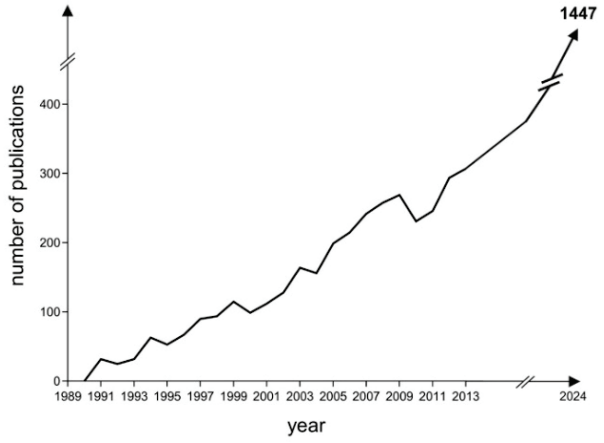

Fig. 2. Number of clinical and experimental papers dealing with „sex and heart AND female heart. From 1989 to 2024. Source: Web of Science.

The present review attempts to summarize the progress in cardiovascular research on sex-related differences in cardiac tolerance to oxygen deprivation during the last 40 years, i.e. from the first experimental observation. Particular attention was paid to the sex-related changes of the normal heart, sex-dependent tolerance to ischemia-reperfusion injury, role of hormones in sex-dependent variation in cardiac sensitivity to ischemia and, finally, to the possible role of cardiac mitochondria.

Sex differences of the normal heart

Sex-related cardiac differences are apparent even in healthy individuals (reviewed in [6]). Although there are no differences in the weight of the cardiac muscle during early phases of ontogenetic development, an increase in myocardial weight occurs in males at puberty; this change makes the male heart 15-20 % heavier than female heart [7]. The initial number of cardiomyocytes is comparable in both sexes; however, during ontogenetic development the number of cardiomyocytes in female hearts remains stable, whereas the number of myocytes in the male hearts decreases significantly [8]. The loss of cells is accompanied in the male myocardium by an increase in their diameter (by 51 % in male monkeys compared to 8 % in females [9]). This hypertrophic growth response can compensate to some extent for the decrease in the number of cardiac cells, but as the cells enlarge, the distance between the capillaries also increases, creating a potential source of insufficient oxygen supply to the cells. Surprising is the finding that the incidence of programmed cell death - apoptosis - is three times higher in the heart and coronary arteries of healthy men than in women; age did not influence this difference [10,11]. The average heart rate for women is approximately 3-5 beats/min more than for the males [12,13]. Moreover, the female heart has longer action potential duration, longer QT interval and a shorter sinus node recovery time as compared to the male heart [14]. In men under the age of 60 years, the average systolic and diastolic pressure is higher by 6–7mm Hg and 3-5mm Hg respectively, as compared to age-matched women. In post-menopausal women, the systolic blood pressure increases, to the extent that the incidence of hypertension is more prevalent in women than in men [15, 16].

Data on the myocardial contractile performance are controversial and not concise. For example, Schwertz

et al. [17 - 18] and Machuki *et al.* [19] have observed that female cardiomyocytes have a larger contraction and greater Ca^{2+} transient amplitude as compared to male cardiomyocytes, whereas Farrel *et al.* [20] have failed to confirm these findings. These contrasting observations were suggested by Machuki *et al.* [19] to be, at least partly, due to the use of whole ventricular myocytes versus left ventricular apical cardiomyocytes, particularly since differences in apical versus basal Ca^{2+} current have been reported in rabbit heart [21]. An important element of cardiomyocyte contraction is the cAMP-PKA-L-type Ca^{2+} channel pathway. Machuki *et al.* [19] have reported that intracellular cAMP, Ca^{2+} channel density and Ca^{2+} transient were larger in female than in male cardiomyocytes. These authors have also suggested that estrogen can regulate the expression of genes for the cAMP–L-type calcium channel pathway and contribute to sex differences in cardiac contraction.

Over the last years, the number of studies describing myocardial sex differences at the molecular and cellular level has increased (for rev. see [22]); their enumeration exceeds the possibilities of this review. For the purpose of this chapter, we have briefly summarized sex differences in cardiac calcium metabolism. It may be noted that Ca^{2+} homeostasis is regulated as a function of the estrous cycle [23], and myofilament Ca^{2+} density is increased in hearts of ovariectomized female rats. Interestingly, Ca^{2+} homeostasis is also regulated by testosterone, which activates phospholipase C and subsequent production of inositol-3-phosphate, which in turn mediates the release of Ca^{2+} from the sarcoplasmic reticulum and increases intracellular Ca^{2+} [24]. Higher expression of sarcolemmal and mitochondrial ATPsensitive potassium (KATP) channels has been reported in the female myocardium; their inhibition during ischemia increases the extent of tissue injury [25]. Estrogen regulates also the expression of phospholamban and ryanodine receptors. In this regard, the higher levels of ryanodine receptors in female cardiomyocytes are linked to higher Ca^{2+} release from the sarcoplasmic reticulum [26]. Interestingly, no sex differences have been observed in SERCA (Ca^{2+}-pump ATPase) expression level [18]. Compared to myocytes, little is known about cellular sex differences in the non-myocytes of the heart [21]: while cardiac myocytes constitute 70 %, they constitute only about 30 % of the total cell number.

Sex differences, with respect to cardiac structure, function and cellular mechanisms during aging, have been summarized by Keller and Howlett [27] and Sapp and Howlett [28]. Dworatzek *et al.* [29] observed agedependent sex differences also in myocardial collagen composition: type I, III, and VI collagens were significantly lower in aged female hearts. Similarly, Arellano *et al.* [30] revealed a specific down-regulation of sirtuins (Sirt1 and Sirt3) in aged female human hearts, which was accompanied by a decline in the mitochondrial anti-oxidative defense system.

Sex differences in cardiac tolerance to ischemia/reperfusion injury

The mentioned sex differences, characteristic of the normal myocardium, create a logical presumption of a possible different response of the heart muscle to various pathogenic stimuli, including ischemia/ reperfusion (I/R) injury. Among cardiovascular diseases, ischemic heart disease (IHD) is the single most frequent cause of death among men and women and is responsible for significant number from all cardiovascular events [31]. Even though IHD is the major cause of mortality in both women and men, it has largely been considered as a “male disease” and, therefore, the majority of experimental and clinical studies have been conducted in men. The information that women are discriminated in diagnostics and treatment of cardiovascular diseases was actually first indicated in the late 1980 [32], noting that women with signs of coronary artery disease required less intensive treatment than men. Another communication from the same research team [33] pointed out the problems associated with the indication of women for coronary surgical intervention. In the same year, the first comprehensive book on IHD in women was published [34].

Epidemiological studies have unequivocally demonstrated that in women before menopause, IHD begins about 10 years later than in men, and the occurrence of myocardial infarction is delayed by even 20 years. However, after menopause, the incidence of this disease increases more than 10 times in women, while in men of the same age it is only 4.5 times [35,36]. The cause are apparently sex differences in the development of atherosclerotic changes during development, which were already pointed out by Fejfar [37]; it is approximately the aforementioned 10 years; this fact is also supported by lower LDL-cholesterol levels and higher HDL-cholesterol values in postmenopausal women [38].

The vast majority of experimental studies confirm clinical observations (for an overview see [39,44]). As it has been mentioned in the Introduction, we found already 40 years ago [1] that the isolated right ventricle of the female laboratory rat is significantly more resistant to acute oxygen deprivation than the right ventricle of males. However, intensive research on this question began many years later. Higher resistance of female myocardium to I/R injury has been demonstrated in various species of laboratory animals (e.g. [24,44-46]). Females were found to have better recovery of contractile function and a lower incidence of reperfusion arrhythmias [47- 49]; Przyklenk *et al.* [50], however, did not observe this difference. Better functional recovery in females was accompanied by a smaller extent of ischemic damage, a lower level of lactate dehydrogenase and a lower production of inflammatory cytokines [51]. Similarly, transgenic females with increased expression of Na/Ca exchanger and β-adrenergic receptors [52,53] had less I/R injury and increased contractility compared to transgenic males. We have observed that sex differences in I/R injury also exist in spontaneously hypertensive rats: postischemic reparation of contractility was significantly higher in hypertensive females, despite the fact that the blood pressure level was comparable in both sexes [54]. Sex differences also exist in obese animals: infarct size was significantly larger in males than in females [55]. Experimental and clinical studies describe significant sex differences in remodeling after myocardial infarction [56-58]: in males, healing was slower with more frequent cardiac ruptures, apparently caused by premature degradation of the extracellular matrix by activation of metalloproteinases [57].

The development of cardiac resistance to oxygen deprivation has a characteristic ontogenetic development: after birth, the resistance of the hearts of male and female laboratory rats does not differ. From the beginning of sexual maturity, the resistance of the male heart decreases, while it does not change in females; thus, significant sex difference arises in adulthood [59]. It is interesting that interventions induced during early stages of ontogenetic development can significantly affect the resistance of the adult myocardium to ischemia in sex-dependent manner. We have observed that perinatal hypoxia increases the resistance of adult female heart to ischemia; on the contrary, in adult males was I/R injury significantly more expressed than in males kept under normoxic conditions [5,43,59] (Fig. 3) These results support the hypothesis that perinatal hypoxia represents a primary programming stimulus for the heart that may lead to sex-dependent sensitivity of the adult heart to ischemia. This fact may be clinically important in patients who have undergone a prolonged hypoxic period in the early stages of development, e.g. in children with hypoxemic congenital heart disease.

In this context, the question arises whether the high resistance of the female heart to hypoxia can be further increased by some of the known cardioprotective phenomena. However, the answer is not simple: the experimental work that dealt with this issue is rare and, moreover, not concise; we did not find clinical observations in the literature. We have observed that adaptation to chronic hypoxia increases cardiac resistance in both sexes; however, the sex difference observed in normoxic animals was preserved [1]. Data on the effect of ischemic preconditioning are contradictory: e.g. Humphreys *et al.* [61] observed the same degree of protection in male and female rats, whereas Wang *et al.* [62] failed to increase the resistance of female rabbit myocardium. Song *et al.* [63] found that the protective effect of preconditioning was lower in females than in males; similar conclusions were reached by Crisostomo *et al.* [64] in the case of ischemic postconditioning. Moreover, Lieder *et al.* [65] observed that sex is not decisive for the cardioprotective effect of pre- and postconditioning. The most plausible explanation seems to be the observation of Turcato *et al.* [66]: they did not find a protective effect of ischemic preconditioning in young females, whose resistance was primarily relatively high; with a decrease in tolerance to ischemia in older individuals, the effect of ischemic preconditioning appeared.

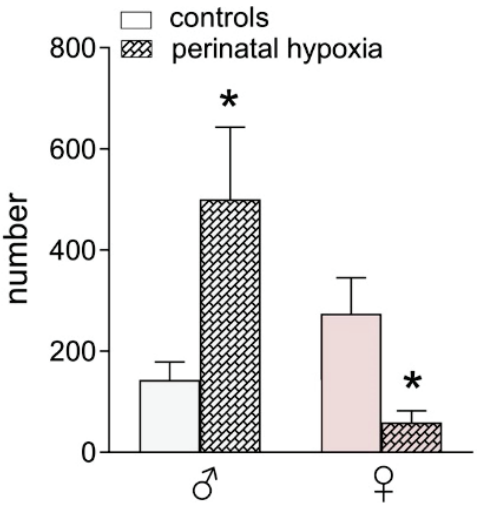

Fig. 3. Effect of perinatal hypoxia on the number of ischemic arrhythmias in adult males and females. * p<0.01; data from [60].

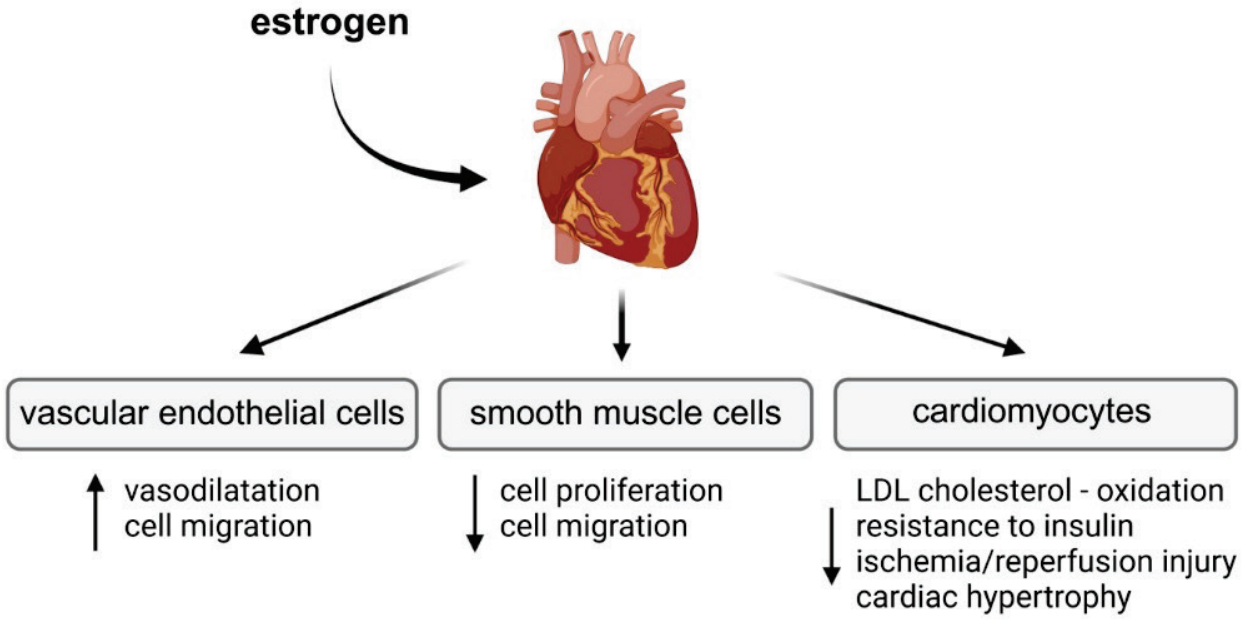

Fig. 4. Effect of estrogen on the heart; according to [68].

It seems to us that this observation belongs to the general biological phenomenon; the degree of cardiac resistance apparently has its threshold. Indeed, we observed a similar effect in the hearts of newborn rats; their high resistance could not be further increased either by adaptation to chronic hypoxia or by ischemic preconditioning; the protective phenomenon appeared only with a decrease in natural resistance during further stages of ontogenetic development [67]. In this context, it is necessary to recall the results of the CONDI-2/ERIC PPCI clinical study, which did not demonstrate a cardio-protective effect of remote ischemic preconditioning in patients with acute myocardial infarction, regardless of sex [68].

It follows that sex significantly affects cardiac resistance to I/R injury. However, we are still waiting for explanation of the pathogenetic mechanisms involved in this process. Let's try to briefly summarize the existing hypotheses.

Role of hormones in sex-dependent differences in cardiac sensitivity to ischemia-reperfusion injury

The most frequently mentioned cause of differences are sex hormones, especially estrogen. Its level changes during the ovarian cycle, during pregnancy, during hormonal contraception; it affects, among others, the function of blood vessels, the inflammatory response, the sensitivity of myocytes to insulin or the degree of development of cardiac muscle hypertrophy [69]. It is, therefore, understandable that experimental studies have focused on elucidating the role of estrogen in the cardiac tolerance to oxygen deprivation (Fig. 4).

There is clear evidence that ovariectomy in female rats increases the infarct size; on the contrary, the administration of estrogens has a protective effect on the male cardiac muscle [51]. Most of the protective effects of estrogens are attributed to their binding to estrogen receptors α and β, which have been demonstrated in female and male heart cells, fibroblasts and vascular smooth muscle [69,70], but are also found in cell membranes and mitochondria [71]. Their affinity for binding to 17βestradiol is the same in both sexes. Experimental studies show that these receptors play an important role in protection against I/R damage [72]. Unfortunately, there is still no consensus on which of the two receptors is responsible for the higher resistance of the female heart. However, there is a third membrane estrogen receptor, identified as G-protein-coupled estrogen receptor (GPER) [73]; it was found to inhibit the opening of the mitochondrial permeability transition pore (PTP) localized on the inner mitochondrial membrane [74]; the latter is involved in the development of ischemic damage (see later).

The binding of estrogens to receptors induces gene expression of a number of functional and structural proteins (the so-called "genomic effect"). In addition to the genomic effects, there are also the so-called "nongenomic" effects of estrogen; they occur rapidly and

independently of protein synthesis [75]. One of the many factors that can influence the response of the female myocardium is nitric oxide; its concentration is higher in female than in male myocardium. Blockade of NO synthase (eNOS) with L-NAME abolishes sex differences in susceptibility to I/R injury. It should be mentioned that a higher concentration of eNOS is also associated with Snitrosylation of L-type calcium channels, which significantly reduces I/R injury in females by decreasing the calcium overload of the cell [76]. In addition, estrogen activates phosphatidylinositol 3-kinase (PI3K) activity, which is considered to play a role in cardioprotection in females [77]. Taken together, it can be suggested that the protective effect of estrogen could be attributed to changes in the expression of specific proteins or altered post translational protein modifications. However, these are apparently not the only mechanisms involved in the cardiac ischemic protection in females. It seems that also e.g. sarcolemmal and mitochondrial KATP channels [78], higher activity of serine/threonine protein kinase (Akt), protein kinase Cε (PKCε) levels [79] or inhibition of proinflammatory tumor necrosis factor α (TNF α) in ischemic myocardium [80,81] may play a significant role. In all these considerations we must take into account the possible role of significant sex differences in cellular calcium metabolism, as discussed above.

The vast majority of experimental laboratories have chosen only one of the sex hormones - estrogen. It is clear that the cardiovascular system is influenced by at least one other powerful player, androgen. Both estrogenic and androgenic hormones are present in both sexes, although in different concentrations and ratios. Testosterone activates androgen receptors, which are expressed in myocytes; it increases the level of homocysteine and endothelin-1 and, by stimulating thyroxine hydrolase, increases the synthesis of catecholamines. Opinions on the effect of testosterone on cardiovascular function vary, both adverse and beneficial effects of testosterone on the heart have been reported [82,83]. It has been found that testosterone can increase the susceptibility to IHD in men [84], higher doses of androgenic steroids increased the development of atheroma [85]. However, there is no experimental evidence that physiological concentrations of testosterone induce myocardial ischemic damage. In contrast, other clinical work shows that testosterone can have a positive effect on the heart muscle [84]. This effect is apparently caused indirectly by conversion to dihydrotestosterone or 17β-estradiol. It was found, for example, that administration of testosterone to ovariectomized females reduced the extent of ischemic damage [86]. Furthermore, Ghimire *et al.* [87] showed that low doses of testosterone have a protective effect against I/R injury in older mice. Cavasin *et al.* [88] demonstrated in a mouse model of myocardial infarction that whereas estrogens prevent maladaptive chronic remodeling and further deterioration of cardiac performance, testosterone adversely affects myocardial healing (as indicated by a higher rate of cardiac rupture), and thus contributed to cardiac dysfunction as well as to adverse cardiac remodeling. On the other hand, Tsang *et al.* [89] observed that testosterone conferred cardioprotection by up regulating the cardiac α1adrenoceptor; this beneficial effect was abolished or attenuated by blockade of androgen receptors. These conflicting results obviously need further experimental analyses under precisely defined and thus comparable conditions: experimental model, form of steroid hormone, dosage, timing and evaluation. It is, however, necessary to stress, that precise understanding is complicated also by the fact that steroid hormone receptors do not act alone but interact with a broad spectrum of co-regulatory proteins to alter transcription [90].

Possible role of mitochondria

The number of different hypotheses trying to explain the causes of sex differences in the cardiac resistance to oxygen deficiency is increasing. In recent years a new promising opinion has appeared, suggesting that mitochondria, organelles responsible for oxygen handling, may be significantly involved in this effect [91-93]. Mitochondrial sexual dimorphism has been described in a number of organs such as liver, heart, brain and adipose tissue.

Cardiomyocytes from female rats exhibit lower mitochondrial content, but are more efficient and more differentiated than male mitochondria [94]. Moreover, they generate less reactive oxygen species (ROS) than male ones and have higher capacity of antioxidant defence [51]. At baseline, no difference in oxygen consumption rate and cardiolipin content is observed between mitochondria from male and female rats [95]. Subsarcolemmal and intermyofibrillar isolated mitochondria from female hearts have the same respiration rates as the male ones except for glutamate-malatestimulated respiration which is lower in females,

while the ADP/O ratio is higher [96]. Taken together, these results suggest that cardiac mitochondria from females have higher specific activity than the male ones but lower mitochondrial content, explaining the similar oxidative capacity in males and females [92]. Recently, Cao *et al.* [97] have observed that cardiac mitochondrial DNA levels and function tend to be reduced in females as compared to males; on the other hand, the expression of genes, encoding mitochondrial proteins, are higher in males than females.

Murphy and Steenbergen [45] suggested that mitochondria are major targets of cardioprotective signalling. Lagranha *et al.* [51] have observed that cardioprotection in females was associated with altered mitochondrial proteins. They found that mitochondria isolated from females exhibited a number of post-translational modifications in mitochondrial enzymes involved in regulating the generation of ROS and oxidative metabolism. Therefore, females exhibit reduced ROS generation and oxidative metabolism. Morkuniene *et al.* [98] and Pavón *et al.* [99] described the relevance of estrogens in maintaining proper mitochondrial function in response to the instability of mitochondrial membrane potential and PTP opening after I/R. They observed that the opening of this pore can be blocked by physiological concentrations of estrogens, similar to blockade with the classic inhibitor cyclosporine.

Significant sex differences were also found in the mitochondrial uptake of Ca^{2+}: mitochondria from female hearts have lower Ca^{2+} uptake rates and improved recovery of mitochondrial membrane potential from Ca^{2+} - induced depolarization [100]. They cope more successfully with external calcium load by decreasing the rate of calcium influx by the calcium uniporter (MCU). The interaction between MCU and calcium uptake regulatory proteins M1CU1, M1CU2, MCUR1, SLC25A23, and EMRE may be here of crucial importance [101]. In addition, Chweih *et al.* [102] have observed that the concentration threshold for net mitochondrial Ca^{2+} uptake was higher in the female heart than in male myocardium. All these findings suggest that female heart mitochondria are less prone to Ca^{2+} overload upon its effect [103-105].

It has been known for a long time that mitochondria become leaky, uncoupled, and massively swollen if they are exposed to high Ca^{2+} concentrations, especially in the presence of phosphate and when accompanied by oxidative stress. The collapse of mitochondrial membrane potential due to opening of permeability transition pore (PTP), localized on the inner mitochondrial membrane, has been implicated in the molecular mechanism of cardiac I/R injury [106,107]. PTP is closed during ischemia due to the low pH (<7.0), but it opens during the first minutes of reperfusion, together with normalization of pH, ROS accumulation, and rise in intracellular calcium. PTP opening accompanied by matrix swelling, leads finally to myocardial cell death [107]. Initial support for the role of PTP in I/R injury was provided by pharmacology: the blockade of PTP by cyclosporine A and sanglifehrin A in perfused heart was cardioprotective in most animal models of cardiac I/R injury [108]. Cyclosporine A was cardioprotective also in small groups of patients with myocardial infarction undergoing percutaneous coronary intervention [108]. However, a large multicenter clinical trial (CIRCUS) revealed no protective effect of cyclosporine A on clinical outcome in patients with myocardial infarction [110,111]. Several factors such as the severity of infarction, a quite narrow window of protection, route of application, and timing of administration as well as comorbidities may be responsible for the lack of cardioprotection in the CIRCUS trial. Nevertheless, these studies challenge the clinical use of cyclosporine A and the possible cyclophiline D (CypD) inhibitors for cardioprotection, and emphasize the importance of further studies to clarify whether CypD is a feasible target for inhibition that can protect the heart from I/R injury [112].

We have tested the hypothesis whether the role of mitochondrial PTP in the pathogenesis of I/R damage to the heart muscle is dependent on sex [42,93,113]. We found that cardiac mitochondria of females are significantly more resistant to swelling induced by higher calcium concentration, indicating their greater resistance to MPTP opening (Fig. 5). Since the opening of the pore is closely related to the development of I/R damage, the higher resistance of this structure to calcium is one possible explanation for the higher tolerance of the female heart. In this context, the question arises as to whether the protein composition of PTP is responsible for these sex differences. Our experiments showed that there is no sex difference in substrate oxidation or ATP formation, which indicates a comparable content of respiratory chain enzymes. This observation was confirmed by quantitative immunodetection: female and male mitochondria contain comparable amounts of ATP synthase (the protein complex responsible for mitochondrial PTP function), as well as the regulatory

protein cyclophilin D. Interestingly, we observed similar results in our previous study, comparing the role of the mitochondrial PTP in highly hypoxic resistant neonatal and adult hearts of laboratory rats [114]. Therefore, it seems that the protein composition of mitochondrial PTP is not responsible for sex differences in cardiac tolerance to oxygen deprivation, but rather reflects sex differences in the regulation of its function, probably together with regulation of CypD by posttranslational modifications [109]. Cyclophilin D thus remains an attractive target for both experimental and clinical studies looking for possible mitochondrial PTP blockers as a way to reduce myocardial I/R damage [115]. It may be, therefore, concluded that mitochondria are significantly involved in the mechanism of sex differences in cardiac tolerance to I/R injury.

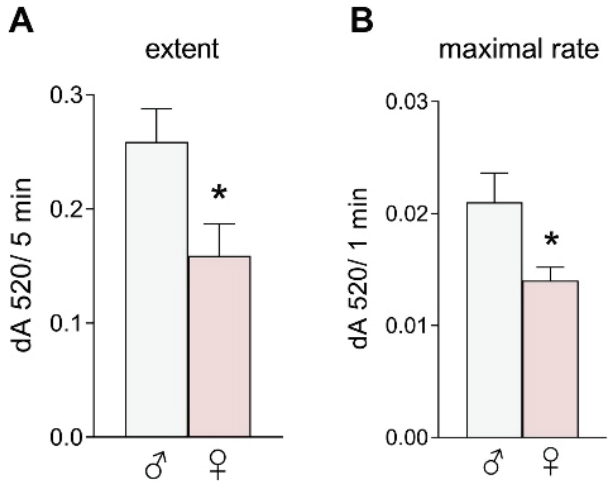

Fig. 5. Calcium induced swelling by rat heart mitochondria from male and female rats. (**A**) Extent of swelling was calculated from the swelling curves and expressed as the decrease of absorbance at 520 nm during 5 min after addition of 200 μM $CaCl_2$. (**B**) Maximum rate of swelling was calculated from curves obtained after derivatization of data of the extent of swelling. * $p<0.01$; data from [113].

Sex differences today

Unfortunately, despite a growing body of evidence, the distinct contribution of biological sex and the sociocultural dimension of gender to the manifestations and outcomes of IHD remain unknown. Moreover, the relative contribution of purely biological factors, such as genes and hormones, to cardiovascular phenotypes and outcomes is not yet fully understood [116]. In spite of the increasing awareness of sex differences in the management of patients with IHD in Europe, a recent study by Hellgren *et al.* [117] confirmed that women still receive guideline-recommended therapies less often than men. Sex-based disparities in outcomes and quality of care were summarized by Aggarwal *el al.* [118]. They include higher morbidity and mortality, delayed presentation, fewer revascularization, less cardiac rehabilitation and less intense pharmacotherapy in females. Recognition of sex differences in presentation, pathophysiology, treatment and outcomes accentuates the need for sex-specific research. Underrepresentation of females results in male outcomes being extrapolated to females, which does not consider sex and gender differences. In conclusion, although women develop IHD later in life than men, the underestimation of women-specific IHD pathophysiology, including biological and sociocultural components, the lack of early recognition and the lack of women-specific treatments increase the risk and mortality of IHD in women [40,116].

Similarly, sex differences are relatively understudied also in animal experiments; many studies fail to report the sex of the cells also in *in vitro* experiments. Moreover, most of experimental studies use exclusively males [40]. On the other hand, it is necessary to admit that experimental approach contributed significantly to our present knowledge on the mechanisms involved in the sex differences of the normal and ischemic heart. The observation that cells from males and females are inherently different is becoming increasingly clear – either due to acquired differences from hormones and other factors or due to intrinsic differences in genotype (XX or XY). In myocardial diseases, sex differences have been described at the tissue level [119]. However, in cells obtained from adults it is difficult to distinguish genetically determined sex differences that exist at birth from sex differences developed during the disease course and are the result of hormones or the environment.

A significant progress in our understanding of the development of sex differences brought the promising studies published by Shi *et al.* [120] and Deegan *et al.* [121], demonstrating that sex chromosome-specific differences in cardiomyocytes exist even before gonads are activated in the embryo. This finding confirm that cardiac sex-related disparities can occur at the early stages of heart development, before gonad formation, and are therefore independent of the influence of sex hormones or the environment. Moreover, identifying how hormones influence sex chromosome effects, whether

antagonistically or synergistically, will enhance our understanding how sex disparities are established. These studies support the view that purely biological mechanisms – genes and sex steroids - contribute to sexrelated differences in IHD and thereby emphasize the importance of sex-specific experimental research on human disease [116].

Conclusions

It follows from the data available that male and female cardiovascular system differ significantly in many characteristics under both physiological and pathological conditions. These differences should be considered by the selection of optimum diagnostic and therapeutic procedures in clinical practice. However, their detailed mechanisms are still poorly understood and the evidence available to date regarding sex-specific aspects of management and outcomes in cardiovascular diseases is still rather limited. Nevertheless, one is clear already today: sex differences in cardiac tolerance to ischemic injury are so important that they should be taken into consideration both in experimental and clinical cardiology.

Conflict of Interest

There is no conflict of interest.

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References

1. Ostadal B, Prochazka J, Pelouch V, Urbanova D, Widimsky J. Comparison of cardiopulmonary responses of male and female rats to intermittent high altitude hypoxia. Physiol Bohemoslov 1984;33:129-138.
2. Ou LC, Sardella GL, Leiter JC, Brinck-Johnsen T, Smith RP. Role of sex hormones in development of chronic mountain sickness in rats. J Appl Physiol (1985) 1994;77:427-433. https://doi.org/10.1152/jappl.1994.77.1.427
3. Kolodgie FD, Farb A, Litovsky SH, Narula J, Jeffers LA, Lee SJ, Virmani R. Myocardial protection of contractile function after global ischemia by physiologic estrogen replacement in the ovariectomized rat. J Mol Cell Cardiol 1997;29:2403-2414. https://doi.org/10.1006/jmcc.1997.0476
4. Moolman JA. Unravelling the cardioprotective mechanism of action of estrogens. Cardiovasc Res 2006;69:777-780. https://doi.org/10.1016/j.cardiores.2006.01.001
5. Netuka I, Szarszoi O, Maly J,Riha H, Turek D, Ostadalova I, Ostadal B. Late effect of early hypoxic disturbances in the rat heart: gender differences. Physiol Res 2010;59:127-131. https://doi.org/10.33549/physiolres.931833
6. Ostadal B, Netuka I, Maly J, Besik J, Ostadalova I. Gender differences in cardiac ischemic injury and protection--experimental aspects. Exp Biol Med (Maywood) 2009;234:1011-1019. https://doi.org/10.3181/0812-MR-362
7. Legato MJ, Leghe JK. Gender and the heart: sex-specific differences in the normal myocardial anatomy and physiology. Principles of Gender Specific Medicine. New York: Elsevier; 2010.
8. de Simone G, Devereux RB, Daniels SR, Meyer RA. Gender differences in left ventricular growth. Hypertension 1995;26:979-983. https://doi.org/10.1161/01.HYP.26.6.979
9. Olivetti G, Giordano G, Corradi D, Melissari M, Lagrasta C, Gambert SR, Anversa P. Gender differences and aging: effects on the human heart. J Am Coll Cardiol 1995;26:1068-1079. https://doi.org/10.1016/0735-1097(95)00282-8
10. Zhang XP, Vatner SF, Shen YT, Rossi F, Tian Y, Peppas A, Resuello RR, Natividad FF, Vatner DE. Increased apoptosis and myocyte enlargement with decreased cardiac mass; distinctive features of the aging male, but not female, monkey heart. J Mol Cell Cardiol 2007;43:487-491. https://doi.org/10.1016/j.yjmcc.2007.07.048
11. Mallat Z, Fornes P, Costagliola R, Esposito B, Belmin J, Lecomte D, Tedgui A. Age and gender effects on cardiomyocyte apoptosis in the normal human heart. J Gerontol A Biol Sci Med Sci 2001;56:M719-723. https://doi.org/10.1093/gerona/56.11.M719

12. Czubryt MP, Espira L, Lamoureux L, Abrenica B. The role of sex in cardiac function and disease. Can J Physiol Pharmacol 2006;84:93-109. https://doi.org/10.1139/y05-151
13. Burke JH, Goldberger JJ, Ehlert FA, Kruse JT, Parker MA, Kadish AH. Gender differences in heart rate before and after autonomic blockade: evidence against an intrinsic gender effect. Am J Med 1996;100:537-543. https://doi.org/10.1016/S0002-9343(96)00018-6
14. Bazett HC. An analysis of the time-relations of electrocardiograms. . Heart 1920;7:353-370.
15. Jochmann N, Stangl K, Garbe E, Baumann G, Stangl V. Female-specific aspects in the pharmacotherapy of chronic cardiovascular diseases. Eur Heart J 2005;26:1585-1595. https://doi.org/10.1093/eurheartj/ehi397
16. Dubey RK, Oparil S, Imthurn B, Jackson EK. Sex hormones and hypertension. Cardiovasc Res 2002;53:688-708. https://doi.org/10.1016/S0008-6363(01)00527-2
17. Schwertz DW, Vizgirda V, Solaro RJ, Piano MR, Ryjewski C. Sexual dimorphism in rat left atrial function and response to adrenergic stimulation. Mol Cell Biochem 1999;200:143-153. https://doi.org/10.1023/A:1007011807383
18. Schwertz DW, Beck JM, Kowalski JM, Ross JD. Sex differences in the response of rat heart ventricle to calcium. Biol Res Nurs 2004;5:286-298. https://doi.org/10.1177/1099800403262615
19. Machuki JO, Zhang HY, Geng J, Fu L, Adzika GK, Wu L, Shang W, Wu J, Kexue L, Zhao Z, Sun H. Estrogen regulation of cardiac cAMP-L-type Ca(2+) channel pathway modulates sex differences in basal contraction and responses to β(2)AR-mediated stress in left ventricular apical myocytes. Cell Commun Signal 2019;17:34. https://doi.org/10.1186/s12964-019-0346-2
20. Farrell SR, Ross JL, Howlett SE. Sex differences in mechanisms of cardiac excitation-contraction coupling in rat ventricular myocytes. Am J Physiol Heart Circ Physiol 2010;299:H36-45. https://doi.org/10.1152/ajpheart.00299.2010
21. Sims C, Reisenweber S, Viswanathan PC, Choi BR, Walker WH, Salama G. Sex, age, and regional differences in L-type calcium current are important determinants of arrhythmia phenotype in rabbit hearts with drug-induced long QT type 2. Circ Res 2008;102:e86-100. https://doi.org/10.1161/CIRCRESAHA.108.173740
22. Walker CJ, Schroeder ME, Aguado BA, Anseth KS, Leinwand LA. Matters of the heart: Cellular sex differences. J Mol Cell Cardiol 2021;160:42-55. https://doi.org/10.1016/j.yjmcc.2021.04.010
23. MacDonald JK, Pyle WG, Reitz CJ, Howlett SE. Cardiac contraction, calcium transients, and myofilament calcium sensitivity fluctuate with the estrous cycle in young adult female mice. Am J Physiol Heart Circ Physiol 2014;306:H938-953. https://doi.org/10.1152/ajpheart.00730.2013
24. Vicencio JM, Ibarra C, Estrada M, Chiong M, Soto D, Parra V, Diaz-Araya G, Jaimovich E, Lavandero S. Testosterone induces an intracellular calcium increase by a nongenomic mechanism in cultured rat cardiac myocytes. Endocrinology 2006;147:1386-1395. https://doi.org/10.1210/en.2005-1139
25. Johnson MS, Moore RL, Brown DA. Sex differences in myocardial infarct size are abolished by sarcolemmal KATP channel blockade in rat. Am J Physiol Heart Circ Physiol 2006;290:H2644-2647. https://doi.org/10.1152/ajpheart.01291.2005
26. Bhupathy P, Babu GJ, Ito M, Periasamy M. Threonine-5 at the N-terminus can modulate sarcolipin function in cardiac myocytes. J Mol Cell Cardiol 2009;47:723-729. https://doi.org/10.1016/j.yjmcc.2009.07.014
27. Keller KM, Howlett SE. Sex Differences in the Biology and Pathology of the Aging Heart. Can J Cardiol 2016;32:1065-1073. https://doi.org/10.1016/j.cjca.2016.03.017
28. Sapp DG, Howlett SE. The influence of sex and age on responses of isolated ventricular myocytes to simulated ischemia and reperfusion. In: *Sex Differences in Heart Disease* (Ostadal B, Dhalla NS, Eds). Cham: Springer International Publishing; 2020. pp. 67-85. https://doi.org/10.1007/978-3-030-58677-5_4
29. Dworatzek E, Baczko I, Kararigas G. Effects of aging on cardiac extracellular matrix in men and women. Proteomics Clin Appl 2016;10:84-91. https://doi.org/10.1002/prca.201500031
30. Barcena de Arellano ML, Pozdniakova S, Kühl AA, Baczko I, Ladilov Y, Regitz-Zagrosek V. Sex differences in the aging human heart: decreased sirtuins, pro-inflammatory shift and reduced anti-oxidative defense. Aging (Albany NY) 2019;11:1918-1933. https://doi.org/10.18632/aging.101881

31. Steg PG, Greenlaw N, Tardif JC, Tendera M, Ford I, Kääb S, Abergel H, Fox KM, Ferrari R. Women and men with stable coronary artery disease have similar clinical outcomes: insights from the international prospective CLARIFY registry. Eur Heart J 2012;33:2831-2840. https://doi.org/10.1093/eurheartj/ehs289
32. Tobin JN, Wassertheil-Smoller S, Wexler JP, Steingart RM, Budner N, Lense L, Wachspress J. Sex bias in considering coronary bypass surgery. Ann Intern Med 1987;107:19-25. https://doi.org/10.7326/0003-4819-107-1-19
33. Steingart RM, Packer M, Hamm P, Coglianese ME, Gersh B, Geltman EM, Sollano J, Katz S, Moyé L, Basta LL, et al. Sex differences in the management of coronary artery disease. Survival and Ventricular Enlargement Investigators. N Engl J Med 1991;325:226-230. https://doi.org/10.1056/NEJM199107253250402
34. Legato MJ, Colman C. The Female Heart: The Truth about Women and Coronary Artery Disease: Simon & Schuster; 1991.
35. Duvall WL. Cardiovascular disease in women. Mt Sinai J Med 2003;70:293-305.
36. Bassuk SS, Manson JE. Physical activity and cardiovascular disease prevention in women: a review of the epidemiologic evidence. Nutr Metab Cardiovasc Dis 2010;20:467-473. https://doi.org/10.1016/j.numecd.2009.12.015
37. Fejfar Z. Prevention against ischaemic heart disease: a critical review. . In: *Modern trends in cardiology*.(Oliver MF, Ed.). London: Butterworths1975. p. 465-495.
38. Mathur P, Ostadal B, Romeo F, Mehta JL. Gender-Related Differences in Atherosclerosis. Cardiovasc Drugs Ther 2015;29:319-327. https://doi.org/10.1007/s10557-015-6596-3
39. Ostadal P, Ostadal B. Women and the management of acute coronary syndrome. Can J Physiol Pharmacol 2012;90:1151-1159. https://doi.org/10.1139/y2012-033
40. Ostadal B, Ostadal P. Sex-based differences in cardiac ischaemic injury and protection: therapeutic implications. Br J Pharmacol 2014;171:541-554. https://doi.org/10.1111/bph.12270
41. Kolar F, Ostadal B. Sex differences in cardiovascular function. Acta Physiol (Oxf) 2013;207:584-587. https://doi.org/10.1111/apha.12057
42. Ostadal B, Drahota Z, Houstek J, Milerova M, Ostadalova I, Hlavackova M, Kolar F. Developmental and sex differences in cardiac tolerance to ischemia-reperfusion injury: the role of mitochondria. Can J Physiol Pharmacol 2019;97:808-814. https://doi.org/10.1139/cjpp-2019-0060
43. Ostadal B, Ostadalova I, Szarszoi O, Netuka I, Olejnickova V, Hlavackova M. Sex-dependent effect of perinatal hypoxia on cardiac tolerance to oxygen deprivation in adults. Can J Physiol Pharmacol 2021;99:1-8. https://doi.org/10.1139/cjpp-2020-0310
44. Ostadal B, Ostadal P, Neckar J. Sex differences in cardiac ischemia/reperfusion injury. In: *Sex differences in heart disease*. Ostadal B, Dhalla, N.S., Eds. Switzerland: Springer; 2020. pp 25-37. https://doi.org/10.1007/978-3-030-58677-5_2
45. Murphy E, Steenbergen C. Gender-based differences in mechanisms of protection in myocardial ischemia-reperfusion injury. Cardiovasc Res 2007;75:478-486. https://doi.org/10.1016/j.cardiores.2007.03.025
46. Booth EA, Lucchesi BR. Estrogen-mediated protection in myocardial ischemia-reperfusion injury. Cardiovasc Toxicol 2008;8:101-113. https://doi.org/10.1007/s12012-008-9022-2
47. Ross JL, Howlett SE. Age and ovariectomy abolish beneficial effects of female sex on rat ventricular myocytes exposed to simulated ischemia and reperfusion. PLoS One 2012;7:e38425. https://doi.org/10.1371/journal.pone.0038425
48. Bell JR, Porrello ER, Huggins CE, Harrap SB, Delbridge LM. The intrinsic resistance of female hearts to an ischemic insult is abrogated in primary cardiac hypertrophy. Am J Physiol Heart Circ Physiol 2008;294:H1514-1522. https://doi.org/10.1152/ajpheart.01283.2007
49. Lujan HL, Dicarlo SE. Sex differences to myocardial ischemia and beta-adrenergic receptor blockade in conscious rats. Am J Physiol Heart Circ Physiol 2008;294:H1523-1529. https://doi.org/10.1152/ajpheart.01241.2007
50. Przyklenk K, Ovize M, Bauer B, Kloner RA. Gender does not influence acute myocardial infarction in adult dogs. Am Heart J 1995;129:1108-1113. https://doi.org/10.1016/0002-8703(95)90390-9

51. Lagranha CJ, Deschamps A, Aponte A, Steenbergen C, Murphy E. Sex differences in the phosphorylation of mitochondrial proteins result in reduced production of reactive oxygen species and cardioprotection in females. Circ Res 2010;106:1681-1691. https://doi.org/10.1161/CIRCRESAHA.109.213645
52. Cross HR, Lu L, Steenbergen C, Philipson KD, Murphy E. Overexpression of the cardiac Na+/ Ca^{2+} exchanger increases susceptibility to ischemia/reperfusion injury in male, but not female, transgenic mice. Circ Res 1998;83:1215-1223. https://doi.org/10.1161/01.RES.83.12.1215
53. Cross HR, Murphy E, Steenbergen C. Ca(2+) loading and adrenergic stimulation reveal male/female differences in susceptibility to ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 2002;283:H481-489. https://doi.org/10.1152/ajpheart.00790.2001
54. Besík J, Szárszoi O, Kunes J, Netuka I, Malý J, Kolár F, Pirk J, Ostádal B. Tolerance to acute ischemia in adult male and female spontaneously hypertensive rats. Physiol Res 2007;56:267-274. https://doi.org/10.33549/physiolres.930998
55. Clark C, Smith W, Lochner A, du Toit EF. The effects of gender and obesity on myocardial tolerance to ischemia. Physiol Res 2011;60:291-301. https://doi.org/10.33549/physiolres.931999
56. Piro M, Della Bona R, Abbate A, Biasucci LM, Crea F. Sex-related differences in myocardial remodeling. J Am Coll Cardiol 2010;55:1057-1065. https://doi.org/10.1016/j.jacc.2009.09.065
57. Regitz-Zagrosek V, Oertelt-Prigione S, Seeland U, Hetzer R. Sex and gender differences in myocardial hypertrophy and heart failure. Circ J 2010;74:1265-1273. https://doi.org/10.1253/circj.CJ-10-0196
58. Cavasin MA, Tao Z, Menon S, Yang XP. Gender differences in cardiac function during early remodeling after acute myocardial infarction in mice. Life Sci 2004;75:2181-2192. https://doi.org/10.1016/j.lfs.2004.04.024
59. Ostadal B, Ostadalova I, Dhalla NS. Development of cardiac sensitivity to oxygen deficiency: comparative and ontogenetic aspects. Physiol Rev 1999;79:635-659. https://doi.org/10.1152/physrev.1999.79.3.635
60. Netuka I, Szarszoi O, Maly J, Besik J, Neckar J, Kolar F, Ostadalova I, Pirk J, Ostadal B. Effect of perinatal hypoxia on cardiac tolerance to acute ischaemia in adult male and female rats. Clin Exp Pharmacol Physiol 2006;33:714-719. https://doi.org/10.1111/j.1440-1681.2006.04423.x
61. Humphreys RA, Kane KA, Parratt JR. The influence of maturation and gender on the anti-arrhythmic effect of ischaemic preconditioning in rats. Basic Res Cardiol 1999;94:1-8. https://doi.org/10.1007/s003950050120
62. Wang M, Crisostomo P, Wairiuko GM, Meldrum DR. Estrogen receptor-alpha mediates acute myocardial protection in females. Am J Physiol Heart Circ Physiol 2006;290:H2204-2209. https://doi.org/10.1152/ajpheart.01219.2005
63. Song X, Li G, Vaage J, Valen G. Effects of sex, gonadectomy, and oestrogen substitution on ischaemic preconditioning and ischaemia-reperfusion injury in mice. Acta Physiol Scand 2003;177:459-466. https://doi.org/10.1046/j.1365-201X.2003.01068.x
64. Crisostomo PR, Wang M, Wairiuko GM, Terrell AM, Meldrum DR. Postconditioning in females depends on injury severity. J Surg Res 2006;134:342-347. https://doi.org/10.1016/j.jss.2006.01.030
65. Lieder HR, Irmert A, Kamler M, Heusch G, Kleinbongard P. Sex is no determinant of cardioprotection by ischemic preconditioning in rats, but ischemic/reperfused tissue mass is for remote ischemic preconditioning. Physiol Rep 2019;7:e14146. https://doi.org/10.14814/phy2.14146
66. Turcato S, Turnbull L, Wang GY, Honbo N, Simpson PC, Karliner JS, Baker AJ. Ischemic preconditioning depends on age and gender. Basic Res Cardiol 2006;101:235-243. https://doi.org/10.1007/s00395-006-0585-4
67. Ostadalova I, Ostadal B, Kolár F, Parratt JR, Wilson S. Tolerance to ischaemia and ischaemic preconditioning in neonatal rat heart. J Mol Cell Cardiol 1998;30:857-865. https://doi.org/10.1006/jmcc.1998.0653
68. Hausenloy DJ, Kharbanda RK, Møller UK, Ramlall M, Aarøe J, Butler R, Bulluck H, et al. Effect of remote ischaemic conditioning on clinical outcomes in patients with acute myocardial infarction (CONDI-2/ERIC-PPCI): a single-blind randomised controlled trial. Lancet 2019;394:1415-1424. https://doi.org/10.1016/S0140-6736(19)32039-2
69. Murphy E. Estrogen signaling and cardiovascular disease. Circ Res 2011;109:687-696. https://doi.org/10.1161/CIRCRESAHA.110.236687

70. Hutson DD, Gurrala R, Ogola BO, Zimmerman MA, Mostany R, Satou R, Lindsey SH. Estrogen receptor profiles across tissues from male and female Rattus norvegicus. Biol Sex Differ 2019;10:4. https://doi.org/10.1186/s13293-019-0219-9
71. Chen JQ, Yager JD, Russo J. Regulation of mitochondrial respiratory chain structure and function by estrogens/estrogen receptors and potential physiological/pathophysiological implications. Biochim Biophys Acta 2005;1746:1-17. https://doi.org/10.1016/j.bbamcr.2005.08.001
72. Gabel SA, Walker VR, London RE, Steenbergen C, Korach KS, Murphy E. Estrogen receptor beta mediates gender differences in ischemia/reperfusion injury. J Mol Cell Cardiol 2005;38:289-297. https://doi.org/10.1016/j.yjmcc.2004.11.013
73. Deschamps AM, Murphy E. Activation of a novel estrogen receptor, GPER, is cardioprotective in male and female rats. Am J Physiol Heart Circ Physiol 2009;297:H1806-1813. https://doi.org/10.1152/ajpheart.00283.2009
74. Bopassa JC, Eghbali M, Toro L, Stefani E. A novel estrogen receptor GPER inhibits mitochondria permeability transition pore opening and protects the heart against ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 2010;298:H16-23. https://doi.org/10.1152/ajpheart.00588.2009
75. Knowlton AA, Lee AR. Estrogen and the cardiovascular system. Pharmacol Ther 2012;135:54-70. https://doi.org/10.1016/j.pharmthera.2012.03.007
76. Sun J, Picht E, Ginsburg KS, Bers DM, Steenbergen C, Murphy E. Hypercontractile female hearts exhibit increased S-nitrosylation of the L-type Ca^{2+} channel alpha1 subunit and reduced ischemia/reperfusion injury. Circ Res 2006;98:403-411. https://doi.org/10.1161/01.RES.0000202707.79018.0a
77. Tong H, Imahashi K, Steenbergen C, Murphy E. Phosphorylation of glycogen synthase kinase-3beta during preconditioning through a phosphatidylinositol-3-kinase--dependent pathway is cardioprotective. Circ Res 2002;90:377-379. https://doi.org/10.1161/01.RES.0000012567.95445.55
78. Lee TM, Su SF, Tsai CC, Lee YT, Tsai CH. Cardioprotective effects of 17 beta-estradiol produced by activation ofmitochondrial ATP-sensitive K(+)Channels in canine hearts. J Mol Cell Cardiol 2000;32:1147-1158. https://doi.org/10.1006/jmcc.2000.1167
79. Bae S, Zhang L. Gender differences in cardioprotection against ischemia/reperfusion injury in adult rat hearts: focus on Akt and protein kinase C signaling. J Pharmacol Exp Ther 2005;315:1125-1135. https://doi.org/10.1124/jpet.105.090803
80. Xu Y, Williams SJ, O'Brien D, Davidge ST. Hypoxia or nutrient restriction during pregnancy in rats leads to progressive cardiac remodeling and impairs postischemic recovery in adult male offspring. Faseb J 2006;20:1251-1253. https://doi.org/10.1096/fj.05-4917fje
81. Yu Y, Wei SG, Weiss RM, Felder RB. Sex differences in the central and peripheral manifestations of ischemia-induced heart failure in rats. Am J Physiol Heart Circ Physiol 2019;316:H70-h79. https://doi.org/10.1152/ajpheart.00499.2018
82. Apaijai N, Chattipakorn SC, Chattipakorn N. The Roles of Testosterone in Cardiac Ischemia/Reperfusion Injury. In: Ostadal B, Dhalla NS, editors. Sex Differences in Heart Disease. Cham: Springer International Publishing; 2020. p. 39-65. https://doi.org/10.1007/978-3-030-58677-5_3
83. van der Wall EE. Testosterone bad for men, good for women? . Neth Heart J 2011;19:1-2. https://doi.org/10.1007/s12471-010-0058-0
84. Parker MW, Thompson PD. Anabolic-androgenic steroids: worse for the heart than we knew? Circ Heart Fail 2010;3:470-471. https://doi.org/10.1161/CIRCHEARTFAILURE.110.957720
85. Jones TH, Kelly DM. Randomized controlled trials - mechanistic studies of testosterone and the cardiovascular system. Asian J Androl 2018;20:120-130. https://doi.org/10.4103/aja.aja_6_18
86. Maldonado O, Ramos A, Guapillo M, Rivera J, Palma I, Rubio-Gayosso I, Ramirez-Sanchez I, Najera N, Ceballos G, Mendez-Bolaina E. Effects of chronic inhibition of Testosterone metabolism on cardiac remodeling after ischemia/reperfusion-induced myocardial damage in gonadectomized rats. Biol Open 2019;8. https://doi.org/10.1242/bio.041905
87. Ghimire A, Bisset ES, Howlett SE. Ischemia and reperfusion injury following cardioplegic arrest is attenuated by age and testosterone deficiency in male but not female mice. Biol Sex Differ 2019;10:42. https://doi.org/10.1186/s13293-019-0256-4

88. Cavasin MA, Tao ZY, Yu AL, Yang XP. Testosterone enhances early cardiac remodeling after myocardial infarction, causing rupture and degrading cardiac function. Am J Physiol Heart Circ Physiol 2006;290:H2043-2050. https://doi.org/10.1152/ajpheart.01121.2005
89. Tsang S, Wu S, Liu J, Wong TM. Testosterone protects rat hearts against ischaemic insults by enhancing the effects of alpha(1)-adrenoceptor stimulation. Br J Pharmacol 2008;153:693-709. https://doi.org/10.1038/sj.bjp.0707624
90. Mendelsohn ME, Karas RH. Molecular and cellular basis of cardiovascular gender differences. Science 2005;308:1583-1587. https://doi.org/10.1126/science.1112062
91. Bonora M, Bononi A, De Marchi E, Giorgi C, Lebiedzinska M, Marchi S, Patergnani S, Rimessi A, Suski JM, Wojtala A, Wieckowski MR, Kroemer G, Galluzzi L, Pinton P. Role of the c subunit of the FO ATP synthase in mitochondrial permeability transition. Cell Cycle 2013;12:674-683. https://doi.org/10.4161/cc.23599
92. Ventura-Clapier R, Moulin M, Piquereau J, Lemaire C, Mericskay M, Veksler V, Garnier A. Mitochondria: a central target for sex differences in pathologies. Clin Sci (Lond) 2017;131:803-822. https://doi.org/10.1042/CS20160485
93. Drahota Z, Endlicher R, Kučera O, Rychtrmoc D, Červinková Z. Factors affecting the function of the mitochondrial membrane permeability transition pore and their role in evaluation of calcium retention capacity values. Physiol Res 2020;69:491-499. https://doi.org/10.33549/physiolres.934391
94. Colom B, Oliver J, Roca P, Garcia-Palmer FJ. Caloric restriction and gender modulate cardiac muscle mitochondrial H2O2 production and oxidative damage. Cardiovasc Res 2007;74:456-465. https://doi.org/10.1016/j.cardiores.2007.02.001
95. Moulin M, Piquereau J, Mateo P, Fortin D, Rucker-Martin C, Gressette M, Lefebvre F, Gresikova M, Solgadi A, Veksler V, Garnier A, Ventura-Clapier R. Sexual dimorphism of doxorubicin-mediated cardiotoxicity: potential role of energy metabolism remodeling. Circ Heart Fail 2015;8:98-108. https://doi.org/10.1161/CIRCHEARTFAILURE.114.001180
96. Ribeiro RF, Jr., Ronconi KS, Morra EA, Do Val Lima PR, Porto ML, Vassallo DV, Figueiredo SG, Stefanon I. Sex differences in the regulation of spatially distinct cardiac mitochondrial subpopulations. Mol Cell Biochem 2016;419:41-51. https://doi.org/10.1007/s11010-016-2748-4
97. Cao Y, Vergnes L, Wang YC, Pan C, Chella Krishnan K, Moore TM, Rosa-Garrido M, Kimball TH, Zhou Z, Charugundla S, Rau CD, Seldin MM, Wang J, Wang Y, Vondriska TM, Reue K, Lusis AJ. Sex differences in heart mitochondria regulate diastolic dysfunction. Nat Commun 2022;13:3850. https://doi.org/10.1038/s41467-022-31544-5
98. Morkuniene R, Arandarcikaite O, Ivanoviene L, Borutaite V. Estradiol-induced protection against ischemia-induced heart mitochondrial damage and caspase activation is mediated by protein kinase G. Biochim Biophys Acta 2010;1797:1012-1017. https://doi.org/10.1016/j.bbabio.2010.03.027
99. Pavón N, Martínez-Abundis E, Hernández L, Gallardo-Pérez JC, Alvarez-Delgado C, Cerbón M, Pérez-Torres I, Aranda A, Chávez E. Sexual hormones: effects on cardiac and mitochondrial activity after ischemia-reperfusion in adult rats. Gender difference. J Steroid Biochem Mol Biol 2012;132:135-146. https://doi.org/10.1016/j.jsbmb.2012.05.003
100. Arieli Y, Gursahani H, Eaton MM, Hernandez LA, Schaefer S. Gender modulation of Ca(2+) uptake in cardiac mitochondria. J Mol Cell Cardiol 2004;37:507-513. https://doi.org/10.1016/j.yjmcc.2004.04.023
101. Williams GS, Boyman L, Lederer WJ. Mitochondrial calcium and the regulation of metabolism in the heart. J Mol Cell Cardiol 2015;78:35-45. https://doi.org/10.1016/j.yjmcc.2014.10.019
102. Chweih H, Castilho RF, Figueira TR. Tissue and sex specificities in Ca^{2+} handling by isolated mitochondria in conditions avoiding the permeability transition. Exp Physiol 2015;100:1073-1092. https://doi.org/10.1113/EP085248
103. Halestrap AP. A pore way to die: the role of mitochondria in reperfusion injury and cardioprotection. Biochem Soc Trans 2010;38:841-860. https://doi.org/10.1042/BST0380841
104. Drahota Z, Milerova M, Endlicher R, Rychtrmoc D, Cervinkova Z, Ostadal B. Developmental changes of the sensitivity of cardiac and liver mitochondrial permeability transition pore to calcium load and oxidative stress. Physiol Res 2012;61:S165-172. https://doi.org/10.33549/physiolres.932377

105. Halestrap AP, Richardson AP. The mitochondrial permeability transition: a current perspective on its identity and role in ischaemia/reperfusion injury. J Mol Cell Cardiol 2015;78:129-141. https://doi.org/10.1016/j.yjmcc.2014.08.018
106. Di Lisa F, Bernardi P. Mitochondrial function as a determinant of recovery or death in cell response to injury. Mol Cell Biochem 1998;184:379-391. https://doi.org/10.1023/A:1006810523586
107. Halestrap AP. Calcium, mitochondria and reperfusion injury: a pore way to die. Biochem Soc Trans 2006;34:232-237. https://doi.org/10.1042/BST20060232, https://doi.org/10.1042/BST0340232
108. Ong SB, Kalkhoran SB, Cabrera-Fuentes HA, Hausenloy DJ. Mitochondrial fusion and fission proteins as novel therapeutic targets for treating cardiovascular disease. Eur J Pharmacol 2015;763:104-114. https://doi.org/10.1016/j.ejphar.2015.04.056
109. Alam MR, Baetz D, Ovize M. Cyclophilin D and myocardial ischemia-reperfusion injury: a fresh perspective. J Mol Cell Cardiol 2015;78:80-89. https://doi.org/10.1016/j.yjmcc.2014.09.026
110. Mewton N, Dernis A, Bresson D, Zouaghi O, Croisille P, Flocard E, Douek P, Bonnefoy-Cudraz E. Myocardial biomarkers and delayed enhanced cardiac magnetic resonance relationship in clinically suspected myocarditis and insight on clinical outcome. J Cardiovasc Med (Hagerstown) 2015;16:696-703. https://doi.org/10.2459/JCM.0000000000000024
111. Cung TT, Morel O, Cayla G, Rioufol G, Garcia-Dorado D, Angoulvant D, Bonnefoy-Cudraz E, et al. Cyclosporine before PCI in Patients with Acute Myocardial Infarction. N Engl J Med 2015;373:1021-1031. https://doi.org/10.1056/NEJMoa1505489
112. Javadov S, Jang S, Parodi-Rullán R, Khuchua Z, Kuznetsov AV. Mitochondrial permeability transition in cardiac ischemia-reperfusion: whether cyclophilin D is a viable target for cardioprotection? Cell Mol Life Sci 2017;74:2795-2813. https://doi.org/10.1007/s00018-017-2502-4
113. Milerova M, Drahota Z, Chytilova A, Tauchmannova K, Houstek J, Ostadal B. Sex difference in the sensitivity of cardiac mitochondrial permeability transition pore to calcium load. Mol Cell Biochem 2016;412:147-154. https://doi.org/10.1007/s11010-015-2619-4
114. Milerova M, Charvatova Z, Skarka L, Ostadalova I, Drahota Z, Fialova M, Ostadal B. Neonatal cardiac mitochondria and ischemia/reperfusion injury. Mol Cell Biochem 2010;335:147-153. https://doi.org/10.1007/s11010-009-0251-x
115. Bernardi P, Di Lisa F. The mitochondrial permeability transition pore: molecular nature and role as a target in cardioprotection. J Mol Cell Cardiol 2015;78:100-106. https://doi.org/10.1016/j.yjmcc.2014.09.023
116. Regitz-Zagrosek V, Gebhard C. Gender medicine: effects of sex and gender on cardiovascular disease manifestation and outcomes. Nat Rev Cardiol 2023;20:236-247. https://doi.org/10.1038/s41569-022-00797-4
117. Hellgren T, Blöndal M, Jortveit J, Ferenci T, Faxén J, Lewinter C, Eha J, Lõiveke P, Marandi T, Ainla T, Saar A, Veldre G, Andréka P, Halvorsen S, Jánosi A, Edfors R. Sex-related differences in the management and outcomes of patients hospitalized with ST-elevation myocardial infarction: a comparison within four European myocardial infarction registries. Eur Heart J Open 2022;2:oeac042. https://doi.org/10.1093/ehjopen/oeac042
118. Aggarwal NR, Patel HN, Mehta LS, Sanghani RM, Lundberg GP, Lewis SJ, Mendelson MA, Wood MJ, Volgman AS, Mieres JH. Sex differences in ischemic heart disease: advances, obstacles, and next steps. Circ Cardiovasc Qual Outcomes 2018;11:e004437. https://doi.org/10.1161/CIRCOUTCOMES.117.004437
119. Regitz-Zagrosek V, Kararigas G. Mechanistic pathways of sex differences in cardiovascular disease. Physiol Rev 2017;97:1-37. https://doi.org/10.1152/physrev.00021.2015
120. Shi W, Sheng X, Dorr KM, Hutton JE, Emerson JI, Davies HA, Andrade TD, Wasson LK, Greco TM, Hashimoto Y, Federspiel JD, Robbe ZL, Chen X, Arnold AP, Cristea IM, Conlon FL. Cardiac proteomics reveals sex chromosome-dependent differences between males and females that arise prior to gonad formation. Dev Cell 2021;56:3019-3034.e3017. https://doi.org/10.1016/j.devcel.2021.09.022
121. Deegan DF, Nigam P, Engel N. Sexual Dimorphism of the Heart: Genetics, Epigenetics, and Development. Front Cardiovasc Med 2021;8:668252. https://doi.org/10.3389/fcvm.2021.668252

Sex-Linked Differences in Cardiac Atrophy After Heterotopic Heart Transplantation: No Direct Relation to the Actions of Sex Steroid Hormones

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Summary

An important complication of prolonged support of the left ventricle with an assist device when implanted in patients with heart failure is unloading-induced cardiac atrophy. Our recent study suggested that sex-linked differences in the development of atrophy induced by heterotopic heart transplantation (HT_X) do exist, however, the role of the environmental conditions dependent on plasma concentrations of sex hormones remains elusive. We aimed to compare the course of HT_X-induced cardiac atrophy in male and female rats after gonadectomy with substitution of steroid hormones of the opposite sex. In a separate series of experiments, we evaluated the course of unloading-induced cardiac atrophy in the female heart transplanted into a male recipient and vice versa. Cardiac atrophy was assessed as the ratio of the transplanted heart weight to native heart weight (HW), which was determined 14 days after HT_X. In female rats, studied in both experimental variants, HT_x resulted in significantly smaller decreases in whole HW when compared to those observed in male rats exposed to the same experimental conditions (-9 ± 1 and - 11 + 1 vs. -44 ± 2 and -42 ± 2 %, $p<0.05$ in both cases). The dynamic of changes in left and right ventricle was similar as in the whole HW. Our results show that the process of unloading-induced cardiac atrophy exhibits important sex-linked differences and that attenuation of this process in female rats cannot be simply ascribed to the protective effects of estradiol or to the absence of deleterious actions of testosterone.

Keywords

Cardiac atrophy • Sex differences • Gonadectomy • Hormonal substitution • Heterotopic heart transplantation • Mechanical heart unloading

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Introduction

Heart transplantation (HT_x) is the best therapeutic approach for the treatment of patients with end-stage heart failure (HF), however, the scarcity of organ donors limits the number of HT_x performed. Therefore, implantation of the left ventricle assist devices (LVADs) has emerged as an alternative treatment for patients with end-stage HF [1-4]. However, the most harmful effect of long-term LVAD use is probably the development of cardiac atrophy, a consequence of LVAD-induced mechanical unloading. It has been claimed that this may be one of the reasons why the beneficial effects on the biological features of the myocardium have not been so far translated into functional improvement [5-15]. Attempts to minimize unloading-induced cardiac atrophy were usually

unsuccessful, which further necessitates a search for new treatment strategies [8,12,13,16-20]. The prerequisite for finding a treatment approach that would minimize unloading-induced cardiac atrophy is profound understanding the physiology of the process. To meet this need, a model of heterotopic rat HT_x onto the abdominal aorta of an isogenic rat recipient was developed. Many research groups, including our own, performed studies employing this model, which provided ample relevant information [5,9,20-31]. The critically important limitation of such studies is the fact that they were performed in male animals only. Since it is known that there are important sex-related differences in the pathophysiology of HF [32-35], one should also expect the presence of such differences in the process of unloading-induced cardiac atrophy. This prompted us recently to elucidate if, and to what extent, sex-related differences are present in the course of cardiac atrophy after heterotopic HT_x. We found that the development of unloading-induced cardiac atrophy was substantially less pronounced in female than in male rats, and that gonadectomy did not alter the course of HT_x-induced cardiac atrophy, similarly in male and female rats. We concluded that the development of unloading-induced cardiac atrophy is less pronounced in female than in male rats, and that those sex-linked differences were not caused by the activity of sex hormones [36]. However, an ultimate conclusion should not be exclusively based on the classical experimental approach (comparison of intact animals with those after gonadectomy) but also on evaluation of the course of HT_x-induced cardiac atrophy in subjects after gonadectomy with substitution of sex steroid hormones of the opposite sex [34,37,38]. Evidently, an alternative approach should be sought to answer the question if the sex-linked differences in the course of unloading-induced cardiac atrophy are due to the inherent properties of donor´s (i.e. transplanted) heart or to the hormonal environment of the recipient. Therefore one should investigate the response of the female heart transplanted into a male recipient and vice versa. Accordingly, the aim of the present study was to assess sex-linked differences in the development of unloading-induced cardiac atrophy using both aforementioned approaches.

Methods

Ethical approval

The studies were performed in agreement with the guidelines and practices established by the *Animal Care and Use Committee of the Institute for Clinical and Experimental Medicine*, Prague, which accord with the *European Convention on Animal Protection and Guidelines on Research Animal Use* and were approved by this committee and subsequently by the Ministry of Health of the Czech Republic (the decision number for this project is 18680/2020-4/OVZ).

Animals and HT_x model

Adult male and female Lewis rats (Charles River Laboratories, Velaz, Prague, Czech Republic), 8 weeks of initial age, were used. The classical heterotopic HT_x, originally described by Ono and Lindsey [39] and employed and validated by many investigators [9,21,23,25,27,28] was used as the model to simulate the effect of full mechanical unloading of the heart; its modification was established in our laboratory and is routinely employed [29-31,36].

Gonadectomy technique

Gonadectomy or sham-operation was performed under combined anesthesia with intraperitoneal ketamine/midazolam mixture (Calypsol, Gedeon Richter, Hungary, 160 mg/kg of body weight, and Dormicum, Roche, France 160 mg/kg of body weight), this was done 28 days before heterotopic HT_x. The details of the operation were as described in our previous studies [40,41]. Briefly, in female rats, the peritoneal cavity was opened and the ovaries and uterus were removed, thereafter, the peritoneal cavity was cleaned and the muscle wall and skin were sutured. In male rats, orchiectomy was performed: the ductus deferens was isolated and ligated and then each testicle was removed *via* midline incision on the scrotum. Butorphanol (Torbugesic, Fort Dodge Animal Health, Fort Dodge, KS, USA), at the dose of 2 mg/kg of body weight, given every 12 hours, was administered subcutaneously for 48-hour postoperative analgesia. In our earlier studies, the effectiveness of gonadectomy was validated by determining plasma levels of testosterone and estradiol, assessed by radioimmunoassay [40,41].

Hormonal substitution

The experimental design for evaluation of the effectiveness of hormonal substitution with steroid hormones of the opposite sex is outlined in Fig. 1. It shows that female rats were gonadectomized on the day labeled -28 and the substitution with testosterone was immediately initiated and repeated on the day labeled 0; the experiment ended on the day labeled +14. Plasma

levels of testosterone were assessed throughout the study as outlined in Fig. 1A and compared with sham-operated males that served as controls. Fig. 1B shows the experimental design for evaluation of the effectiveness of hormonal substitution by estradiol in goadectomized male rats, which was identical as the design used for testosterone substitution in gonadectomized female rats.

Due to the fact that repeated determination of plasma testosterone and estradiol requires a large volume of blood (400 µl), these assessments were performed initially in separate groups of animals (n = 8 in each). Plasma levels of estradiol and testosterone were assessed by RIA techniques as described in our previous studies [40,41]. The following experimental groups were examined:

1) Sham-operated (i.e., Intact) male Lewis rats
2) Sham-operated (i.e., Intact) female Lewis rats
3) Castrated male Lewis rats
4) Castrated female Lewis rats
5) Castrated female Lewis rats + Testosterone substitution
6) Castrated male Lewis rats + Estradiol substitution

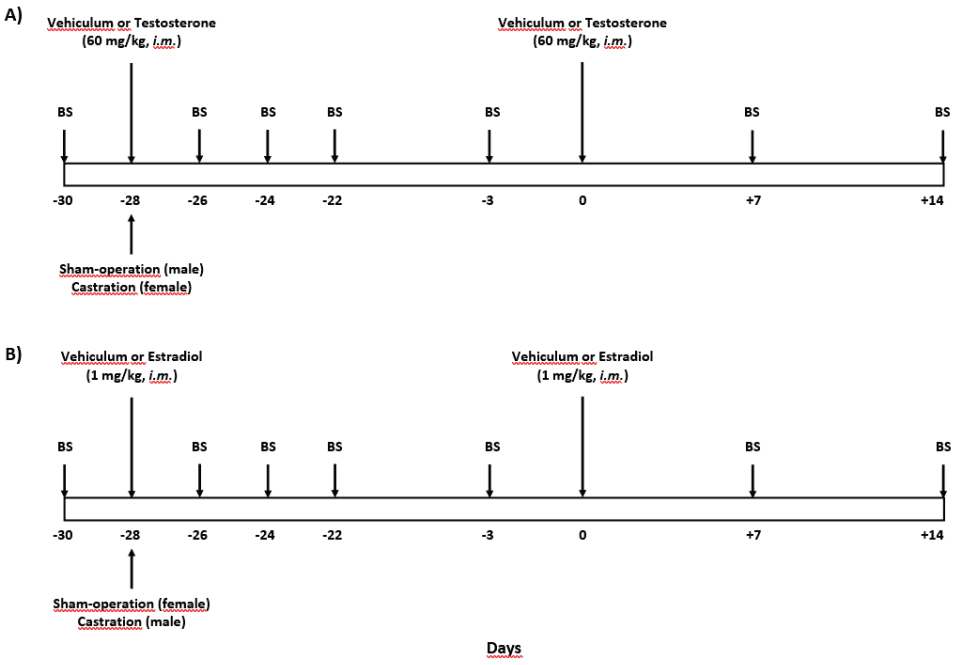

Fig. 1. An outline of the set of experiments in male and female Lewis rats performed for evaluation of the effectiveness of hormonal substitution with steroid hormones of the opposite sex. BS indicates blood sampling from the tail vein.

Detailed experimental design for evaluation of unloading-induced cardiac atrophy.

Series 1: The course of cardiac atrophy after heterotopic HTx in rats after castration and substitution of steroid hormones of the opposite sex

The experimental design used is outlined in Fig. 2. Donor animals were anesthetized by inhalation of 2 % isoflurane (Forane, ABBVie Ltd., Prague, Czech Republic) and the hearts were harvested and transplanted as described previously [29-31,36]. Recipient animals were anesthetized with intraperitoneal thiopental sodium (Thiopental, VUAB Pharma Ltd., Brno Czech Republic, 50 mg/kg of body weight). We and others [9,25,27-31] have demonstrated that the unloading-induced cardiac atrophy develops within the first 14 days after HT_x when a dramatic loss of myocardial mass is seen. The following 40 days is a steady-state period with no further loss of cardiac mass, suggesting stabilization of unloading-induced cardiac atrophy. Therefore, in the present study, the degree of cardiac atrophy was determined 14 days after HT_x. Recipient animals were castrated and the supplementation of steroid sex hormones of the opposite sex was started and performed as described above and validated in the initial above-described studies. The degree of atrophy was assessed from the total heart weight and of its individual structural components [i.e. left ventricle (LV) + septum and right ventricle (RV)]. Explicitly, the index of cardiac atrophy was calculated as the ratio of the weight of the heterotopically transplanted heart to the recipient native normal heart. The degree of cardiac atrophy was expressed as percent decrease in the whole heart weight (HW), LV weight (LVW), and RV weight (RVW) of the hearts after HT_x. Unfortunately,

HW of the donor´s heart before and after HT_x cannot be used for evaluation of the degree of cardiac atrophy because the donor´s heart is immediately placed in cold cardioplegia solution, which precludes precise determination of HW. The following experimental groups were examined:

1. Castrated male Lewis rats (recipient) + estradiol substitution + HT_x of healthy male donor´s heart (14 days after HT_x) (n = 10),
2. Castrated female Lewis rats (recipient) + testosterone substitution + HT_x of healthy female donor´s heart (14 days after HT_x) (n = 10),

At the end of the experiment, the hearts were excised, blood was removed from the chambers by gentle compression, and the hearts´ wet weight was determined.

Series 2: The course of cardiac atrophy after heterotopic HTx to the recipient of the sex opposite to that of the donor´s heart

The experimental design is outlined in Fig. 3 and was virtually the same as described for series 1. However, native heart of the recipient cannot be used as the control for calculation of the index of cardiac atrophy. Evidently, the hearts of male's recipient cannot serve as controls (i.e. 100 %) for the hearts of female´s donor´s heart and vice versa, due to the differences in cardiac mass between males and females. Therefore, the hearts from males and females without HT_x were used as controls (Figs 3C, D). The following experimental groups were examined:

1. Intact female Lewis rats (recipient) + HT_x of healthy male donor´s heart (14 days after HT_x) (n = 10),
2. Intact male Lewis rats (recipient) + HT_x of healthy female donor´s heart (14 days after HT_x) (n = 10),
3. Intact male Lewis rats without HT_x (14 days after sham-operation) (n = 10),
4. Intact female Lewis rats without HT_x (14 days after sham-operation) (n = 10).

Statistical analyses

All values are expressed as mean ± SEM. Using the Graph-Pad Prism software (Graph Pad Software, San Diego, CA, USA), statistical analysis was done by Wilcoxon´s signed-rank test for unpaired data, or one-way analysis of variance (ANOVA) when appropriate. ANOVA analysis was employed for evaluation of differences in plasma concentrations of steroid hormones within the same experimental group over time. The values exceeding 95 % probability limits ($p<0.05$) were considered statistically significant.

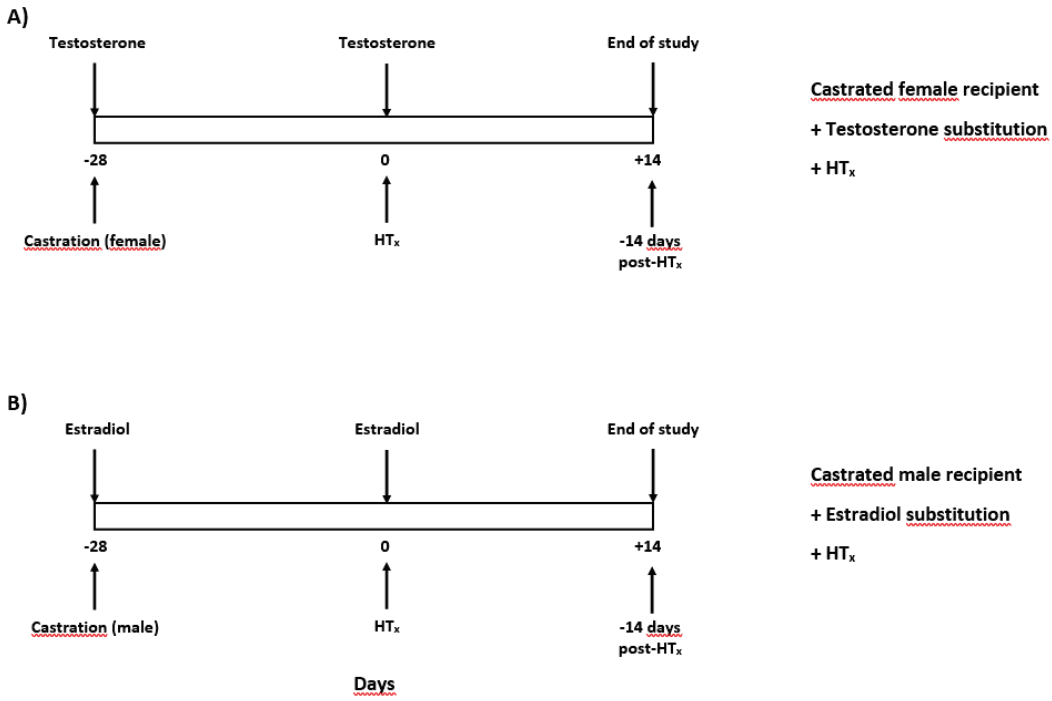

Fig. 2. An outline of the set of experiments evaluating the course of cardiac atrophy after heterotopic heart transplantation (HT_X) in Lewis rats after castration and exposed to substitution of steroid hormones of the opposite sex.

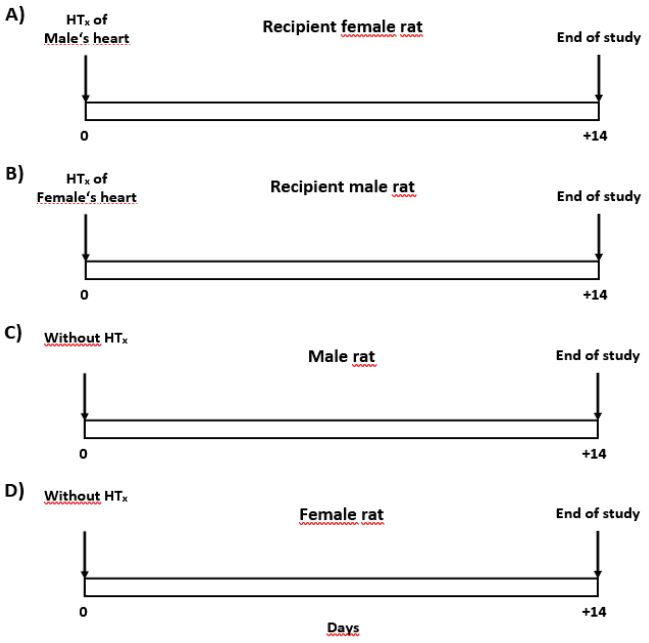

Fig. 3. An outline of the set of experiments evaluating the course of cardiac atrophy after heterotopic heart transplantation (HT_X) to Lewis rat recipients of the sex opposite to that of the donor´s heart.

Results

Fig. 4 summarizes results of our initial studies which evaluated the effectiveness of hormonal substitution by steroid hormones of the opposite sex. Within 7 days after castration of male as well as female Lewis rats, a profound decrease was observed in plasma testosterone, down to levels 6 to 8 times lower than observed in intact female Lewis rats. Testosterone substitution in castrated female Lewis rats increased within 2 days plasma testosterone to levels measured in intact male Lewis rats (Fig. 4A). Likewise, castration of female as well as male Lewis rats markedly decreased plasma estradiol levels, and estradiol substitution in castrated male Lewis rats increased plasma estradiol levels to levels that are comparable to those observed in intact female Lewis rats (Fig. 4B). Again, this occurred within 2 days after initiating of the substitution.

Table 1 collects the absolute values of whole HW, LVW, and RVW of the native and transplanted hearts 14 days after HT_X. The values for the native heart, either in the chest of the castrated recipient or in the chest of the intact control counterpart served as basal values (100 %) for evaluation of the process of cardiac atrophy and, when the same sex was compared, there were no significant differences between the values in the chest of castrated animals versus those in the chest of intact animals. As expected, the weight of the native hearts in the chest of the castrated recipients or in the chest of their intact control counterparts was significantly lower in female Lewis than in male Lewis rats.

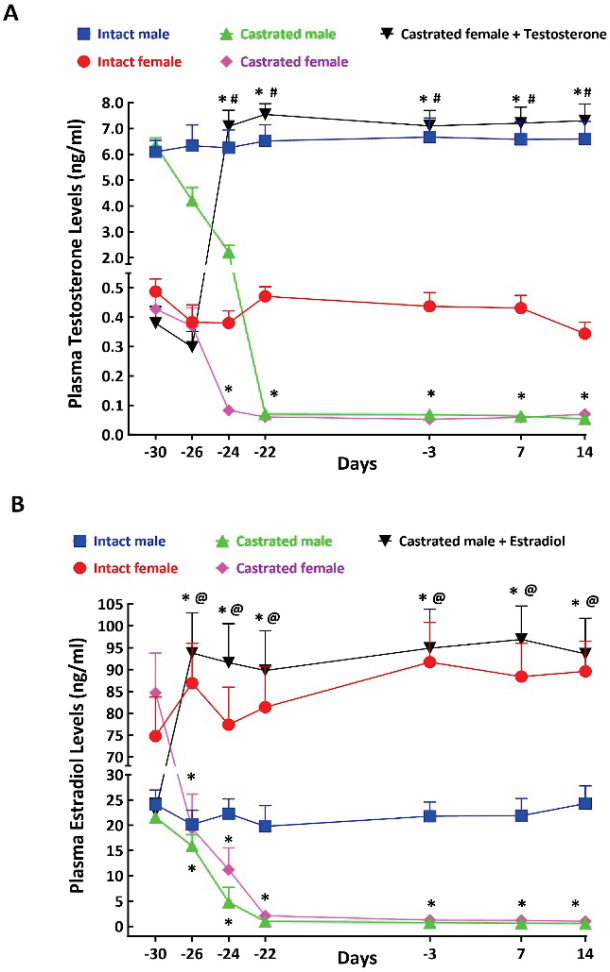

Fig. 4. Plasma levels of testosterone (**A**) and estradiol (**B**) in the series of studies evaluating the effectiveness of hormonal substitution with steroid hormones of the opposite sex. *P<0.05 compared with basal values (day -30, i.e. before castration). #P<0.05 compared with the values of intact female at the same time point. @ P<0.05 compared with the values of intact male at the same time point.

Table 1. The weight of the native (recipient) heart and the transplanted (donor heart and of the individual hert structural components after heterotopic heart transplantation (HT_x). Native heart values served as basal values (100 %) for evaluation of the process of cardiac atrophy in animals after HT_x.

	Parameter					
	HW (mg) (native)	HW (mg) (HT_x)	LVW (mg) (native)	LVW (mg) (HT_x)	RVW (mg) (native)	RVW (mg) (HT_x)
Castrated male recipient + estradiol substitution + HT_x of male donor´s heart	1024 ± 27	568 ± 16	691 ± 18	372 ± 16	182 ± 8	134 ± 5
Intact recipient female + HT_x of male donor´s heart		570 ± 18		374 ± 15		139 ± 8
Intact male without HT_x	1031 ± 29		692 ± 19		181 ± 7	
Castrated female recipient + testosterone supplementation + HT_x of female donor´s heart	762 ± 20*	657 ± 9*	509 ± 8*	447 ± 4*	137 ± 8*	123 ± 7*
Intact recipient male + HT_x of female donor´s heart		660 ± 10*		449 ± 6*		125 ± 5*
Intact female without HT_x	768 ± 23*		512 ± 10*		138 ± 6*	

Values are means ± SEM. HT_x, heterotopic heart transplantation; HW, whole heart weight; LVW, left ventricule weight; RVW, right ventricule weight. . * P<0.05 vs. male (i.e. effects sex differences on the parameter measured).

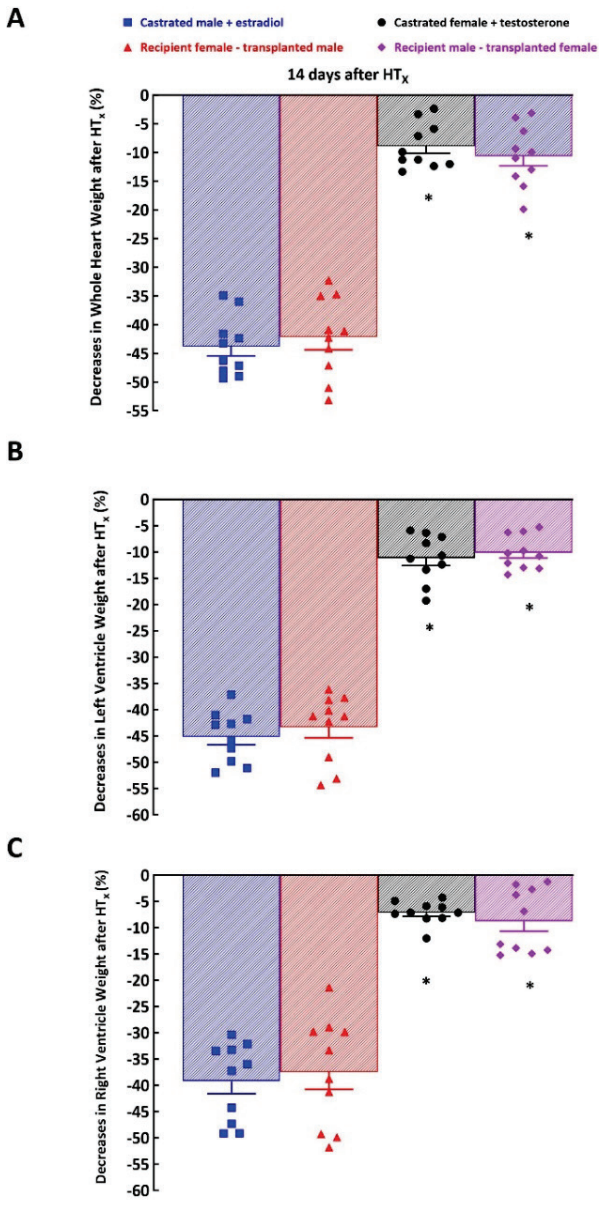

Fig. 5. Effects of either substitution of steroid hormones of the opposite sex in castrated animals or heterotopic heart transplantation (HT_x) into the recipient of the sex opposite to that of the donor´s heart on the course of cardiac atrophy in response to mechanical heart unloading induced by (HT_x) in male and female Lewis rats. Data are expressed as percent decreases compared with the native heart: (**A**) changes in whole heart weight, (**B**) changes in left ventricle weight, (**C**) changes in right ventricle weight. *P<0.05 compared with male animals.

As shown in Fig. 5A, 14 days after mechanical unloading induced by HT_X, female rats under both experimental set-ups (i.e., when the female heart was transplanted to either castrated female recipients exposed to testosterone substitution or to intact male recipients) displayed significantly lower decreases in whole HW when compared to the decreases observed in male rats exposed to the same experimental arrangements (-9 ± 1 and -11 + 1 vs. -44 ± 2 and -42 ± 2 %, p<0.05 in all cases). The dynamics of changes in LVW and RVW after HT_X were quite similar as those in whole HW (Figs 5B and 5C).

Fig. 6A shows whole HW values of the native heart normalized to tibia length (TL). This is the standard approach to assess cardiac mass in groups of animals with significant differences in BW. The HW/TL ratio values show that male rats (in both experimental arrangements) have higher mass of the native hearts when compared to those of female rats (again, in both experimental arrangements). As shown in Fig. 6B, 14 days after HT_X, the decreases in whole HW were more significant in male rats after mechanical unloading induced by HT_X, making this ratio significantly higher in the female than in male rats (again in both experimental arrangements).

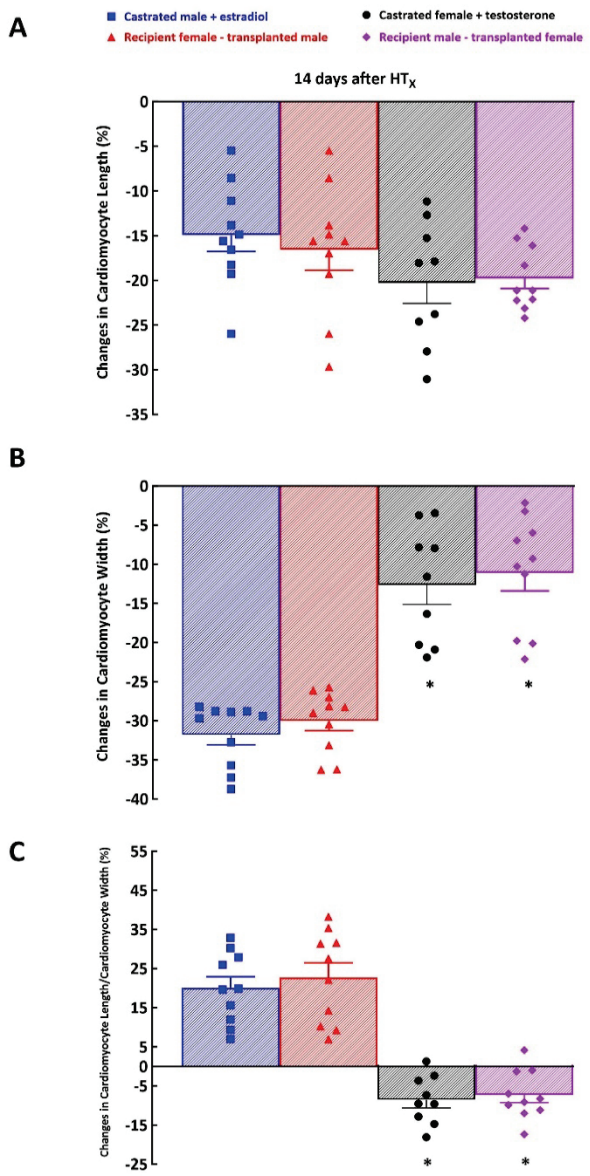

Fig. 6. Effects of either substitution of steroid hormones of the opposite sex in castrated animals or heterotopic heart transplantation (HT_x) into the recipient of the sex opposite to that of the donor´s heart on the course of the whole heart weight to tibia length ratio over 14 days after heterotopic heart transplantation (HT_x) in male and female Lewis rats. (**A**) values in native (i.e. orthotropic) heart, (**B**) values in transplanted (i.e. heterotopic) heart. *P<0.05 compared with male animals.

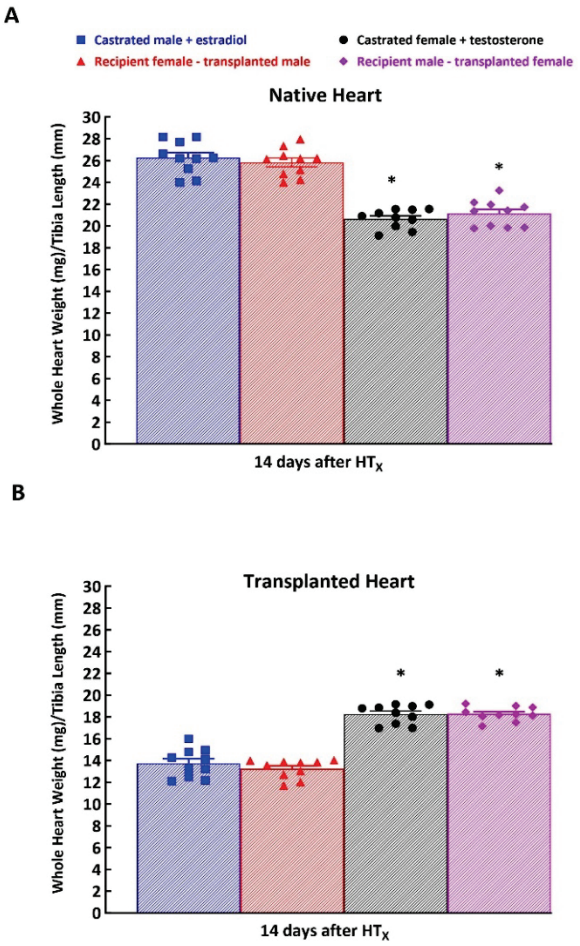

Fig. 7. Effects of either substitution of steroid hormones of the opposite sex in castrated animals or heterotopic heart transplantation (HT_x) into the recipient of the sex opposite to that of the donor´s heart on the course of myocyte size change in the left ventricle in response to mechanical heart unloading induced by heterotopic heart transplantation (HT_x) in male as well as in female Lewis rats. Data are expressed as percent decreases compared with the native heart: (**A**) changes in cardiomyocyte length, (**B**) changes in cardiomyocyte width, (**C**) changes in cardiomyocyte length to width ratio. *P<0.05 compared with male animals.

Table 2 summarizes the absolute values of the parameters of the myocyte size, specifically cardiomyocyte length (CL), cardiomyocyte width (CW), and the ratio of CL to CW in the native and transplanted hearts measured 14 days after HT_X. As can be inferred from the data, there were no significant differences between the values of the CL, CW and the CL to CW ratio in the chest of castrated animals when compared to the values measured in the chest of intact animals, similarly in male and female rats.

As shown in Fig. 7A, in all experimental groups, mechanical unloading induced by HT_X caused similar decreases in CL. In contrast, in female rats under both experimental arrangements (with female heart transplanted to either castrated female recipient exposed to testosterone substitution or to intact male recipients), HT_X caused significantly smaller decreases in CW when compared to male rats exposed to the same experimental conditions (-13 ± 2 and -11 + 1 vs. -32 ± 2 and -31 ± 1 %, $p<0.05$ in all cases) (Fig. 7B). As shown in Fig. 7C, under both experimental arrangements, the augmented decreases in CW in male rats when compared to those of female rats caused significant increases in CL to CW ratios in male rats when compared to female rats (+20 ± 1 and +23 + 2 vs. -7 ± 1 and -7 ± 1 %, $p<0.05$ in all cases).

Table 2. Cardiomyocyte length and cardiomyocyte width of the native (recipient) heart and the transplanted (donor) heart of the left ventricle after heterotopic heart transplantation (HT_x). Native heart values served as baseline (100 %) for evaluation of the process of cardiac atrophy in animals after HT_x.

	Parameter					
	CL (µm) (native)	CL (µm) (HT_x)	CW (µm) (native)	CW (µm) (HT_x)	CL/CW (native)	CL/CW (mg) (HT_x)
Castrated male recipient + estradiol substitution + HT_x of male donor´s heart	124.1 ± 1.7	104.9 ± 2.6	16.3 ± 0.4	10.6 ± 0.6	7.6 ± 0.2	9.9 ± 0.5
Intact recipient female + HT_x of male donor´s heart		103.4 ± 2.8		11.1 ± 0.5		9.4 ± 0.4
Intact male without HT_x	122.4 ± 1.9		16.6 ± 0.5		7.5 ± 0.2	
Castrated female recipient + testosterone supplementation + HT_x of female donor´s heart	119.8 ± 2.1	97.2 ± 2.1	16.2 ± 0.4	13.2 ± 0.3*	7.5 ± 0.3	7.3 ± 0.3*
Intact recipient male + HT_x of female donor´s heart		98.1 ± 1.8		13.4 ± 0.2*		7.4 ± 0.3*
Intact female without HT_x	120.1 ± 1.9		16.1 ± 0.5		7.5 ± 0.3	

Values are means ± SEM. Cl, cardiomyocyte length; CW, cardiomyocyte width; HT_x, heterotopic heart transplantation. * $P<0.05$ vs. Male (i.e. effects sex differences on the parameter measured).

Discussion

The critically important finding of the present study is that attenuation of unloading-induced cardiac atrophy in female rats when compared to male rats does not depend on the actions of sex hormones. If this were the case, the differences in the development of cardiac atrophy would exist in the hormones' presence and disappear after their removal [38].

This conclusion is based on our present results showing that neither castration of female rats combined with substitution of testosterone nor HT_X of female heart into the male recipient worsened the process of unloading-induced cardiac atrophy in female rats. Furthermore, since we showed that gonadectomy did not augment the process of unloading-induced cardiac atrophy induced by HT_X in female rats [36], we can conclude that attenuation of unloading-induced cardiac atrophy in female rats is not related to protective effects of estradiol or due to the absence of testosterone at concentrations observed in their male counterparts.

Of particular interest is our finding that testosterone substitution in castrated female rats did not exhibit any detrimental effects on the course of cardiac atrophy. Moreover, our present results show that neither castration of male rats combined with substitution of estradiol nor HT_X of male heart into the female recipient attenuated the process of unloading-induced cardiac atrophy in male rats. In this context, our recent study showed that gonadectomy did not diminish the process of unloading-induced cardiac atrophy after HT_X [36]. Thus, we can conclude that the augmented unloading-induced cardiac atrophy in male rats is not associated with detrimental actions of testosterone or the lack of protective effects of estradiol.

Our pertinent recent and present findings at the whole organ level are corroborated at the cardiomyocyte level. Again, this was seen under native conditions and also under conditions of each of the three experimental arrangements: in animals after gonadectomy, animals after gonadectomy with substitution of steroid hormone of the opposite sex, and in the experiment with the heart transplanted to the recipient of the opposite sex.

Based on the results of our present and our most recent study [36], we are convinced that sex-linked differences in the process of unloading-induced cardiac atrophy, specifically augmentation of this process in male animals, cannot be simply ascribed to the deleterious actions of testosterone in males or to the protective effects of estradiol in females. We are aware that this conclusion refers only to "activational" effects of sex hormones as described above and not to "organizational" effects of sex hormones, which persist long after sex hormones have been removed from circulation. It is believed that the latter effects are dominantly mediated by changes in DNA structure and chromatin remodeling i.e. by different epigenetic modification of DNA [34,38]. Our recent and present study aimed, first, to establish whether sex-linked differences in the development of unloading-induced cardiac atrophy do exist and, if this was the case, if they simply depend on environmental conditions dependent on plasma concentrations of sex hormones. Therefore, we cannot provide any further information regarding the mechanisms responsible for the

sex-linked differences in the course of the atrophy. Nevertheless, it is now recognized that numerous genetic as well as epigenetic mechanisms play a major role in mediating the sex differences [34,38] so that future studies are needed to address this very complex issue.

Our studies of animals which were either post gonadectomy on steroid hormone substitution of the opposite sex, or after HT_X to the recipient of the opposite sex, have unexpectedly generated noteworthy information. Transgender medical care is an area that is rapidly expanding, despite the fact that it is disregarded in the health care system. However, as reported from the Los Angeles Williams Institute of the University of California, about 0.6 % of the US adult population is transgender (https://williamsinstitute.law.ucla.edu/publications/trans-adults-united-states). Transgender individuals often undergo so called "gender-affirming hormone therapy" (GAHT), intended to elicit secondary sexual character-istics of the affirmed gender. It will be noticed that in those individuals, sex steroids are administered at large doses that are highly unphysiological [42]. There is growing concern about the long-term adverse effects of GATH, because recent evidence suggests that such treatment is associated with an increased risk of cardiovascular disease [42-45]. However, because of only limited information about the effects of cross-sex steroid application on the cardiovascular system, a call for more clinical as well as basic research in the field of transgender medicine is rising [42]. Our present findings clearly show that un-physiological concentrations of steroid hormones incompatible with the biological sex does not have any beneficial or detrimental effect on the course of unloading-induced cardiac atrophy, which suggests that the course of cardiac atrophy after HT_x is dominantly dependent on inherent properties of the donor´s (i.e. transplanted) heart. Our data suggests that in transgender individuals the course of cardiac atrophy after long-term LVAD would correspond to the actual biological sex and would not be altered by GAHT. However, it is important to admit that our present studies are relatively short-term and that in the long-term perspective GAHT could exhibit some detrimental effects either at the cellular or functional level. If our preclinical results are confirmed by clinical studies, this would imply that not only in women but also in so called "trans men" (sometimes identified as of male gender but assigned female sex at birth) undergoing GAHT, LVAD-induced cardiac atrophy would be attenuated when compared to men as well as so called "trans women" (someone who identifies as of female gender but was assigned male sex at birth).

Sex-linked differences in function of the human healthy heart and in HT: general considerations

It is known that after puberty LV mass shows sex-dependent differences, as the male cardiomyocyte undergoes greater hypertrophy than in the female. Moreover, the female heart is generally smaller but in proportion to smaller body size. There are also sex-linked differences in electrophysiology of the heart, e.g. in the action potential of parameters and ionic currents of cardiomyocytes. Specifically, the duration of action potential is longer in female cardiomyocytes, consistent with clinical observation that women have longer rate-corrected QT interval. Furthermore, there are multiple sex-linked differences in the Ca^{2+} handling in cardiomyocytes, leading to the differences in excitation-contracting coupling and contractility of cardiomyocytes. The sex differences in the healthy heart were recently appraised in a comprehensive review [46].

Regarding the sex-linked differences in HT_x, it should first be noted that in human medicine, the terms of sex and gender are often used interchangeably, which results in some misleading interpretations. *Sex* refers to an organism´s biological sex, while *gender* refers to roles associated with the sex of an individual and with social and cultural roles of the person. There are important sex-linked as well as gender-based differences of approach in the HT_x program. This issue is excellently handled in a recent review [47] and therefore only the most important sex- and gender-based differences are highlighted here. Sex-specific considerations in HT_X are divided into "Pre-Transplant", "Peri-Transplant" and "Post-Transplant" categories.

In the "Pre-Transplant" phase, women are often referred with a considerable delay to an advanced HF specialist, which results in a lower rate of LVAD implantation indications and translating to an unconscious bias against women in candidate selection for HT_x. These are gender-related rather than sex-related differences. In addition, in this phase women exhibit higher risk of allosensitization, which is related to a history of pregnancy. Moreover, women also display markedly higher challenges in gaining access to temporary mechanical circulatory support, and are exposed to a higher risk of complications. Such divergences can be described as typically sex-linked.

In the "Peri-Transplant" phase the major issue was the finding that if a female donor was used for a male recipient, reduced post-HT_x survival was reported. However, such sex-mismatching proved a misconception: it was later found that if appropriately sized female donor heart was used (similar size heart from a male donor was employed for male recipient), the outcomes observed would be equally good.

In the "Post-Transplant" phase, higher rates and severity of rejection in women is observed. On the contrary, women exhibit lower cardiac allograft vasculopathy and a lower rate of malignancies and cancer-related deaths. However, it should be noticed that the males undergoing HT_x tend to be older and have higher prevalence of obesity, diabetes, dyslipidemia and, in particular, a history of tobacco use. Therefore, it seems that in such cases we have to deal with gender-related rather than sex-related differences. Thus, it is apparent that sex-specific characteristics must be considered before application of HT_x in humans. Evidently, experimental, as well as clinical studies are needed to evaluate sex-linked differences in the treatment of advanced HF.

It is worthwhile to emphasize again that sex-linked differences in the cardiovascular system and cardiovascular diseases should not be solely ascribed to actions of estradiol and testosterone and the activity level of their receptors. Alternative mechanisms, such as the influence of other sex hormones, sex chromosome-linked genes, incomplete X-chromosomal gene inactivation, histone and DNA modifications, interactions of sex hormones with different neurohormonal systems in different organs must be taken into consideration. This extremely complex issue of sex differences in cardiovascular system was recently appraised in some comprehensive reviews [34,38,48].

Conclusions, merits and perspectives

Our present data clearly shows that the enhanced development of unloading-induced cardiac atrophy in female as compared to male rats does not depend on activational actions of sex hormones. Future studies are needed to evaluate more thoroughly the mechanisms responsible for the sex-linked differences in the course of unloading-induced cardiac atrophy. The information obtained in our recent and present preclinical studies should be considered in prospective clinical studies designed to explore the development of cardiac atrophy in response to LVAD-induced mechanical unloading and in the search for new therapeutic measures against this process.

Author contribution

All authors have read and approved the final version of the manuscript. D.M.K., I.M., and M.P. performed all experiments related to heterotopic heart transplantation and evaluation of plasma concentrations of sex hormones. P.K. performed all histological evaluations. P.Š. performed all sham operations and gonadectomy and participated in the preparation of tissue samples for histological evaluation. Z.V. performed all RIA analyses of steroid hormones. J.S., L.Č., and I.N. were responsible for conceptualization, data evaluation, writing of the original draft, and review of the manuscript. I.N. is also responsible for funding acquisition.

Conflict of Interest

There is no conflict of interest.

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All data and materials employed in the present study are available from the corresponding author upon reasonable request.

References

1. Frigerio M. Left Ventricular assist device: indication, timing, and management. Heart Fail Clin. 2021; 17: 619-634. https://doi.org/10.1016/j.hfc.2021.05.007
2. Varshney AS, DeFilippis EM, Cowger JA, et al. Trends and outcomes of left ventricular assist device therapy: JACC Focus Seminar. J Am Coll Cardiol. 2022; 79: 1092-1107. https://doi.org/10.1016/j.jacc.2022.01.017

3. Shah P, Yuzefpolskaya M, Hickey GW, et al. Twelfth Interagency Registry for Mechanically Assisted Circulatory Support Report: Readmissions After Left Ventricular Assist Device. Ann Thorac Surg. 2022; 113: 722-737. https://doi.org/10.1016/j.athoracsur.2021.12.011
4. Mehra MR, Nayak A, Desai AS. Life-Prolonging Benefits of LVAD Therapy in advanced heart failure: a clinician's action and communication aid. JACC Heart Fail. 2023; 11: 1011-1017. https://doi.org/10.1016/j.jchf.2023.05.013
5. Brinks H, Tevaearai H, Mühlfeld C, et al. The contractile function is preserved in unloaded hearts despite atrophic remodeling. J Thorac Cardiovasc Surg. 2009; 137: 742-6. https://doi.org/10.1016/j.jtcvs.2008.09.020
6. Diakos NA, Selzman CH, Sachse FB, et al. Myocardial atrophy and chronic mechanical unloading of the failing human heart: implications for cardiac assist device-induced myocardial recovery. J Am Coll Cardiol. 2014; 64: 1602-12. https://doi.org/10.1016/j.jacc.2014.05.073
7. Miyagawa S, Toda K, Nakamura T, et al. Building a bridge to recovery: the pathophysiology of LVAD-induced reverse modeling in heart failure. Surg Today. 2016; 46: 149-154. https://doi.org/10.1007/s00595-015-1149-8
8. Pokorný M, Cervenka L, Netuka I, et al. Ventricular assist devices in heart failure: how to support the heart but prevent atrophy? Physiol Res. 2014; 63: 147-56. https://doi.org/10.33549/physiolres.932617
9. Brinks H, Giraud MN, Segiser A, et al. Dynamic patterns of ventricular remodeling and apoptosis in hearts unloaded by heterotopic transplantation. J Heart Lung Transplant. 2014; 33: 203-10. https://doi.org/10.1016/j.healun.2013.10.006
10. Heckle MR, Flatt DM, Sun Y, et al. Atrophied cardiomyocytes and their potential for rescue and recovery of ventricular function. Heart Fail Rev. 2016; 21: 191-8. https://doi.org/10.1007/s10741-016-9535-x
11. Pham BN, Chaparro SV. Left ventricular assist device recovery: does duration of mechanical support matter?. Heart Fail Rev. 2019; 24: 237-244. https://doi.org/10.1007/s10741-018-9744-6
12. Burkhoff D, Topkara VK, Sayer G, Uriel N. Reverse remodeling with left ventricular assist devices. Circ Res. 2021; 128: 1594-1612. https://doi.org/10.1161/CIRCRESAHA.121.318160
13. Pamias-Lopez B, Ibrahim ME, Pitoulis FG. Cardiac mechanics and reverse remodelling under mechanical support from left ventricular assist devices. Front Cardiovasc Med. 2023; 10: 1212875. https://doi.org/10.3389/fcvm.2023.1212875
14. Drakos SG, Badolia R, Makaju A, et al. Distinct Transcriptomic and Proteomic Profile Specifies Patients Who Have Heart Failure With Potential of Myocardial Recovery on Mechanical Unloading and Circulatory Support. Circulation. 2023; 147: 409-424. https://doi.org/10.1161/CIRCULATIONAHA.121.056600
15. Chrysakis N, Xanthopoulos A, Magouliotis D, et al. Myocardial Recovery. Diagnostics (Basel). 2023; 13: 1504. https://doi.org/10.3390/diagnostics13081504
16. Soloff LA. Atrophy of myocardium and its myocytes by left ventricular assist device. Circulation. 1999; 100: 1012. https://doi.org/10.1161/circ.100.9.1011/-b
17. Tsuneyoshi H, Oriyanhan W, Kanemitsu H, et al. Does the beta2-agonist clenbuterol help to maintain the myocardial potential to recover during mechanical unloading? Circulation. 2005; 112 (Suppl): I51-6. https://doi.org/10.1161/CIRCULATIONAHA.104.525097
18. Birks EJ, Tansley PD, Hardy J, et al. Left ventricular assist device and drug therapy for the reversal of heart failure. N Engl J Med. 2006; 355: 1873-84. https://doi.org/10.1056/NEJMoa053063
19. Navaratnarajah M, Siedlecka U, Ibrahim M, et al. Impact of combined clenbuterol and metoprolol therapy on reverse remodeling during mechanical unloading. PLoS One. 2014; 9: e92909. https://doi.org/10.1371/journal.pone.0092909
20. Hu D, Li H, Yu H, et al. Clenbuterol Prevents Mechanical Unloading-Induced Myocardial Atrophy via Upregulation of Transient Receptor Potential Channel-3. Int Heart J. 2023; 64: 901-909. https://doi.org/10.1536/ihj.21-129
21. Rakusan K, Heron MI, Kolar F, Korecky B. Transplantation-induced atrophy of normal and hypertrophic rat hearts: effect on cardiac myocytes and capillaries. J Mol Cell Cardiol. 1997; 29: 1045-54. https://doi.org/10.1006/jmcc.1996.0350

22. Navaratnarajah M, Siedlecka U, Ibrahim M, et al. Impact of combined clenbuterol and metoprolol therapy on reverse remodeling during mechanical unloading. PLoS One. 2014; 9: e92909. https://doi.org/10.1371/journal.pone.0092909
23. Didié M, Biermann D, Buchert R, et al. Preservation of left ventricular function and morphology in volume-loaded versus volume-unloaded heterotopic heart transplants. Am J Physiol Heart Circ Physiol. 2013; 305: H533-41. https://doi.org/10.1152/ajpheart.00218.2013
24. Liu Y, Maureira P, Gauchotte G, et al. Effect of chronic left ventricular unloading on myocardial remodeling: Multimodal assessment of two heterotopic heart transplantation techniques. J Heart Lung Transplant. 2015; 34: 594-603. https://doi.org/10.1016/j.healun.2014.11.015
25. Oriyanhan W, Tsuneyoshi H, Nishina T, et al. Determination of optimal duration of mechanical unloading for failing hearts to achieve bridge to recovery in a rat heterotopic heart transplantation model. J Heart Lung Transplant. 2007; 26: 16-23. https://doi.org/10.1016/j.healun.2006.10.016
26. Muranaka H, Marui A, Tsukashita M, et al. Prolonged mechanical unloading preserves myocardial contractility but impairs relaxation in rat heart of dilated cardiomyopathy accompanied by myocardial stiffness and apoptosis. J Thorac Cardiovasc Surg. 2010; 140: 916-22. https://doi.org/10.1016/j.jtcvs.2010.02.006
27. Fu X, Segiser A, Carrel TP, et al. Rat heterotopic heart transplantation model to investigate unloading-induced myocardial remodeling. Front Cardiovasc Med. 2016; 3: 34. https://doi.org/10.3389/fcvm.2016.00034
28. Benke K, Sayour AA, Mátyás C, et al. Heterotopic Abdominal Rat Heart Transplantation as a Model to Investigate Volume Dependency of Myocardial Remodeling. Transplantation. 2017; 101: 498-505. https://doi.org/10.1097/TP.0000000000001585
29. Pokorný M, Mrázová I, Malý J, et al. Effects of increased myocardial tissue concentration of myristic, palmitic and palmitoleic acids on the course of cardiac atrophy of the failing heart unloaded by heterotopic transplantation. Physiol Res. 2018; 67:13-30. https://doi.org/10.33549/physiolres.933637
30. Pokorný M, Mrázová I, Kubátová H, et al. Intraventricular placement of a spring expander does not attenuate cardiac atrophy of the healthy heart induced by unloading via heterotopic heart transplantation. Physiol Res. 2019; 68: 567-580. https://doi.org/10.33549/physiolres.933936
31. Pokorný M, Mrázová I, Šochman J, et al. Isovolumic loading of the failing heart by intraventricular placement of a spring expander attenuates cardiac atrophy after heterotopic heart transplantation. Biosci Rep. 2018; 38: BSR20180371. https://doi.org/10.1042/BSR20180371
32. McDonagh TA, Metra M, Adamo M, et al. 2021 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure. Eur Heart J. 2021; 42: 3599-3726.
33. Clayton JA, Gaugh MD. Sex as a Biological Variable in Cardiovascular Diseases: JACC Focus Seminar 1/7. J Am Coll Cardiol. 2022; 79: 1388-1397. https://doi.org/10.1016/j.jacc.2021.10.050
34. Reue K, Wiese CB. Illuminating the mechanisms underlying sex differences in cardiovascular disease. Circ Res. 2022; 130: 1747-1762. https://doi.org/10.1161/CIRCRESAHA.122.320259
35. Regitz-Zagrosek V, Gebhard C. Gender medicine: effects of sex and gender on cardiovascular disease manifestation and outcomes. Nat Rev Cardiol. 2023; 20: 236-247. https://doi.org/10.1038/s41569-022-00797-4
36. Kolesár DM, Kujal P, Mrázová I, Pokorný M, Škaroupková P, Sadowski J, Červenka L, Netuka I. Sex-linked differences in cardiac atrophy after mechanical unloading induced by heterotopic heart transplantation. Physiol Res. 2024;73:9-25. https://doi.org/10.33549/physiolres.935217
37. Ostadal B, Netuka I, Maly J, et al. Gender differences in cardiac ischemic injury and protection--experimental aspects. Exp Biol Med (Maywood). 2009; 234: 1011-9. https://doi.org/10.3181/0812-MR-362
38. Regitz-Zagrosek V, Kararigas G. Mechanistic Pathways of Sex Differences in Cardiovascular Disease. Physiol Rev. 2017; 97: 1-37. https://doi.org/10.1152/physrev.00021.2015
39. Ono K, Lindsey ES. Improved technique of heart transplantation in rats. J Thorac Cardiovasc Surg. 1969; 57: 225-9. https://doi.org/10.1016/S0022-5223(19)42744-X
40. Vaněčková I, Husková Z, Vaňourková Z, Cervenka L. Castration has antihypertensive and organoprotective effects in male but not in female heterozygous Ren-2 rats. Kidney Blood Press Res. 2011; 34: 46-52. https://doi.org/10.1159/000322618

41. Koblihová E, Mrázová I, Vaňourková Z, et al. Sex-linked differences in the course of thioacetamide-induced acute liver failure in Lewis rats. Physiol Res. 2020; 69: 835-845. https://doi.org/10.33549/physiolres.934499
42. Shawky NM, Reckelhoff JF, Alexander BT, Yanes Cardozo LL. Insights Into the Cardiomodulatory Effects of Sex Hormones: Implications in Transgender Care. Hypertension. 2023; 80: 1810-1820. https://doi.org/10.1161/HYPERTENSIONAHA.123.19501
43. de Blok CJ, Wiepjes CM, van Velzen DM, et al. Mortality trends over five decades in adult transgender people receiving hormone treatment: a report from the Amsterdam cohort of gender dysphoria. Lancet Diabetes Endocrinol. 2021; 9: 663-670. https://doi.org/10.1016/S2213-8587(21)00185-6
44. Getahun D, Nash R, Flanders WD, et al. Cross-sex Hormones and Acute Cardiovascular Events in Transgender Persons: A Cohort Study. Ann Intern Med. 2018; 169: 205-213. https://doi.org/10.7326/M17-2785
45. Jackson SS, Brown J, Pfeiffer RM, et al. Analysis of Mortality Among Transgender and Gender Diverse Adults in England. JAMA Netw Open. 2023; 6: e2253687. https://doi.org/10.1001/jamanetworkopen.2022.53687
46. Prajapati C, Koivumäki J, Pekkanen-Mattila M, Aalto-Setälä K. Sex differences in heart: from basics to clinics. Eur J Med Res. 2022; 27: 241. https://doi.org/10.1186/s40001-022-00880-z
47. DeFilippis EM, Nikolova A, Holzhauser L, Khush KK. Understanding and Investigating Sex-Based Differences in Heart Transplantation: A Call to Action. JACC Heart Fail. 2023;11: 1181-1188. https://doi.org/10.1016/j.jchf.2023.06.030
48. Drury ER, Wu J, Gigliotti JC, Le TH. Sex differences in blood pressure regulation and hypertension: renal, hemodynamic, and hormonal mechanisms. Physiol Rev. 2024; 104: 199-251. https://doi.org/10.1152/physrev.00041.2022

Long-Term Adverse Effects of Perinatal Hypoxia on the Adult Pulmonary Circulation Vary Between Males and Females in a Murine Model

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Summary

Adverse events during the perinatal period are associated with an increased risk to develop cardiometabolic diseases later in life. We established a murine model to study long-term effects of perinatal hypoxia (PH) on the pulmonary circulation. We previously demonstrated that PH led to an impaired regulation of pulmonary vascular tone in adulthood, linked to alterations in K^+ channels in males and in the nitric oxide (NO)/cyclic guanosine monophosphate pathway in females. Moreover, simultaneous administration of inhaled NO (iNO) during PH exposure prevented adverse effects of PH on adult pulmonary vasculature in females. The present study showed that PH induced a significant increase in right ventricular pressure in males and females, and an enhanced sensitivity to acute hypoxia in females. PH significantly reduced acetylcholine-induced relaxation in pulmonary artery, to a greater extent in females than in males. PH led to right ventricular hypertrophy in adulthood, appearing earlier in males than in females. Morphometric measurements showed a significant increase in the number of 25-75-µm pulmonary vessels in male lungs following PH, probably resulting in increased pulmonary vascular resistance. The effects of prolonged hypoxia in adulthood differed between males and females. Perinatal iNO during PH prevented PH-induced alterations in the cardiopulmonary system, whereas perinatal iNO alone could have some adverse effects. Therefore, PH led to long-lasting alterations in the regulation of adult pulmonary circulation, which vary between males and females. In males, the increased pulmonary vascular resistance was associated with morphological changes besides functional alterations, whereas females showed an important pulmonary vascular dysfunction.

Keywords

Perinatal hypoxia • Pulmonary circulation • Endothelium-dependent relaxation • Phosphodiesterases • Sex differences

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Introduction

Adverse events occurring *in utero* or soon after birth are associated with an increased risk of developing cardiometabolic diseases later in life [1]. Chronic pulmonary vascular diseases and abnormal pulmonary vasoreactivity in adulthood may be associated with a hypoxic insult occurring around birth [2,3]. Individuals born in a hypoxic environment show later in life an exaggerated pulmonary hypertensive response following a re-exposure to hypoxia [2,4-8].

Jan Herget and colleagues have extensively studied the effects of perinatal and adult exposure to chronic hypoxia on the pulmonary circulation in rats. They were particularly interested in the nitric oxide (NO) signaling pathway, which plays a key role in the regulation of pulmonary vascular tone and the response to hypoxia, as well as in adult pulmonary arterial hypertension (PAH) and persistent pulmonary hypertension of the newborn (PPHN) [9]. They found increased production of reactive oxygen species (ROS) [10,11] and NO, due to transient induction of inducible NO synthase (iNOS) in the pulmonary vascular wall [12,13], during early phase of chronic hypoxia in adulthood. NO can exert both protective and adverse effects, depending on the balance between NO and ROS [9]. The antioxidant N-acetylcysteine, administered during early phase of chronic hypoxia, or a combined

treatment with L-arginine and the phosphodiesterase 5 (PDE5) inhibitor sildenafil during hypoxic exposure were able to limit adverse effects of chronic hypoxia in adulthood [10,14]. They also demonstrated long-term effects of perinatal hypoxia (PH) on adult pulmonary circulation, by increasing pulmonary vessels' basal tone and reducing their reactivity to angiotensin II [15]. They showed that chronic prenatal hypoxia increased fetoplacental vascular resistance and vasoconstrictive response to angiotensin II or acute hypoxia, which could lead to placental hypoperfusion and impaired fetal nutrition and growth [16]. They were also interested in sex differences in the regulation and alterations of pulmonary circulation. They showed that females are more sensitive than males to late effects of PH on pulmonary vasculature and that these effects are blunted by the presence of ovaries during maturation [17].

Despite extensive research, the mechanisms contributing to the long-term effects of PH on the pulmonary circulation are still incompletely understood. Moreover, sex differences remain largely understudied.

We have previously established a murine model to investigate long-lasting effects of PH. We showed that PH resulted in impaired regulation of pulmonary vascular tone in adulthood [18-20]. PH triggered alterations in K^+ channels of adult pulmonary artery smooth muscle cells in males [18,19], and in the NO/ cyclic guanosine monophosphate (cGMP) pathway in females [20]. Adult females born in hypoxia displayed higher systolic right ventricular pressure (RVP) in normoxia and an increased sensitivity to acute hypoxia compared to controls [20]. Moreover, PH decreased the acetylcholine-induced endothelium-dependent relaxation of pulmonary artery (PA) in females. The vasorelaxant response to acetylcholine was restored by pirenzepine or telenzepine, selective antagonists of the muscarinic receptor M1 (M1AChR), which mediates vasoconstrictive effects of acetylcholine. The PDE1 inhibitor vinpocetine also reversed the decrease in endothelium-dependent relaxation following PH, suggesting that M1AChR-mediated impairment of acetylcholine-induced relaxation was due to activation of the calcium-dependent PDE1 [20]. Therefore, a perinatal reduction in oxygen supply leads to pulmonary vascular dysfunction, which seems associated with permanent alterations in muscarinic receptors and their effectors, in particular PDEs.

We also showed that simultaneous administration of inhaled NO (iNO) during the perinatal exposure to hypoxia was able to prevent adverse effects of PH on adult pulmonary vasculature in females [21]. Perinatal iNO restored acetylcholine-induced relaxation and prevented the development of right ventricular (RV) hypertrophy in old females [21].

A transient exposure to hypoxia during a critical period of development of the lung vasculature resulted in a definitive imprint leading to altered regulation of pulmonary vascular tone later in life. Consequently, individuals having suffered from PH could be at risk to have an altered regulation of pulmonary circulation and to develop pulmonary vascular pathologies in adulthood. This is more likely to occur during/following re-exposure to clinical conditions associated with hypoxia, such as sleep apnea, chronic or acute pulmonary diseases, or exposure to high altitude.

The present study was designed to further investigate long-term effects of PH on adult pulmonary circulation in males and females. The effects of adult (re-)exposure to acute or prolonged hypoxia and/or aging were assessed on several parameters.

Methods

Animal model

All experimental procedures were approved and carried out in accordance with the Swiss Veterinarian Animal Care Office (authorization numbers VD1454, VD2170, VD2622). C57BL/6 pregnant mice were purchased from Harlan (Horst, Netherlands). They were all fed *ad libitum* and exposed to day-night cycles. PH was induced as previously described [18,20,21]. Pregnant mice were placed under hypoxia (13 % O_2) 5 days before delivery (from D16 of gestation) and left with their litter for 5 days after birth. Pups were then bred in normoxia (21 % O_2) until adulthood. The timing of exposure to hypoxia was chosen to cover the lung vasculogenesis, during which the functional units of gas exchange develop [22]. Pups born and grown in normoxia were used as controls (Ctr). Mice were studied at 5-6 months ("young") or 12-15 months ("old"). Mice from different litters and cages were randomly assigned to the different experiments.

To investigate the effects of chronic hypoxia in adulthood, some adult mice were (re-)exposed to 13 % O_2 for 5 days and studied at the end of the exposure period.

Some mice were treated with iNO (10ppm) during the 10-day perinatal hypoxic/normoxic exposure, as previously published [21].

The different experimental groups are presented at Table 1.

Table 1. Experimental groups and related treatments

Group	Ctr	PH	Ctr+AH	PH+AH	Ctr+NO	PH+NO
Perinatal exposure (10 days)	21 % O_2	12 % O_2	21 % O_2	12 % O_2	21 % O_2 NO 10ppm	12 % O_2 NO 10ppm
Adult hypoxia (5days)	-	-	12 % O_2	12 % O_2	-	-

Anatomical data

The number of alive pups per litter was recorded at postnatal day 5 (P5). Body weight was recorded at P5 or in adulthood. After removal of the auricles, the right ventricle (RV) was separated from the left ventricle plus septum (LV+S) and both were weighed to determine the RV/(LV+S) ratio (Fulton index), which is used as an index of RV hypertrophy.

Hemodynamic measurements

Hemodynamic measurements were performed as previously described [20]. Adult mice were anaesthetized with ketamine/xylazine (100 mg/kg and 10 mg/kg i.p., respectively) and placed on a heating board, to prevent hypothermia, under a small Plexiglas® hood to control oxygen administration. Body temperature was monitored using a rectal probe (ADInstruments, Hastings, UK). Closed-chest measurements were performed using a Millar mikro-tip® 1.4 F catheter (Millar Instruments, Houston, TX) inserted into the right jugular vein and advanced into the RV for measurement of RVP. The catheter was connected to a pressure transducer, and pressure signals recorded using a MacLab A/D converter (ADInstruments). Systolic RVP is used as an indirect indicator of pulmonary systolic artery pressure [23]. Baseline systolic RVP was recorded in normoxia (21 % O_2) and under acute hypoxia (→2 % O_2). Gas mixture was controlled using an oxygen monitor mono2® (Roche, Bioelectronics, Switzerland). The response to iNO was also assessed by adding NO (10-60ppm) to the 12 % O_2 gas mixture. NO concetration was controlled using a SensorNox® analyzer (SensorMedics BV, Bilthoven, Netherlands).

Isolated vessel tension studies

The PA vasoreactivity was assessed as previously described [18,20]. Adult mice were administered a lethal dose of pentobarbital (1 g/kg i.p.) and the main PA was immediately harvested. Vascular rings were suspended into vertical organ chambers filled with 10 ml modified Krebs-Ringer solution maintained at 37 °C and aerated with 95 % O_2-5 % CO_2 (pH 7.4) [20]. Vessels were brought to their optimal resting tension after two 0.5g stretches. After equilibration, N^G-nitro-L-arginine (NLA, 10^{-4}M; except for dose-response to acetylcholine) and/or indomethacin (10^{-5}M) were added to exclude possible interference of endogenous NO and prostanoids. Vascular rings were then pre-constricted with phenylephrine (10^{-5}M) before addition of cumulative doses of acetylcholine or 8-bromo-cGMP. In some experiments, acetylcholine-induced relaxation was tested after preincubation with the PDE inhibitors 3-isobutyl-1-methylxanthine (IBMX, 10^{-6}M), 8-methoxymethyl-3-isobutyl-1-methylxanthine (8-MM-IBMX, $3x10^{-6}$M), sildenafil (10^{-8}M) or milrinone ($3x10^{-7}$M). Change in tension induced by the vasodilator was expressed as percentage of the initial contraction induced by phenyl-ephrine. Area under the curve (AUC) was calculated from each dose-response curve using GraphPad Prism 10.2.3 (GraphPad Software).

Cardiopulmonary parameters assessment

Cardiopulmonary parameters were recorded using a non-invasive pulse oximeter (MouseOx®, Red Box Direct Limited) during the transition from normoxia to hypoxia (13 % O_2) in anaesthetized mice placed on a heating board under a small Plexiglas® hood, or in conscious mice placed in a restraining tube. This non-invasive sensor clip, specially designed for small rodents, allowed simultaneous recordings of arterial O_2 saturation (O_2-sat), pulse rate and breath rate. The clip was placed on the leg in anesthetized mice and on tail in conscious mice.

Cardiopulmonary data were also recorded in anesthetized mice following a 30-min swim-ming challenge in normoxic or hypoxic (13 % O_2) conditions.

Table 2. Anatomical data

	Females						Males					
group	**Ctr**	**PH**	**Ctr+AH**	**PH+AH**	**Ctr+NO**	**PH+NO**	**Ctr**	**PH**	**Ctr+AH**	**PH+AH**	**Ctr+NO**	**PH+NO**
Young mice (5-6 months)												
n	25	20	30	21	6	13	42	61	20	20	5	17
Body (g)	22.8 ± 0.4	22.6 ± 0.4	21.9 ± 0.3	21.4 ± 0.2 †	24.1 ± 0.5	23.7 ± 0.4	29.9 ± 0.4 §	30.4 ± 0.4 §	29.3 ± 0.5 §	27.6 ± 0.5 †‡§	33.5 ± 1.1 *§	33.9 ± 0.8 †§
Heart (mg)	100.1 ± 1.8	97.2 ± 1.2	99.5 ± 2.6	96.2 ± 0.8	94.5 ± 1.4 *	92.5 ± 1.5 †	132.1 ± 2.6 §	127.0 ± 1.9 §	132.0 ± 3.2 §	122.0 ± 3.1 ‡§	117.5 ± 2.4 *§	121.2 ± 2.1 †§
RV (mg)	23.0 ± 0.5	22.7 ± 0.6	27.1 ± 1.0 *†	25.5 ± 0.5 †	19.2 ± 0.4 *	20.5 ± 0.6 †	31.4 ± 0.8 §	32.1 ± 0.5 §	35.3 ± 0.9 *†§	31.9 ± 1.2 ‡§	24.8 ± 0.5 *§	25.9 ± 0.6 †§
LV+S (mg)	77.1 ± 1.5	74.5 ± 0.9	72.4 ± 2.0	70.7 ± 0.9 †	75.3 ± 1.4	72.1 ± 1.3	100.7 ± 2.1 §	94.9 ± 1.5 *§	96.6 ± 2.7 §	90.2 ± 2.1 §	92.7 ± 2.0 *§	95.3 ± 1.7 §
RV/LV+S	0.300 ± 0.006	0.305 ± 0.008	0.376 ± 0.010 *†	0.362 ± 0.010 †	0.256 ± 0.008 *	0.285 ± 0.010 ‡	0.314 ± 0.006	0.341 ± 0.006 *§	0.369 ± 0.011 *†	0.353 ± 0.009	0.268 ± 0.005 *	0.273 ± 0.005 †
Old mice (12-15 months)												
n	11	15	15	15	10	17	17	16	24	18	11	
Body (g)	26.8 ± 1.1 #	28.2 ± 0.9 #	25.6 ± 0.9 #	25.2 ± 0.7 †#	27.0 ± 1.0 #	27.3 ± 0.6 #	37.8 ± 1.5 #§	39.2 ± 1.6 #§	35.7 ± 1.1 #§	36.5 ± 1.3 #§	36.2 ± 1.0 §	
Heart (mg)	106.6 ± 3.0	107.3 ± 1.9 #	102.1 ± 3.1	109.9 ± 3.7 #	102.2 ± 3.5	108.7 ± 1.8 #	140.9 ± 4.2 §	141.7 ± 3.7 #§	137.9 ± 4.1 §	135.4 ± 5.6 #§	124.8 ± 2.8 *§	
RV (mg)	23.1 ± 1.0	25.2 ± 0.4 #	24.4 ± 0.8 #	28.2 ± 1.2 †‡#	21.3 ± 0.6 #	23.2 ± 0.5 †‡#	32.1 ± 1.2 §	33.8 ± 1.2 §	33.1 ± 1.0 §	31.4 ± 1.2	27.0 ± 1.0 *§	
LV+S (mg)	83.5 ± 2.3 #	82.1 ± 1.8 #	77.7 ± 2.3	81.7 ± 2.7 #	80.9 ± 3.0	85.5 ± 1.5 #	108.9 ± 3.2 #§	108.0 ± 3.3 #§	104.8 ± 3.3 §	104.0 ± 4.6 #§	97.8 ± 2.0 *§	
RV/LV+S	0.276 ± 0.009 #	0.309 ± 0.008 *	0.314 ± 0.006 *#	0.345 ± 0.007 †‡	0.264 ± 0.006	0.271 ± 0.004 †	0.295 ± 0.008	0.316 ± 0.012	0.319 ± 0.008 *#	0.304 ± 0.007 #§	0.276 ± 0.007	

Ctr, control mice; PH, perinatal hypoxia; AH, adult chronic hypoxia; NO, perinatal inhaled nitric oxide; RV, right ventricle; LV+S, left ventricle plus septum.
Statistical difference between two groups was determined using an unpaired t test with Welch's correction. Significant difference ($p<0.05$): * PH or Ctr+AH or Ctr+NO versus Ctr; † PH+AH or Ctr+AH or PH+NO versus PH; ‡ PH+AH versus Ctr+AH or PH+NO versus Ctr+NO; # old versus young; § males versus female

Lung morphometry

Adult mice were anaesthetized with ketamine/xylazine (100 mg/kg and 10 mg/kg i.p., respectively) and placed in a supine position. Lungs were prepared as previously published [24]. After careful punctuation of the diaphragm to collapse the lungs, the air space was filled by tracheal instillation with freshly prepared paraformaldehyde 4 % in phosphate buffered saline (PBS) at a constant pressure of 20 cm H_2O. This water pressure allows the lung to reach roughly its mid-respiratory volume. The pressure was maintained throughout the preparation to prevent the lungs from recoiling. Once the lungs were filled, the trachea was ligated, and the heart-lung unit retrieved. After a 2h fixation in paraformaldehyde, the lungs were progressively dehydrated using ethanol and Histoclear before paraffin-embedding. Five-µm lung sections were immunostained using an alpha-smooth muscle actin monoclonal antibody (Sigma, A-2457, 1:400, mouse), aminoethylcarbazol and hematoxylin. Areas of 1.5 x 1.0 mm of lung parenchyma were systematically, randomly photographed in a meandering order on the Polyvar microscope using the 10x objective [25]. After image analysis, the vessels were categorized by size. Data are expressed as number of vessels per area analyzed.

Statistical analysis

Data are expressed as mean ± SEM, unless otherwise specified. Results were analyzed using GraphPad Prism 10.2.3. The tests used for statistical analyses were mentioned in the legend of each figure or table. $P<0.05$ was considered statistically significant.

Results

Anatomical data

The number of alive pups per litter recorded at P5 was lower in PH than Ctr offspring (median (range): 6 (0-10) in Ctr (n=186) and 4 (0-8) in PH (n=265), $p<0.0001$, Mann-Whitney test). At P5, PH pups had a reduced body weight (2.90±0.03 g in Ctr (n=119) and 2.60±0.07 g in PH (n=46), p=0.0002, Mann-Whitney test) and an increased hematocrit (20.9±0.6 % in Ctr (n=8) and 26.7±0.6 % in PH (n=7), p=0.0003, Mann-Whitney test).

Table 2 presents anatomical data from 5-6-month-old ("young") and 12-15-month-old ("old") mice. The difference in body weight between Ctr and PH mice was no longer present in adulthood. At 5-6 months, the RV/(LV+S) ratio was significantly increased following PH in males, whereas no significant difference was found between PH and Ctr females. At 12–15 months however, the Fulton index was significantly higher in PH than Ctr females, probably because of significant increase in RV weight with aging in PH, but not Ctr females.

At both ages, adult hypoxia (AH) significantly increased RV weight and the Fulton index compared to normoxia in all groups except in PH males. These parameters were significantly higher in Ctr+AH than PH mice. AH significantly reduced body weight in young and old PH+AH females and young PH+AH males compared to PH mice, but not in Ctr+AH mice.

Heart weight was significantly increased with aging only in PH mice, with or without re-exposure to hypoxia.

Perinatal exposure to iNO resulted in several anatomical changes compared to Ctr or PH mice. In young mice exposed to perinatal iNO, body weight was significantly increased in males, but not in females, while heart weight and RV weight were significantly decreased in Ctr+NO and PH+NO mice. The Fulton index was significantly reduced in Ctr+NO males and females, and PH+NO males, but not in PH+NO females. In old mice however, the Fulton index and RV weight were significantly reduced in PH+NO females.

Hemodynamic measurements

RVP measured in PH mice, under normoxia or acute hypoxia, was significantly higher than in Ctr mice (Fig.1A). In normoxia, RVP was higher in Ctr females than Ctr males, but lower in PH females than PH males, whereas no significant difference was found between males and females under hypoxia (Fig.1A). A 5-day (re-)exposure to hypoxia resulted in a significant rise in RVP in Ctr+AH and PH+AH males, under normoxia or acute hypoxia (Fig.1B). In normoxia, RVP was significantly higher in Ctr+AH than PH males but not under hypoxia. An acute exposure to hypoxia during hemodynamic measurements induced in all groups a significant increase in RVP compared to normoxia (Fig.1A-B). However, this increase was significantly higher in PH females than in other groups, whereas in males, acute hypoxia led to a similar increase in all groups (Fig.1C). In males, increasing concentrations of iNO under hypoxia led to a decrease in RVP in all groups, but did not allow to reach RVP values below those measured in normoxia (Fig. 1D). When applied in normoxia, iNO did not influence RVP in all male groups (data not shown).

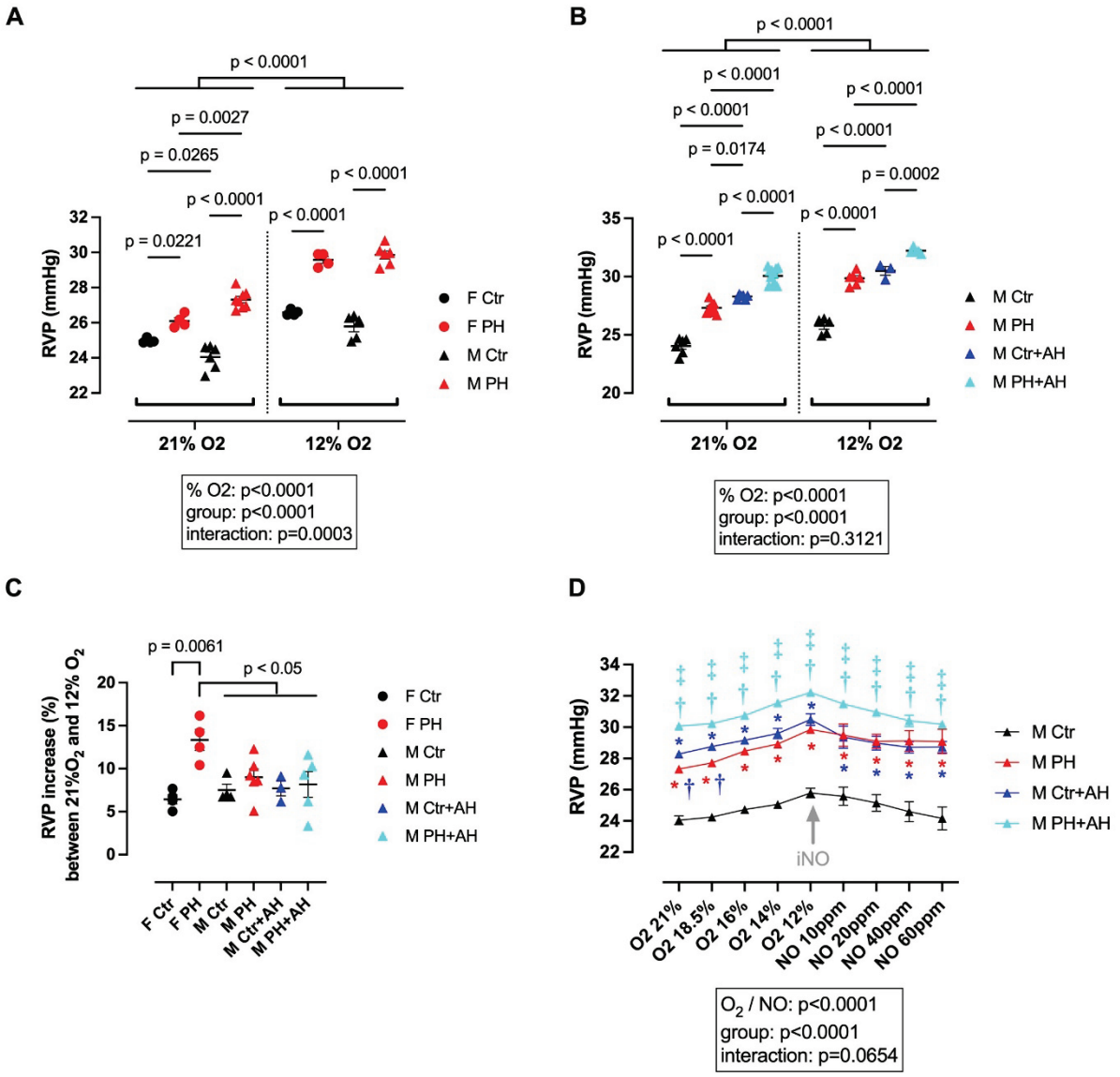

Fig. 1. Systolic right ventricular pressure (RVP) measured under normoxia (21 % O_2) or acute hypoxia (12 % O_2) in males (M) and females (F). Closed-chest measurements were performed in adult mice born in normoxia (Ctr) or hypoxia (PH), with or without a 5-day exposure to chronic hypoxia in adulthood (AH). Graphs **A-C** present individual values with bar at mean ± SEM. (**A**) RVP in Ctr and PH males and females (n=4-8); (**B**) RVP in Ctr and PH males with or without AH (n=3-11); data were analyzed by two-way ANOVA (results are shown below) with Sidak's multiple comparison test (significant p values are reported on the graph). (**C**) RVP increase between normoxia and acute hypoxia, expressed as percentage of RVP in normoxia (n=3-6); data were analyzed by one-way ANOVA and unpaired t tests with Welch's correction to compare two groups (significant p values are reported on the graph). (**D**) RVP measured in males during the transition from normoxia to acute hypoxia, followed by addition of increasing concentrations of inhaled nitric oxide (iNO) in the 12 % O_2 gas mixture; data are expressed as mean ± SEM (n=3-11) and were analyzed by two-way ANOVA (results are shown below) with Sidak's multiple comparison test; significant difference (p<0.05): * PH or Ctr+AH versus Ctr; † PH+AH or Ctr+AH versus PH; ‡ PH+AH versus Ctr+AH.

Isolated vessel tension studies

The resting tension measured in PA rings was similar between Ctr and PH mice (0.38±0.02 g in Ctr females (n=7), 0.37±0.02 g in PH females (n=6), 0.38±0.02 g in Ctr males (n=7), 0.40±0.01 g in PH males (n=16)). Acetylcholine-induced relaxation was significantly reduced in PA of PH versus Ctr mice (Fig. 2A-B). The decrease observed in AUC between Ctr and PH mice was greater in females (-28 %) than in males (-14 %). A 5-day (re-)exposure to hypoxia led to similar dose-response curves in Ctr+AH and PH+AH mice (Fig. 2A-B). The resulting relaxation was significantly improved in PH+AH versus PH mice, and Ctr+AH versus PH mice (Fig. 2A-B); in contrast, the response to low concentrations of acetylcholine was reduced in Ctr+AH versus Ctr females (significant difference at $3x10^{-8}$ and 10^{-7} M) (Fig. 2A), although AUC did not significantly differ between the two groups (p=0.0519).

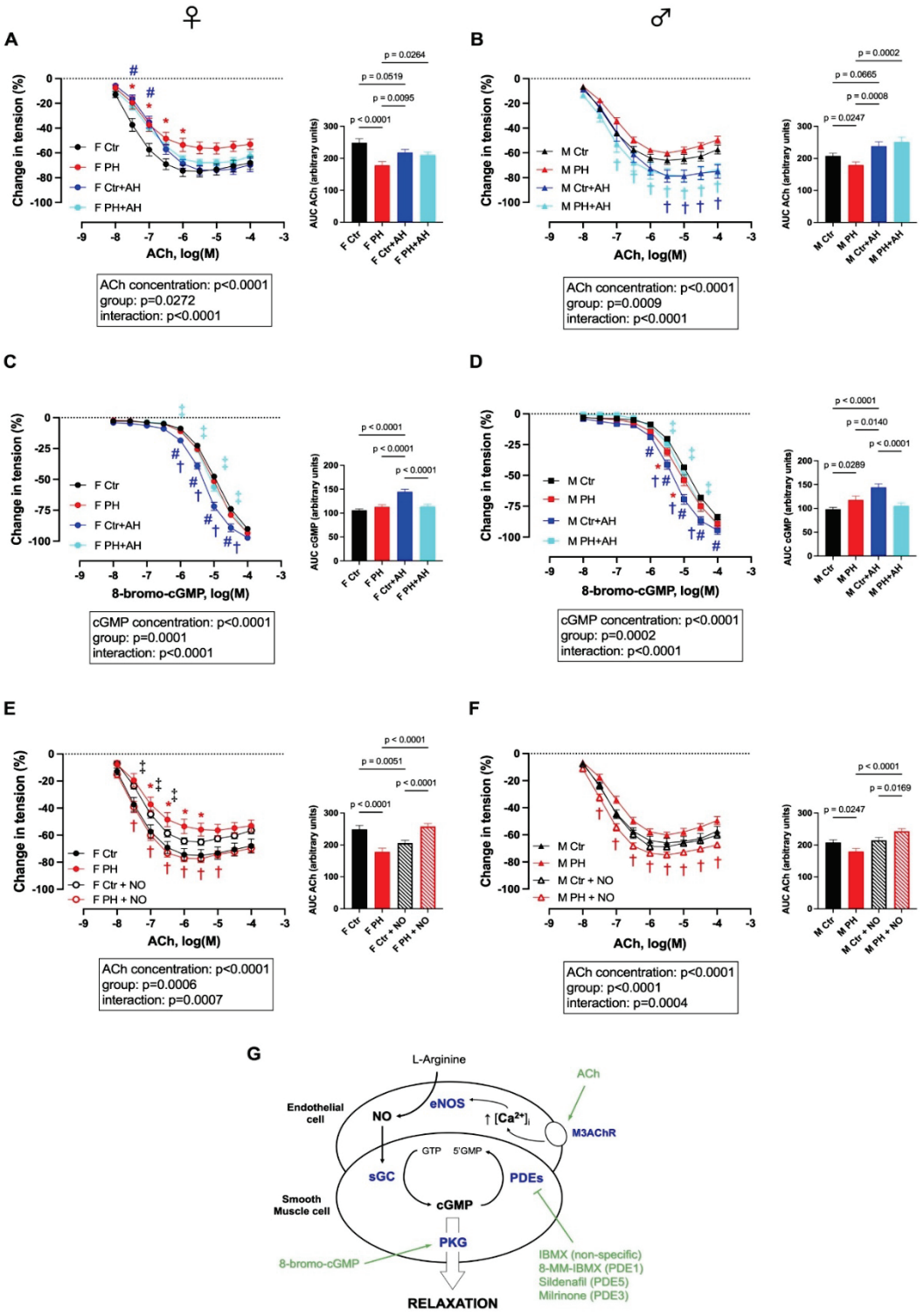

Fig. 2. Relaxation induced by cumulative doses of acetylcholine (ACh) or 8-bromo-cGMP in pulmonary arteries of males (M) and females (F) born in normoxia (Ctr) or hypoxia (PH), with or without a 5-day exposure to chronic hypoxia in adulthood (AH), or with perinatal exposure to inhaled nitric oxide (NO). Vascular rings were pre-constricted with phenylephrine 10^{-5}M. Data are expressed as mean ± SEM of the percentage of change in tension induced by the vasodilator (**A** n=6-8; **B** n=7-16; **C** n=5-9; **D** n=7-9; **E** n=6-14; **F** n=7-16). Data were analyzed by two-way ANOVA (results are shown below each graph) with Sidak's multiple comparison test; significant difference ($p<0.05$): * PH versus Ctr; # Ctr+AH vs Ctr; † PH+AH or Ctr+AH or PH+NO versus PH; ‡ PH+AH versus Ctr+AH or PH+NO versus Ctr+NO. The corresponding AUC were presented next to each graph; data are expressed as mean ± SEM and were analyzed by unpaired t tests with Welch's correction to compare two groups (significant p values are reported on each graph) (**G**). Schematic representation of the NO/cGMP relaxing pathway and interactions with pharmacological agents. eNOS, endothelial nitric oxide synthase; M3AChR, muscarinic acetylcholine receptor M3; PDEs, phosphodiesterases; PKG, cGMP-dependent protein kinase; sGC, soluble guanylyl cyclase. Dose-response curves to ACh assessed in Ctr and PH females were previously published in Peyter *et al.* [20]. Dose-response curves to ACh assessed in Ctr, PH and PH+NO females were previously published in Peyter *et al.* [21].

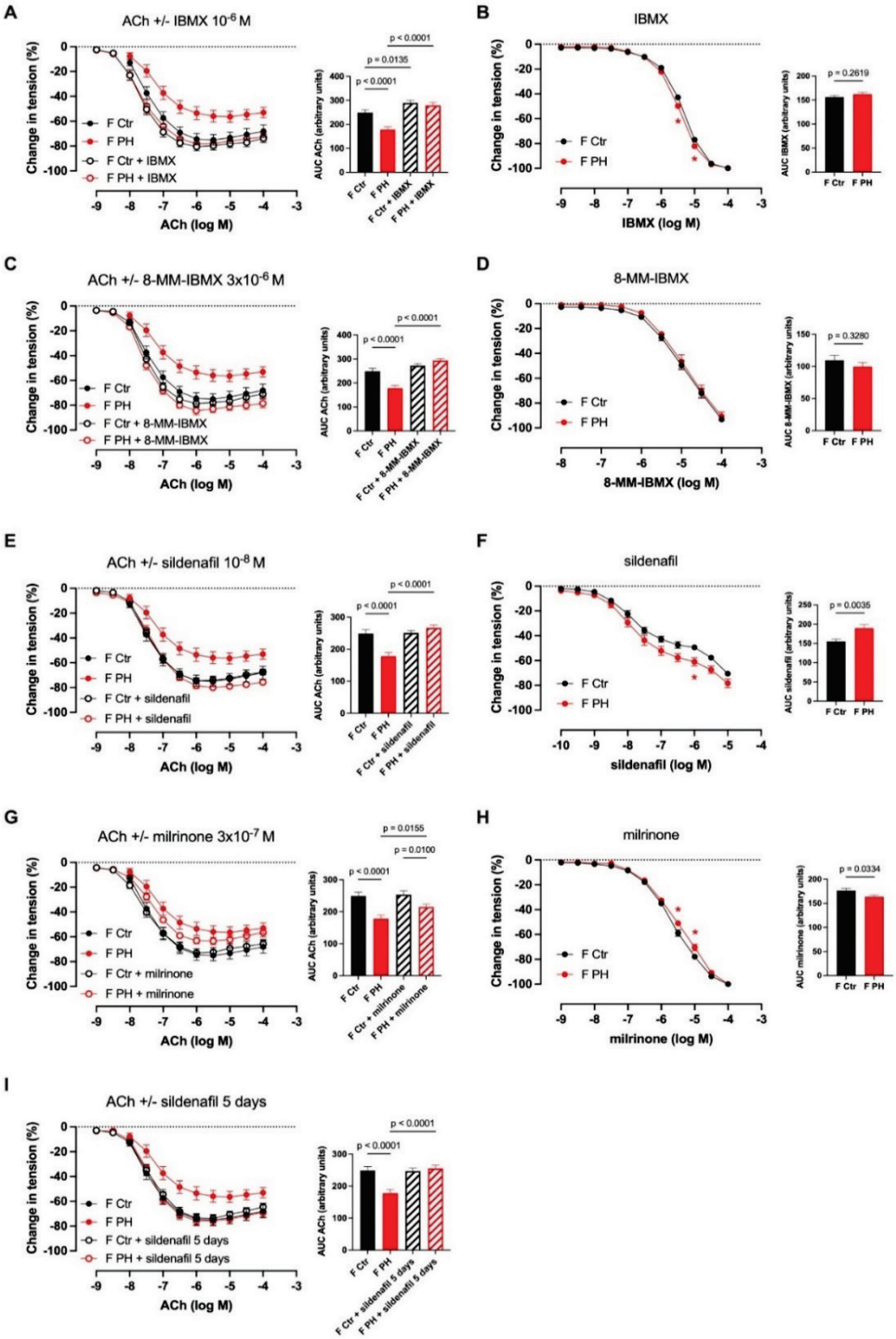

Fig. 3. Relaxation induced by cumulative doses of acetylcholine (ACh) in pulmonary arteries of females (F) born in normoxia (Ctr) or hypoxia (PH). Vascular rings were pre-incubated with phosphodiesterase (PDE) inhibitors (**A**, IBMX; **C**, 8-MM-IBMX; **E**, sildenafil; **G**, milrinone) and pre-constricted with phenylephrine 10^{-5}M. Some mice were treated with sildenafil during 5 days before testing the reactivity of their pulmonary arteries in the absence of PDE inhibitor (**I**). Data are expressed as mean ± SEM of the percentage of change in tension induced by ACh (**A** n=6-8; **C** n=6-7; **E** n=6-11; **G** n=6-11; **I** n=6-8). Data were analyzed by two-way ANOVA (results are shown below each graph) with Sidak's multiple comparison test; significant difference ($p<0.05$): * PH versus Ctr; † PH+PDE inhibitor versus PH. The corresponding AUC were presented next to each graph; data are expressed as mean ± SEM and were analyzed by unpaired t tests with Welch's correction to compare two groups (significant p values are reported on each graph). Dose-response curves to the PDE inhibitors (**B**, IBMX; **D**, 8-MM-IBMX; **F**, sildenafil; **H**, milrinone) were established in pulmonary arteries pre-constricted with phenylephrine 10^{-5}M. Data are expressed as mean ± SEM of the percentage of change in tension induced by the PDE inhibitor (**B** n=8; **D** n=5-11; **F** n=8; **H** n=7-8). Data were analyzed by two-way ANOVA with Sidak's multiple comparison test; * significant difference between PH and Ctr mice. The corresponding AUC were presented next to each graph; data are expressed as mean ± SEM and were analyzed by unpaired t tests with Welch's correction. Dose-response curves to ACh assessed in Ctr and PH females were previously published in Peyter *et al.* [20].

The PDE-resistant cGMP analog 8-bromo-cGMP induced similar relaxation in Ctr, PH and PH+AH females, but a significantly better relaxant response in Ctr+AH females (Fig. 2C). In males, 8-bromo-cGMP-induced relaxation was significantly increased in PH versus Ctr males, whereas Ctr+AH males relaxed significantly more than the other groups (Fig. 2D).

Simultaneous exposure to iNO and PH was able to preserve the relaxant response to acetylcholine in females and males, thus resulting in a significantly increased relaxation compared to PH mice (Fig. 2E-F). However, perinatal exposure to iNO alone resulted in a decreased acetylcholine-induced relaxation in Ctr+NO versus Ctr females (Fig. 2E), but did not affect Ctr+NO males (Fig. 2F).

Several PDE inhibitors were studied in females. The non-specific PDE inhibitor IBMX significantly improved acetylcholine-induced relaxation in Ctr and PH females, which then showed similar dose-response curves (Fig. 3A). The PDE1 inhibitor 8-MM-IBMX completely restored acetylcholine-induced relaxation in PH females but had no effect in Ctr females (Fig. 3C). The PDE5 inhibitor sildenafil also completely reversed the alteration in acetylcholine-induced relaxation observed in PH females but did not influence the relaxation in Ctr females (Fig. 3E). A 5-day treatment with sildenafil led to similar results (Fig. 3I). Finally, the PDE3 inhibitor milrinone significantly increased the response to acetylcholine in PH females, but did not restore complete acetylcholine-induced relaxation, and had no effect in Ctr females (Fig. 3G). The dose-response curves to IBMX and 8-MM-IBMX were similar between both groups (Fig. 3B-D). The relaxation induced by sildenafil was biphasic and significantly increased in PH versus Ctr females (Fig. 3F). In contrast, milrinone induced a lower relaxation in PH than Ctr females (Fig. 3H).

Cardiopulmonary parameters at rest and after exercise

In anesthetized females at rest, the transition from normoxia to acute hypoxia induced a significant decrease in O_2-sat and increase in pulse rate and breath rate in all study groups (Fig. 4A-C). In normoxia, O_2-sat was significantly lower in PH than Ctr females, whereas pulse rate and breath rate were similar between both groups (Fig. 4A-C). Under acute hypoxia, breath rate was significantly higher in PH than Ctr females and lower in PH+NO than PH females, without any significant difference in O_2-sat or pulse rate (Fig. 4A-C).

In conscious females at rest, transition from normoxia to hypoxia induced a significant decrease in O_2-sat in all groups (Fig. 4D), but a significant increase in pulse rate in PH and Ctr+NO females (Fig. 4E), and in breath rate in Ctr+NO females (Fig. 4F). No significant difference was found between Ctr and PH females in these conditions. Ctr+NO females showed a significant decrease in O_2-sat, pulse rate and breath rate compared to Ctr females in normoxia, as well as in O_2-sat under hypoxia (Fig. 4D-F). In normoxia, pulse rate and breath rate were significantly higher in PH+NO than Ctr+NO females (Fig. 4E-F); under hypoxia, O_2-sat was significantly higher in PH+NO than Ctr+NO females (Fig. 4D).

A swimming challenge under hypoxia led, in all groups, to significantly lower O_2-sat than exercise in normoxia (Fig. 4G), without change in pulse rate and breath rate (Fig. 4H-I). O_2-sat was significantly higher in PH+NO than PH females (Fig. 4G).

Lung morphometry

Morphometric measurements on adult male lungs showed a significant increase in the number of 25-75-µm pulmonary vessels per area in PH versus Ctr males, whereas no significant difference was found for smaller vessels (<25 µm) or larger vessels (>75 µm) (Fig. 5A). The total number of vessels per area did not significantly differ between groups (Fig. 5B). The number of small and medium vessels tended to be higher in PH than Ctr+AH males, although the difference was not significant, probably due to the small number of mice analyzed. A similar trend was found when comparing total pulm-onary vessels per area in PH males with the other groups.

Discussion

The present study showed that PH leads to long-term adverse effects in adult pulmonary circulation, which vary between males and females (Table 3).

The reduction in litter size and pup weight observed at P5 showed that PH resulted in fetal growth restriction and increased perinatal mortality, which is consistent with previous reports [16].

The higher RVP measured in 5-6-month-old PH versus Ctr mice likely reflects the development of pulmonary hypertension. RVP was further increased by acute hypoxia, to a greater extent in PH females than in other groups, suggesting that PH induced an increased sensitivity to acute hypoxia in the pulmonary vasculature of adult females.

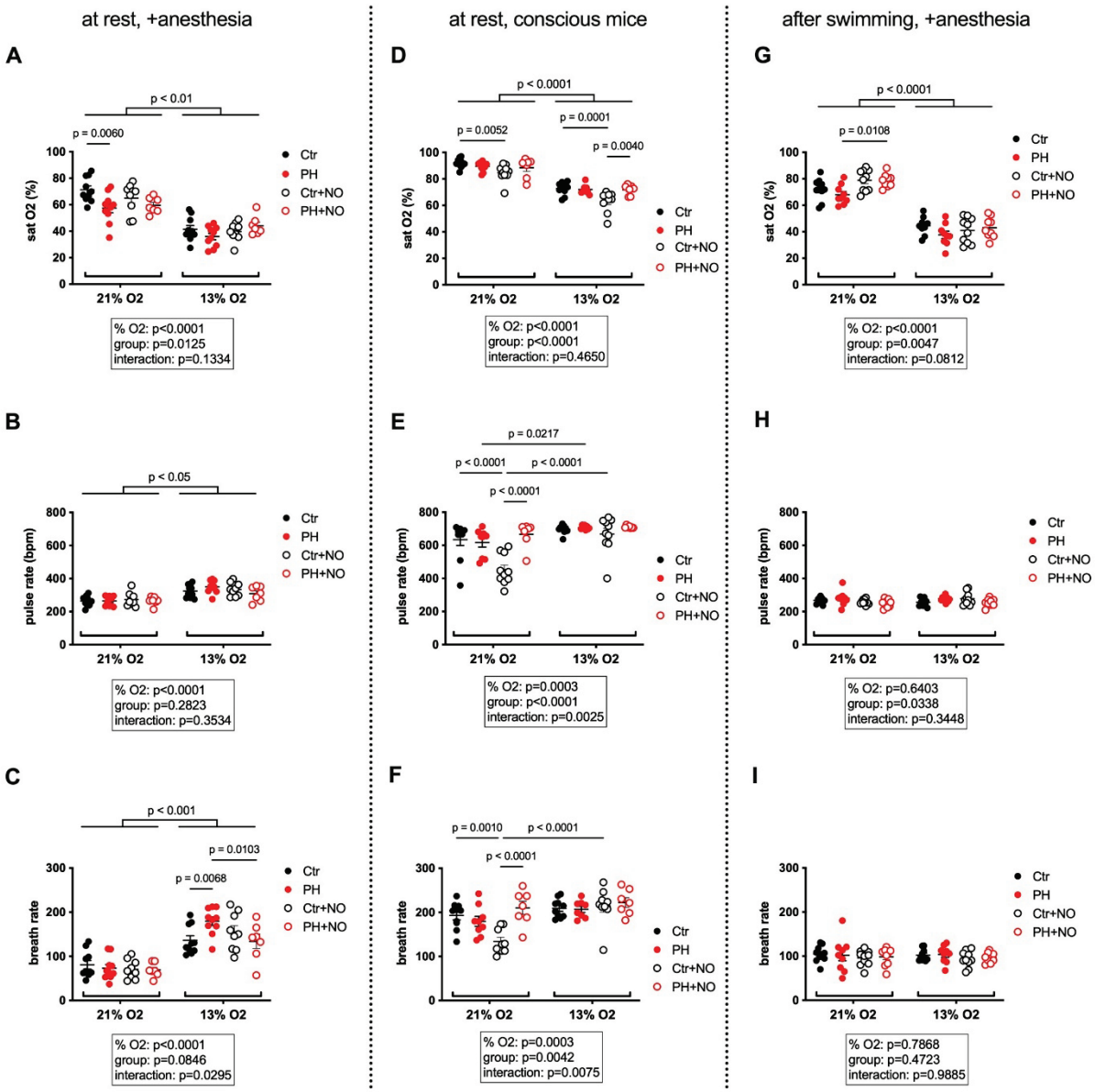

Fig. 4. Cardiopulmonary parameters at rest and after a 30-min swimming challenge in females born in normoxia (Ctr) or hypoxia (PH), with or without perinatal exposure to inhaled nitric oxide (NO). Oxygen saturation (sat O2) (**A, D, G**), pulse rate (**B, E, H**) and breath rate (**C, F, I**) were recorded in anesthetized females at rest (**A-C**), conscious females at rest (**D-F**) and females anesthetized after 30-min swimming in normoxic or hypoxic conditions (**G-I**). For females at rest, cardiopulmonary data were recorded during the transition from normoxia (21 % O_2) to acute hypoxia (12 % O_2); for females submitted to exercise, the recordings were performed in the same condition (normoxia or hypoxia) as the swimming challenge. Individual values are presented with bar at mean ± SEM (**A-C** n=7-10; **D-F** n=7-10; **G-I** n=9-10). Data were analyzed by two-way ANOVA (results are shown below each graph) with Sidak's multiple comparison test (significant p values are reported on the graph).

Acute iNO did not modify RVP in normoxia and simply reversed the effect of acute hypoxia on RVP in adult males, without lowering RVP below the value measured in normoxia.

Ex vivo investigation of PA vasoreactivity showed that endothelium-dependent relaxation was significantly reduced in PH versus Ctr mice, to a greater extent in females than in males. We previously demonstrated that this pulmonary vascular dysfunction was linked to a reduced eNOS protein content in PH versus Ctr females and alterations in M1AChR and PDE1 [20]. Here, we showed that preincubation with the non-specific PDE inhibitor IBMX, the PDE1 inhibitor 8-MM-IBMX or the PDE5 inhibitor sildenafil completely restored the relaxant response to acetylcholine in PH females. Similarly, a 5-day treatment with sildenafil also abolished the alteration of acetylcholine-induced relaxation in PH females. These data suggest that PDEs contribute to the pulmonary vascular dysfunction following PH, and that PDE1 and PDE5 inhibitors could

be promising agents to counterbalance adverse effects of PH in adulthood.

Our pharmacological and hemodynamic data suggested that PH had a greater impact on the adult pulmonary circulation in females than in males. Nevertheless, at 5-6 months, the Fulton index was significantly increased only in PH males, whereas at 12-15 months this index was significantly higher in PH than Ctr females, suggesting that the functional alterations observed in young females could lead to RV hypertrophy later in life. Therefore, PH leads to RV hypertrophy in adulthood, which appeared earlier in males than in females. Given that RVP was significantly increased in both young PH males and females, sex hormones could contribute to the delayed development of RV hypertrophy in females. This is supported by the observation that female rats were more sensitive than males to late effects of PH on pulmonary circulation and that neonatal – but not adult – gonadectomy exacerbated pulmonary hypertensive effects of PH only in females [17]. In humans, incidence of PAH is higher in young women than in men, although women have a better prognosis and survival, while these differences disappear after menopause [26].

Morphometric measurements were performed to explain RV hypertrophy already found in young PH males, despite lower vascular dysfunction in PH males than females. We observed a significant increase in the number of 25-75-µm pulmonary vessels in adult male lungs following PH, which could result in increased pulmonary vascular resistance, thus leading to RV hypertrophy. Studying angiogenic factors in neonatal lungs could be useful to characterize pulmonary vascular remodeling following PH. Investigation of pulmonary vascular density over time could help to determine whether PH directly induced an increase in number of pulmonary vessels or rather a decrease followed by an increase in vessel formation as a compensatory mechanism. Comparison with other studies is difficult because hypoxia-induced pulmonary vascular remodeling is a complex process, depending on the species, sex, and developmental stage at which the exposure to hypoxia occurred, but also on the site along the pulmonary vasculature [27].

Prolonged (re-)exposure to hypoxia in adulthood resulted in a significant increase in the Fulton index in all groups except in PH males - which already had a high index without re-exposure – and modified PA vasoreactivity. Long-term consequences of PH significantly differed from the effects of AH, which is consistent with previous reports [7,17], probably because PH occurred during a plastic phase of development thus leading to permanent adaptations/alterations.

Simultaneous administration of iNO during PH was able to prevent adverse effects of PH on endothelium-dependent relaxation of PA and the development of RV hypertrophy in females [21]. Here we showed that perinatal iNO also normalized some cardiopulmonary parameters in PH females, and protected males from impaired acetylcholine-induced relaxation and RV hypertrophy. However, perinatal iNO alone induced some changes in anatomical and cardiopulmonary parameters in adult mice, and decreased endothelium-dependent relaxation in Ctr+NO versus Ctr females, but not in males. Therefore, anything affecting cardiopulmonary hemodynamics during the perinatal period may have long-term consequences.

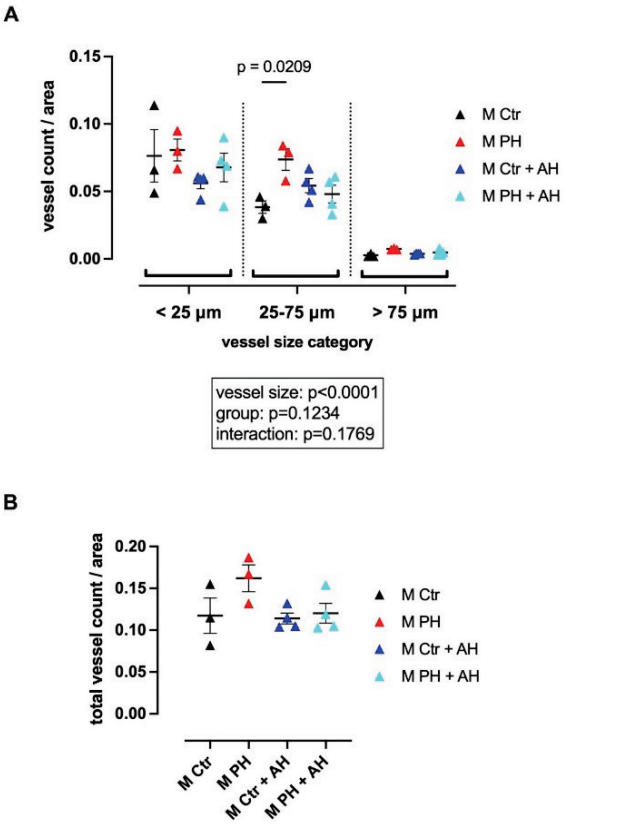

Fig. 5. Morphometric analysis of adult lungs from males (M) born in normoxia (Ctr) or hypoxia (PH), with or without a 5-day exposure to chronic hypoxia in adulthood (AH). Individual values are presented with bar at mean ± SEM (n=3-4). (**A**) The counted vessels were categorized by size; data are expressed as number of vessels per area analyzed; data were analyzed by two-way ANOVA (results are shown below) with Sidak's multiple comparison test (significant p values are reported on the graph). (**B**) Total number of vessels per area analyzed; data were analyzed by two-way ANOVA followed by Sidak's multiple comparison test.

Table 3. Summary of the main results

	Females	**Males**
RV/LV+S	PH = Ctr	PH > Ctr
in young mice	Ctr+AH > Ctr, PH+AH > PH, Ctr+AH > PH	Ctr+AH > Ctr, PH+AH = PH, Ctr+AH > PH
(Table 2)	Ctr+NO < Ctr, Ctr+NO < PH+NO	Ctr+NO < Ctr, PH+NO < PH
RV/LV+S	PH > Ctr	PH = Ctr
in old mice	Ctr+AH > Ctr, PH+AH > PH	Ctr+AH > Ctr, PH+AH = PH
(Table 2)	Ctr+NO = Ctr, PH+NO < PH	Ctr+NO = Ctr
RVP	PH > Ctr	PH > Ctr
	RVP ↗ by acute hypoxia	Ctr+AH > Ctr, PH+AH > PH, PH+AH >
	Increased sensitivity to acute hypoxia in PH	Ctr+AH, Ctr+AH > PH
(Fig.1)	females	RVP ↗ by acute hypoxia
ACh-induced relaxation	PH < Ctr	PH < Ctr
(Fig.2)	Ctr+AH < Ctr, PH+AH > PH, Ctr+AH > PH	PH+AH > PH, Ctr+AH > PH
	Ctr+NO < Ctr, PH+NO > PH, PH+NO > Ctr+NO	PH+NO > PH, PH+NO > Ctr+NO
cGMP-induced	PH = Ctr	PH > Ctr
relaxation	Ctr+AH > Ctr, Ctr+AH > PH+AH, Ctr+AH > PH	Ctr+AH > Ctr, Ctr+AH > PH+AH, Ctr+AH > PH
(Fig.2)		
Effects of PDE	IBMX, 8-MM-IBMX and sildenafil completely	–
inhibitors on response	restore ACh-induced relaxation in PH	
to ACh		
(Fig.3)		
O_2 saturation	↘ by acute hypoxia in all groups and conditions	–
	At rest, anesthesia: PH < Ctr in normoxia	
	At rest, conscious: Ctr+NO < Ctr in normoxia and	
	hypoxia; Ctr+NO < PH+NO in hypoxia	
(Fig.4)	After exercise: PH+NO > PH in normoxia	
Pulse rate	At rest, anesthesia: ↗ by acute hypoxia in all	–
	groups	
	After exercise: no difference between normoxia	
(Fig.4)	and hypoxia	
Breath rate	At rest, anesthesia: ↗ by acute hypoxia in all	–
	groups	
	After exercise: no difference between normoxia	
(Fig.4)	and hypoxia	
Lung morphometry	–	medium vessels density: PH > Ctr
(Fig.5)		

In vivo assessment of O_2-sat, pulse rate and breath rate, at rest or after a swimming challenge, showed that PH impacted the whole cardiopulmonary system. In females, perinatal exposure to hypoxia and/or to iNO differentially impacted cardiorespiratory parameters at rest or after exercise, probably as a result of functional alterations induced by PH and/or iNO in female pulmonary circulation. Acute hypoxia induced a decrease in O_2-sat in all groups and conditions, and an increase in pulse rate and breath rate at rest but not after swimming.

Taken together, our data demonstrated that PH led to long-lasting alterations in the regulation of pulmonary vascular tone. Males and females are differentially impacted by several factors acting on the cardiopulmonary system, like PH, perinatal iNO, adult prolonged or acute hypoxia, and aging (Table 3).

There are few reports directly comparing long-term effects of PH between males and females. In

humans, reanalysis of data of [2] showed that hypoxic exposure at high altitude led to exaggerated pulmonary vasoconstriction in males but not in females; however, number of females was very low (n=3-4 females, n=6-7 males) [28]. Neonatal exposure to hypoxia in swine induced a more severe pulmonary vascular disease in male than female piglets [28], and an impaired endothelium-dependent relaxation in pulmonary small arteries, but male and female data were not separately analyzed [29]. In contrast, female rats were more sensitive than males to late effects of PH on pulmonary circulation and these effects are blunted by the presence of ovaries during maturation [17].

Limitations and perspectives

Our study has several limitations. First, not all parameters were assessed in both males and females. Missing data need therefore to be completed to provide a comprehensive overview. Namely, lung morphometry should be performed in females, but also following perinatal iNO to determine if iNO during PH can prevent/reverse pulmonary vascular changes.

Then, some experiments were performed on a limited number of animals, in particular lung morphometry. Increasing sample size would provide more accurate and probably more significant results.

Another limitation was that estrous cycle was not monitored. However, mice were randomly assigned to the different experiments over time, thus mitigating variability due to litters, cages, and estrous cycle stages. The similar variability between male and female data strongly suggests that randomization was sufficient to limit the effects of estrous cycle on measurements.

Moreover, due to size limitation, mice only allow to use the main PA for isolated vessel tension studies. However, PH likely impacts the whole pulmonary vascular tree, including pulmonary veins (PVs), thus contributing to the increase in RVP and Fulton index observed in PH mice. PH-induced alterations may vary among the different kinds of pulmonary vessels [27,30]. Another animal model like rat could help to better investigate such alterations in PA and PV. Indeed, PVs play a crucial role in bringing freshly oxygenated blood to the heart [22,30]. Investigating alterations occurring in both PA and PV would help to better understand the mechanisms implicated and to design effective therapeutic strategies with limited side-effects. Indeed, if a treatment only improves PA or PV reactivity, it may lead to an imbalance between arterial and venous pulmonary circulation, resulting in altered hemodynamics.

Our pharmacological experiments highlighted PDE inhibitors as promising drugs to improve pulmonary vascular relaxation, in particular PDE5 and PDE1 inhibitors. This is consistent with clinical data in humans, where combined therapies using iNO and/or PDE inhibitors showed encouraging results in the treatment of pulmonary vascular diseases [31,32]. Another way to improve NO/cGMP-mediated relaxation will be to limit cGMP efflux, which appeared as important as cGMP hydrolysis in cGMP clearance [33]. The multidrug resistance-associated protein 4 (MRP4) was found to contribute to cGMP efflux and MRP4 inhibitors are currently under investigation to treat several vascular disorders like erectile dysfunction and pulmonary hypertension [34-36]. It would be therefore interesting to study the contribution of cGMP efflux in our model and to test whether MRP4 inhibitors could help to reverse pulmonary alterations following PH. Other stimulators of the NO/cGMP-mediated relaxation, like riociguat, could also be studied [32,37].

Cardiopulmonary parameters and exercise tolerance should be assessed after treatment with PDE or MRP4 inhibitors. Early interventions using PDE or MRP4 inhibitors, or Resveratrol, an antioxidant with PDE inhibition properties, during PH exposure could be of interest, to inhibit pulmonary vascular remodeling and prevent functional alterations. It would be interesting to test whether perinatal iNO at a lower dose would also be beneficial for PH mice, without adverse effects in Ctr animals.

Studying several proteins implicated in the NO/cGMP pathway is needed to better characterize the molecular alterations and elucidate the mechanisms implicated.

In conclusion, a transient exposure to hypoxia during the perinatal period results in long-term adverse effects on the adult pulmonary circulation, which vary between males and females. In males, the increased pulmonary vascular resistance seems to be associated with morphological changes besides functional alterations, whereas females have an important pulmonary vascular dysfunction. The effects of chronic hypoxia in adulthood also vary between males and females, and along life. Moreover, simultaneous administration of iNO during the PH exposure has a protective effect against long-term alterations induced by PH on the cardiopulmonary system. However,

perinatal iNO alone could have some adverse effects in Ctr mice. Therefore, any intervention in the perinatal period can leave a definitive imprint that could influence the regulation of the cardiovascular system later in life.

Further investigations are needed to better understand the mechanisms contributing to the differential alteration of adult pulmonary circulation following PH in males and females.

More broadly, our data emphasize the need to consider sex as an important biological variable in cardiovascular research.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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References

1. Barker DJ. In utero programming of cardiovascular disease. Theriogenology 2000;53:555-574. https://doi.org/10.1016/S0093-691X(99)00258-7
2. Sartori C, Allemann Y, Trueb L, Delabays A, Nicod P, Scherrer U. Augmented vasoreactivity in adult life associated with perinatal vascular insult. Lancet 1999;353:2205-2207. https://doi.org/10.1016/S0140-6736(98)08352-4
3. Grover RF, Vogel JHK, Voigt GC, Blount SG. Reversal of high altitude pulmonary hypertension. Am J Cardiol 1966;18:928-932. https://doi.org/10.1016/0002-9149(66)90443-7
4. Caslin A, Heath D, Smith P. Influence of hypobaric hypoxia in infancy on the subsequent development of vasoconstrictive pulmonary vascular disease in the Wistar albino rat. Journal of Pathology 1991;163:133-141. https://doi.org/10.1002/path.1711630209
5. Hakim TS, Mortola JP. Pulmonary vascular resistance in adult rats exposed to hypoxia in the neonatal period. Can J Physiol Pharmacol 1990;68:419-424. https://doi.org/10.1139/y90-059
6. Hampl V, Herget J. Perinatal hypoxia increases hypoxic pulmonary vasoconstriction in adult rats recovering from chronic exposure to hypoxia. Am Rev Respir Dis 1990;142:619-624. https://doi.org/10.1164/ajrccm/142.3.619
7. Tang JR, Le Cras TD, Morris KG, Jr., Abman SH. Brief perinatal hypoxia increases severity of pulmonary hypertension after reexposure to hypoxia in infant rats. Am J Physiol Lung Cell Mol Physiol 2000;278:L356-364. https://doi.org/10.1152/ajplung.2000.278.2.L356
8. Steenhorst JJ, Hirsch A, Verzijl A, Wielopolski P, de Wijs-Meijler D, Duncker DJ, Reiss IKM, Merkus D. Exercise and hypoxia unmask pulmonary vascular disease and right ventricular dysfunction in a 10- to 12-week-old swine model of neonatal oxidative injury. J Physiol 2022;600:3931-3950. https://doi.org/10.1113/JP282906
9. Hampl V, Herget J. Role of nitric oxide in the pathogenesis of chronic pulmonary hypertension. Physiol Rev 2000;80:1337-1372. https://doi.org/10.1152/physrev.2000.80.4.1337
10. Lachmanova V, Hnilickova O, Povysilova V, Hampl V, Herget J. N-acetylcysteine inhibits hypoxic pulmonary hypertension most effectively in the initial phase of chronic hypoxia. Life Sci 2005;77:175-182. https://doi.org/10.1016/j.lfs.2004.11.027
11. Hodyc D, Johnson E, Skoumalova A, Tkaczyk J, Maxova H, Vizek M, Herget J. Reactive oxygen species production in the early and later stage of chronic ventilatory hypoxia. Physiol Res 2012;61:145-151. https://doi.org/10.33549/physiolres.932206
12. Koubsky K, Durisova J, Mikova D, Herget J. Chronic hypoxia inhibits tetrahydrobiopterin-induced NO production in rat lungs. Respir Physiol Neurobiol 2013;185:547-552. https://doi.org/10.1016/j.resp.2012.11.012

13. Hampl V, Bibova J, Banasova A, Uhlik J, Mikova D, Hnilickova O, Lachmanova V, Herget J. Pulmonary vascular iNOS induction participates in the onset of chronic hypoxic pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol 2006;290:L11-20. https://doi.org/10.1152/ajplung.00023.2005
14. Al-Hiti H, Chovanec M, Melenovsky V, Vajnerova O, Banasova A, Kautzner J, Herget J. L-arginine in combination with sildenafil potentiates the attenuation of hypoxic pulmonary hypertension in rats. Physiol Res 2013;62:589-595. https://doi.org/10.33549/physiolres.932463
15. Hampl V, Bibova J, Herget J. Perinatal history of hypoxia leads to lower vascular pressures and hyporeactivity to angiotensin II in isolated lungs of adult rats. Physiol Res 2000;49:567-575.
16. Jakoubek V, Bibova J, Herget J, Hampl V. Chronic hypoxia increases fetoplacental vascular resistance and vasoconstrictor reactivity in the rat. Am J Physiol Heart Circ Physiol 2008;294:H1638-1644. https://doi.org/10.1152/ajpheart.01120.2007
17. Hampl V, Bibova J, Ostadalova I, Povysilova V, Herget J. Gender differences in the long-term effects of perinatal hypoxia on pulmonary circulation in rats. Am J Physiol Lung Cell Mol Physiol 2003;285:L386-392. https://doi.org/10.1152/ajplung.00389.2002
18. Marino M, Beny JL, Peyter AC, Bychkov R, Diaceri G, Tolsa JF. Perinatal hypoxia triggers alterations in K+ channels of adult pulmonary artery smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 2007;293:L1171-1182. https://doi.org/10.1152/ajplung.00126.2007
19. Marino M, Beny JL, Peyter AC, Diaceri G, Tolsa JF. Perinatal Hypoxia Enhances Cyclic Adenosine Monophosphate-mediated BKCa Channel Activation in Adult Murine Pulmonary Artery. J Cardiovasc Pharmacol 2011;57:154-165. https://doi.org/10.1097/FJC.0b013e3182016adf
20. Peyter AC, Muehlethaler V, Liaudet L, Marino M, Di Bernardo S, Diaceri G, Tolsa JF. Muscarinic receptor M1 and phosphodiesterase 1 are key determinants in pulmonary vascular dysfunction following perinatal hypoxia in mice. Am J Physiol Lung Cell Mol Physiol 2008;295:L201-213. https://doi.org/10.1152/ajplung.00264.2007
21. Peyter AC, Delhaes F, Diaceri G, Menetrey S, Tolsa JF. Perinatal nitric oxide therapy prevents adverse effects of perinatal hypoxia on the adult pulmonary circulation. Biomed Res Int 2014;2014:949361. https://doi.org/10.1155/2014/949361
22. Gao Y, Cornfield DN, Stenmark KR, Thebaud B, Abman SH, Raj JU. Unique aspects of the developing lung circulation: structural development and regulation of vasomotor tone. Pulm Circ 2016;6:407-425. https://doi.org/10.1086/688890
23. Fujita M, Mason RJ, Cool C, Shannon JM, Hara N, Fagan KA. Pulmonary hypertension in TNF-alpha-overexpressing mice is associated with decreased VEGF gene expression. J Appl Physiol 2002;93:2162-2170. https://doi.org/10.1152/japplphysiol.00083.2002
24. Mund SI, Stampanoni M, Schittny JC. Developmental alveolarization of the mouse lung. Dev Dyn 2008;237:2108-2116. https://doi.org/10.1002/dvdy.21633
25. Schittny JC, Djonov V, Fine A, Burri PH. Programmed cell death contributes to postnatal lung development. Am J Respir Cell Mol Biol 1998;18:786-793. https://doi.org/10.1165/ajrcmb.18.6.3031
26. Morris H, Denver N, Gaw R, Labazi H, Mair K, MacLean MR. Sex differences in pulmonary hypertension. Clin Chest Med 2021;42:217-228. https://doi.org/10.1016/j.ccm.2020.10.005
27. Stenmark KR, Fagan KA, Frid MG. Hypoxia-induced pulmonary vascular remodeling: cellular and molecular mechanisms. Circ Res 2006;99:675-691. https://doi.org/10.1161/01.RES.0000243584.45145.3f
28. de Wijs-Meijler DPM, van Duin RWB, Duncker DJ, Scherrer U, Sartori C, Reiss IKM, Merkus D. Structural and functional changes of the pulmonary vasculature after hypoxia exposure in the neonatal period: a new swine model of pulmonary vascular disease. Am J Physiol Heart Circ Physiol 2018;314:H603-H615. https://doi.org/10.1152/ajpheart.00362.2017
29. de Wijs-Meijler DPM, Duncker DJ, Danser AHJ, Reiss IKM, Merkus D. Changes in the nitric oxide pathway of the pulmonary vasculature after exposure to hypoxia in swine model of neonatal pulmonary vascular disease. Physiol Rep 2018;6:e13889. https://doi.org/10.14814/phy2.13889
30. Gao Y, Raj JU. Role of veins in regulation of pulmonary circulation. Am J Physiol Lung Cell Mol Physiol 2005;288:L213-226. https://doi.org/10.1152/ajplung.00103.2004

31. Fei Q, Pan J, Zhang F, Lin Y, Yuan T. Comparison of Different Treatments of Persistent Pulmonary Hypertension of the Newborn: A Systematic Review and Network Meta-Analysis. Crit Care Med 2024. https://doi.org/10.1097/CCM.0000000000006227
32. D'Agostino A, Lanzafame LG, Buono L, Crisci G, D'Assante R, Leone I, De Vito L, Bossone E, Cittadini A, Marra AM. Modulating NO-GC Pathway in Pulmonary Arterial Hypertension. Int J Mol Sci 2023;25. https://doi.org/10.3390/ijms25010036
33. Krawutschke C, Koesling D, Russwurm M. Cyclic GMP in vascular relaxation: export is of similar importance as degradation. Arterioscler Thromb Vasc Biol 2015;35:2011-2019. https://doi.org/10.1161/ATVBAHA.115.306133
34. Belleville-Rolland T, Sassi Y, Decouture B, Dreano E, Hulot JS, Gaussem P, Bachelot-Loza C. MRP4 (ABCC4) as a potential pharmacologic target for cardiovascular disease. Pharmacol Res 2016;107:381-389. https://doi.org/10.1016/j.phrs.2016.04.002
35. Hara Y, Sassi Y, Guibert C, Gambaryan N, Dorfmuller P, Eddahibi S, Lompre AM, Humbert M, Hulot JS. Inhibition of MRP4 prevents and reverses pulmonary hypertension in mice. J Clin Invest 2011;121:2888-2897. https://doi.org/10.1172/JCI45023
36. de Oliveira MG, Passos GR, de Gomes ET, Leonardi GR, Zapparoli A, Antunes E, Monica FZ. Inhibition of multidrug resistance proteins by MK571 restored the erectile function in obese mice through cGMP accumulation. Andrology 2023;11:611-620. https://doi.org/10.1111/andr.13340
37. Kraehling JR, Sessa WC. Contemporary approaches to modulating the nitric oxide-cGMP pathway in cardiovascular disease. Circ Res 2017;120:1174-1182. https://doi.org/10.1161/CIRCRESAHA.117.303776

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REVIEW

Influence of Hypoxia on the Airway Epithelium

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Summary

The necessity of oxygen for metabolic processes means that hypoxia can lead to serious cell and tissue damage. On the other hand, in some situations, hypoxia occurs under physiological conditions and serves as an important regulation factor. The airway epithelium is specific in that it gains oxygen not only from the blood supply but also directly from the luminal air. Many respiratory diseases are associated with airway obstruction or excessive mucus production thus leading to luminal hypoxia. The main goal of this review is to point out how the airway epithelium reacts to hypoxic conditions. Cells detect low oxygen levels using molecular mechanisms involving hypoxia-inducible factors (HIFs). In addition, the cells of the airway epithelium appear to overexpress HIFs in hypoxic conditions. HIFs then regulate many aspects of epithelial cell functions. The effects of hypoxia include secretory cell stimulation and hyperplasia, epithelial barrier changes, and ciliogenesis impairment. All the changes can impair mucociliary clearance, exacerbate infection, and promote inflammation leading to damage of airway epithelium and subsequent airway wall remodeling. The modulation of hypoxia regulatory mechanisms may be one of the strategies for the treatment of obstructive respiratory diseases or diseases with mucus hyperproduction.

Keywords

Secretory cells • Motile cilia • Epithelial barrier • Oxygenation • Obstructive respiratory diseases

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Introduction

The continuous cellular need for oxygen requires the maintenance of oxygen homeostasis. While in very simple microscopic organisms diffusion is enough to distribute oxygen sufficiently, in vertebrates, including humans, the increase of body mass led to the development of a complicated system for oxygen distribution [1,2]. Lack of oxygen in the environment or failure of the oxygen distribution system can lead to hypoxia.

Hypoxia is defined as deprivation of oxygen level in a tissue. The term was probably used for the first time in 1938 [3]. In his recent review, Sargon divided hypoxia into four groups: hypoxemic hypoxia, ischemic hypoxia, anemic hypoxia and histotoxic hypoxia [4]. The relationship between the terms hypoxia and ischemia should be mentioned, too. Whereas hypoxia is the result of disproportion between oxygen supply and demand, regardless of the cause, ischemia is defined as reduction or interruption of the blood flow leading to hypoxia of perfused tissue [5]. The necessity of oxygen for metabolic processes means that hypoxia, due to its severity and duration, can lead to serious cell and tissue damage. On the other hand, in some situations, hypoxia occurs under physiological conditions and in this case, hypoxia serves as an important regulation factor, e.g. in the intrauterine period [6,7], in the intestinal epithelium [8] or in renal medulla, bone marrow, lymphoid tissue and placenta [9]. General hypoxia affecting the whole organism can be caused by high altitudes, by various pathological conditions including respiratory, circulatory, hemato-logical or infectious disorders, or by artificial conditions in animal experiments. Local hypoxia can develop in many situations of local failure of natural oxygenation [10].

In the respiratory system, hypoxia influences many processes. They include pulmonary vascular remodeling, shifts in immune response and changes in the

airway and alveolar wall. However, the airway epithelium is specific in that it gains oxygen not only from the blood supply but that the majority of oxygen transported into epithelial cells is provided directly by the luminal flowing air [11–13]. Many respiratory diseases are associated with airway obstruction or excessive mucus production and thus with epithelial hypoxia. The main goal of this review is to point out how the airway epithelium reacts to hypoxic conditions.

Cellular hypoxia

The need for oxygen homeostasis led to the evolution of many regulative mechanisms. The quick adaptation involves increasing respiration, blood flow, and survival responses. Longer hypoxia activates mechanisms either increasing the oxygen supply or allowing the adaptation to hypoxia. The effect of hypoxia on cells is an important research field. In 2019 Nobel Prize in Physiology or Medicine "for their discoveries of how cells sense and adapt to oxygen availability" [14] was awarded to G. Semenza, P. J. Ratcliffe and W. G. Kaelin.

Cells detect low oxygen levels using molecular mechanisms involving hypoxia-inducible factors (HIFs) [15]. These mechanisms are linked to oxygen-sensing prolyl hydroxylase domain proteins (PHDs), which hydroxylate proline in α subunits of HIFs [16], and are summarized in Fig. 1. HIFs are heterodimeric transcription factors, consisting of α (1α or 2α) and β subunits [1]. Under normoxic conditions, the α subunit is hydroxylated by PHDs using oxygen as a substrate. Hydroxylated HIFα interacts with Von Hippel-Lindau protein (VHL) and this complex undergoes ubiquitination. Under hypoxic conditions, the hydroxylation is arrested. This leads to stabilization of the α subunit, transport to the nucleus, and dimerization with the β subunit. This HIF complex then acts as a transcription factor for many genes [17], with VEGF, leptin and TGF-β3 among the regulated genes. The number of genes known to be directly or indirectly regulated by HIF-1 gradually increases - more than 800 genes had been described by 2015 [18], and they accounted for over 2 % of known genes in 2017, respectively [19]. Main HIF-2 targets are EPO (erythropoietin) and EDN1 (endothelin 1). HIF-2 also plays a role in NO metabolism [17]. Currently, in the HIF family, three HIFs are described - HIF-1, HIF-2 and HIF-3. HIF-3 was for some time considered to be only a negative mediator of HIF-regulated genes [20] but later findings have shown different functions of HIF-3 [21]. HIF-3 is particularly important in chronic hypoxia [22,23].

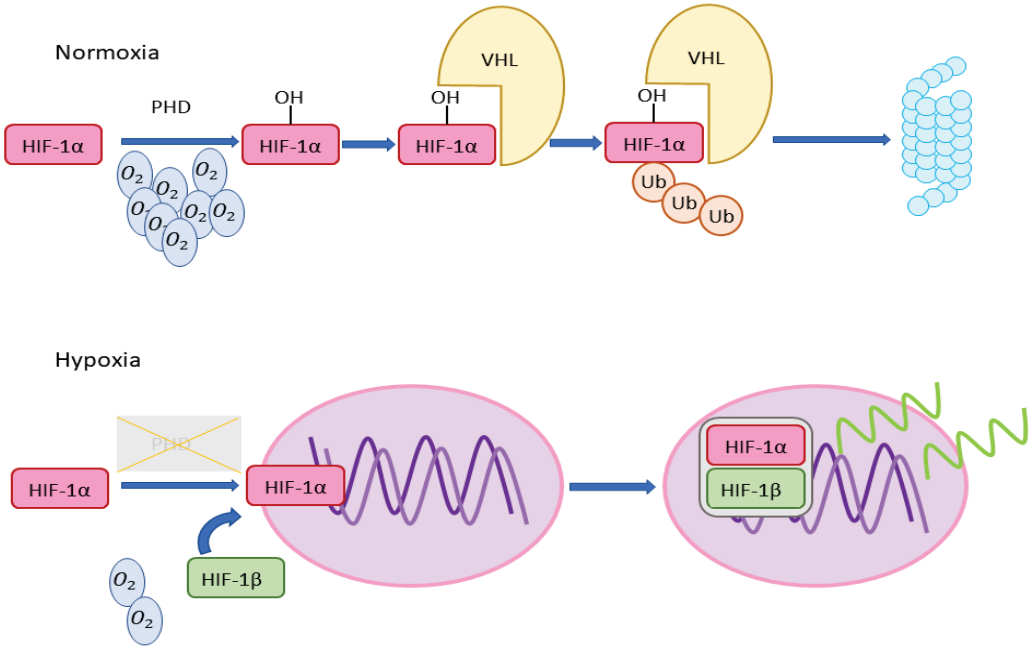

Fig. 1. In normoxia, prolyl hydroxylase domain proteins with O_2 as a substrate hydroxylate HIF-1α prolines. Hydroxylated HIF-1α subunit forms a complex with VHL and this complex is subjected to ubiquitination and proteasome digestion. In hypoxia, HIF-1α gets not hydroxylated and it leads to stabilization of this subunit and its transport to the nucleus. In the nucleus, the α subunit dimerizes with the β subunit and this HIF complex then functions as a transcription factor. HIF-1α - α-subunit of the hypoxia-inducible factor 1; PHD - prolyl hydroxylase domain proteins; VHL - Von Hippel-Lindau protein; Ub - ubiquitin; HIF-1β - β-subunit of the hypoxia-inducible factor 1

Stabilization of HIFs is the main way cells detect low oxygen levels. However, low oxygen levels can be also detected in other stress pathways as well as changes in metabolite levels and the generation of reactive oxygen species by mitochondria [10]. Some genes are influenced by hypoxia without the HIFs [24].

Under normoxic conditions, HIF-1α is the target of VHL-mediated degradation [25]. VHL protein is the main regulation factor of HIFs in normoxia [26]. The importance of VHL for this regulation can be shown in a genetic condition called Von Hippel-Lindau disease. Von Hippel-Lindau disease is a tumor predisposition characterized by the occurrence of highly vascularized tumors in the CNS, retina and visceral organs. The VHL protein (pVHL), is the product of the VHL gene. Protein pVHL influences many cellular processes, especially the cellular adaptive response to hypoxia [27]. Highly vascularized tumors in Von Hippel-Lindau disease overproduce angiogenic polypeptides such as VEGF. The situation when the pVHL protein is non-functional (as here due to genetic condition in Von Hippel-Lindau disease) has analogous consequences as physiological hypoxia [18]. And vice versa, hypoxia can show tumorigenic effects [28–30].

VHL also interferes with other processes. It has an impact on extracellular matrix organization [31–33]. It also affects microtubules [34–36] and through the control of microtubule growth orientation and stabilization influences the ciliogenesis of primary cilia [37,38]. Hypoxia, which stabilizes HIFs, weakens the primary cilia [39] and induces their elongation [40]. The mechanism connecting hypoxia and primary cilia formation is probably VHL regulation of primary cilia formation [41]. Primary cilia are important for embryogenesis, too, because they are deeply involved in the mediation of intercellular [42] and intracellular signaling [43]. A relationship exists between the ciliogenesis of primary cilia and that of motile cilia, as seen in some types of ciliopathies where the gene mutation affects both primary and motile cilia [44].

The activity of HIF-1 is regulated by many genes [45,46]. Besides hypoxia, HIF-1 can be activated by other mechanisms, e.g. by lipopolysaccharide [47,48]. In tumor tissue, HIF-1 activation can occur due to mutations in oncogenes and tumor suppressor genes [49]. Rohwer and her co-workers described increased levels of HIF-1 even in cells without hypoxia in intestinal tumorigenesis, showing that non-canonical HIF-1 stabilization through oncogenes exists [50].

Genetic selection as an adaptation to hypoxia at high altitudes was described. The principles of adaptation are lower levels of hemoglobin and the absence of polyglobulia (which otherwise are linked to chronic mountain sickness - Monge's disease) [51]. This adaptation in Tibet is represented by single nucleotide polymorphisms in the endothelial Per-Arndt-Sim domain protein 1 (EPAS1) gene, coding for the HIF-2α subunit, involved then in the stimulation of red blood cell production [52]. Chronic mountain sickness occurs more frequently in Andeans than in Tibetans and the search for this genetic adaptation in Andeans was unsuccessful at first [51]. It may be a consequence of the fact that the population lives in the Andes for a shorter period than in Tibet [53]. However, later the EPAS1 adaptation was found in Andeans as well. Furthermore, a genetic adaptation affecting the G protein-coupled receptor 126 (GPR126) gene was revealed in the Andean population [54]. The Andean EPAS1 adaptation results in a hypomorphic allele [17]. Another genetic adaptation localized in the PHD2 gene was later discovered in both Tibetans and Andeans [55,56]. The relationship between HIF-2α and erythropoietin levels can be also illustrated by inherited HIF-2α mutation in a large pedigree, accompanied by erythrocytosis and an increase of erythropoietin in serum [57].

The relationship between hypoxia and inflammation exists [58–60]. Indirectly, in people with mountain sickness, an increased level of inflammation markers was observed [16,61]. Hypoxia activates HIFs influencing many aspects of immune cells' function [9,62]. HIFs can be stabilized also under normoxic conditions during inflammation and regulate then the metabolism and expression of immune genes, thus HIFs can be seen not only as homeostasis regulators under hypoxic conditions but as well as specific regulators of immune and inflammatory genes [63]. Hypoxia induces airway epithelium to produce compounds influencing innate immunity in surrounding areas and this mechanism can then contribute to chronic pulmonary disease pathogenesis [64,65]. This process might be related to HIF stabilization in myeloid cells [62].

Hypoxia and airway epithelium

The airway epithelium lines the conductive portion of the respiratory tract from the nasal cavity to the smallest bronchioles. Although several cell types are present in both, the airway epithelium differs in large and

small airways. According to classic morphological descriptions, the large airways like nasal cavity, larynx, trachea, and bronchi are lined with a pseudostratified columnar epithelium containing mostly ciliated cells, goblet cells, and basal cells, while the small terminal and respiratory bronchioles are lined with a simple columnar to cuboidal epithelium where mucus-secreting goblet cells are gradually replaced by multifunctional club cells and ciliated and basal cells decrease in number and density [66]. However, further studies and mainly novel single-cell RNA sequencing (scRNA-seq) have uncovered enormous cellular heterogeneity within the airway epithelium. For example, ion-transporting pulmonary ionocytes, several types of tuft cells, variable pulmonary neuroendocrine cells, hillock cells, and pulmonary microfold cells were described [67–69]. Basic cell types and their prospective quantitative changes under hypoxic conditions are visualized in Fig. 2.

Hypoxia in airways can, due to various causes, affect the airway system globally as well as locally. Local hypoxia can arise as the result of obstruction (e.g. the obstruction of nasal sinuses) or the aggregation of mucus (e.g. cystic fibrosis). Mucus hyperproduction and disruption of epithelial barrier function by the production of VEGF and down-regulation of junctional proteins caused by local obstruction of nasal sinuses led to overexpression of HIF-1 in epithelial cells. Moreover, hypoxia-induced inflammation by high-mobility group box 1 (HMGB1) protein translocation into the cytoplasm resulted in the release of IL-8 through a ROS-dependent mechanism in upper airway epithelium [70]. In patients with cystic fibrosis, the thick mucus not only led to partial obstructive luminal hypoxia, but also created particularly hypoxic niches in the airway epithelium. Epithelial hypoxia in this case was potentiated by increased epithelial oxygen consumption associated with increased epithelial Na^+ channel (ENaC) mediated Na^+ absorption [71].

The general hypoxia can influence the airway epithelium from both luminal and basal sides. In our previous studies, we tested the effect of inhalation of a 10 % hypoxic atmosphere on the epithelium of large and small airways in rabbits at the level of transmission electron microscopy [72,73]. After four-day exposure, the most affected cells were tracheal goblet cells, which were stimulated to mucus release. After rapid mucus discharge,

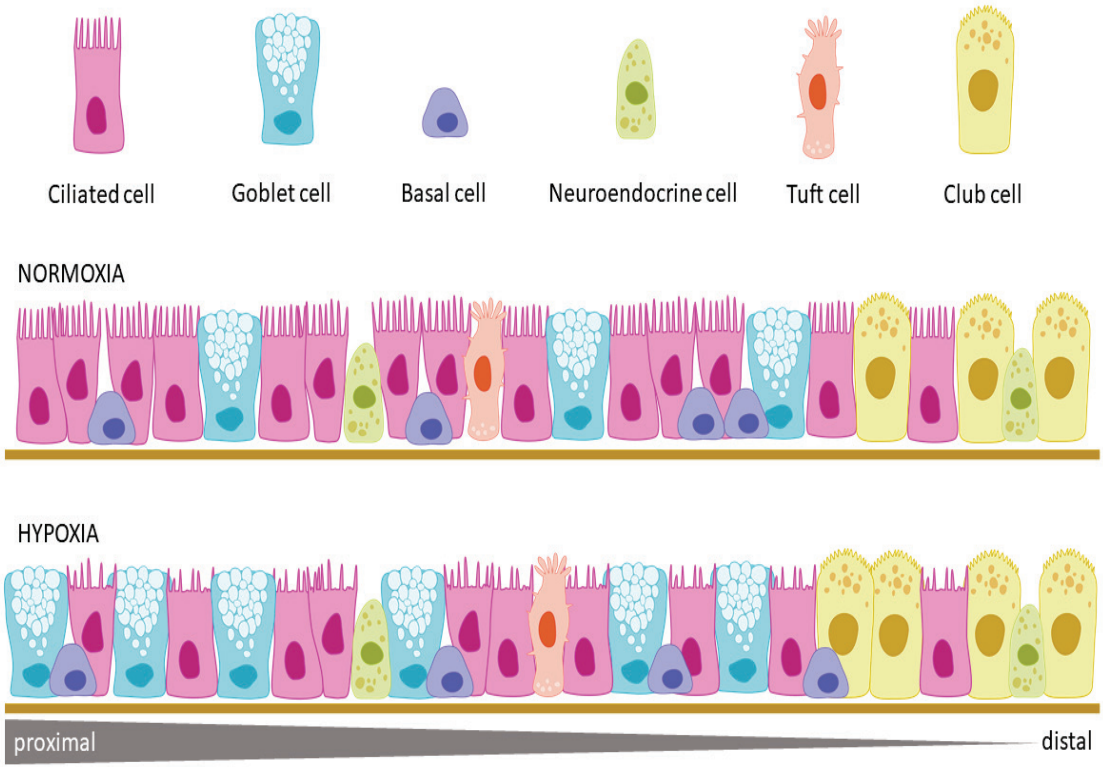

Fig. 2. The normal airway epithelium lining the tracheobronchial tree contains more cell types. The main cell types in the upper part of the tree are represented by ciliated cells, goblet cells and basal cells, in lower branches these types are gradually replaced by club cells. Among main cells the minor cell types are irregularly interspersed. Hypoxia influences both cellular proliferation and cilia formation. The proliferation was observed mainly in the population of secreting cells - goblet cells and club cells. Cilia of ciliated cells under hypoxic conditions become shorter and sparse.

the overstimulated goblet cells mostly did not take part in further secretory cycles but they degenerated and gradually sloughed off. We have demonstrated that a high level of stimulation of secretory cells in the airway epithelium accompanied by degeneration of about 50 % of goblet cells induced a massive differentiation of new secretory elements [74,75]. As the differentiating goblet cells retained the ability to divide [76], the result of this process was hyperplasia of secretory elements followed by changes in their distribution in the epithelium [74,75]. Indeed, differentiating goblet cells represented almost one-fifth of secretory elements and the formation of intra-epithelial mucous glands was recorded [72]. Ultrastructural changes of the epithelium in terminal bronchioles were not so prominent, but they corresponded with the findings described in the tracheal epithelium. Cytoplasmic alteration was found both in ciliated and club cells, but degenerative changes were observed only in some club cells. Observed significant increase of club cell relative number in hypoxic rabbits could reflect their compensatory proliferation [73].

Secretory cells

Although the airway epithelial cells' gross morphology in the light microscope can be described as intact in hypoxic conditions, many metabolic pathways are up- or downregulated [13], which can explain the discrete changes in the cellular ultrastructure found in our studies [72,73]. The secretory cell stimulation and hyperplasia seem to be a common response of the airway epithelium to the hypoxic conditions. The goblet cell hyperplasia mediated by HIF-1α was described in human bronchial epithelial (HBE) cell cultures of patients with chronic obstructive pulmonary disease (COPD) [77]. Hypoxia in mouse bronchial epithelium activated FoxM1 (proliferative factor for club cells) and bronchial cell growth factors RELMα and RELMβ *via* HIF2α. This activation led to a proliferation of bronchial epithelial cells (Ki67 staining has shown proliferation activity in 10-15 % of cells). Detailed analysis revealed that 78 % of them were club cells, 8 % ciliated cells, and the remaining portion was probably represented by basal cells [78]. Hypoxia-induced HIF-1 stabilization also increased the mucin 5AC (MUC5AC) expression in HBE and this mechanism led to the elevation of its secretion [79]. In a recent study, chronic hypoxia of HBE was associated with an increase in mucus concentration and MUC5AC transcription. The high mucus concentration could be also explained by ion-transport dysregulation *via* an epithelial Na+ channel (ENaC) beta and gamma subunits hyperexpression, which is HIF dependent [13]. Accumulation of mucus in airways can cause harm in more ways: by aggregation of pollution and immunomodulatory effect [80], by induction of inflammation and epigenetic regulation of macrophages [81].

Neuroendocrine cells

Shivaraju et al. were concerned with the neuroendocrine (NE) cell population in airways. The authors of this study filtered possible other influences that could mimic or modify the effect of pure hypoxia. Hypoxia led to significant proliferation of NE cells. Although some new NE cells could be derived from solitary NE cells, most newly differentiated NE cells arose from basal stem cells. Moreover, some basal stem cells displayed NE-specific vesicles [82]. On the other hand, NE cell proliferation as a reaction to hypoxia was not observed in the later study [13] and our personal observation did not reveal any increase in NE cell density, either [72,73].

Epithelial barrier

Epithelium represents a complicated barrier between luminal content and tissues that can be influenced by hypoxia. HIFs contribute to the expression of barrier-related genes and act in the regulation of barrier-adaptive responses within the mucosa [83]. Although more was described for the hypoxic effect on epithelial barrier function in the gastrointestinal tract [84], the general principles would probably be similar in epithelia, including the airway epithelium [83]. HIF-1 in the intestinal mucosa impacts two ways of barrier maintenance: 1) continual proliferation and epithelium renewal (*via* expression of regulation genes as WNT/β-catenin and Notch), 2) tight sealing of the barrier (*via* expression regulation of genes involved in mucus composition, permeability and integrity of tight junctions) [8]. Airway epithelium barrier impairment due to hypoxia affecting various mechanisms here described was observed [85–87]. On the other hand, if the barrier function was impaired by other conditions, then hypoxia-activated HIF-1 improved the epithelial barrier function [58].

Cilia

Oxygen is needed for motile cilia differentiation in airway ciliated cells. If cultivated in submersion, hypoxia blocks the cilia growth through impact on Notch signaling pathway activation and influences the expression of multicilin and Forkhead box J1 (FoxJ1) genes, both strongly involved in cilia formation [88] - multicilin in the early regulation stage of ciliogenesis, FoxJ1 gene in the later one [89]. The importance of the Notch signaling pathway for ciliogenesis can be illustrated also in tumors derived from multiciliated cells. Notch activation led to reduced multiciliation in choroid plexus tumors because of Notch inhibition of Geminin Coiled-Coil Domain Containing (GMNC) and multi-ciliate differentiation and DNA synthesis associated cell cycle protein (MCIDAS) [90]. GMNC and MCIDAS were previously described as having a crucial role in the ciliogenesis of multiciliated cells [89,91]. As described above, hypoxia can impact the organization and growth of cytoskeletal elements, including microtubules. Centrosomes, too, as microtubular complexes, can be influenced by hypoxia. Hypoxia plays an important role in centrosome amplification through the polo-like kinase 4 (PLK4) receptor, leading to their abnormal size, shape, number and position [92,93]. As PLK4 plays an important role in the early stages of centrosome duplication [94], there is a possibility that motile cilia could be affected by this mechanism under hypoxic conditions in multiciliated cells. Indeed, a significant decrease in the number of kinocilia/mm^2, an increase in the percentage of altered cilia and morphological signs of impairment of the vital self-cleaning ability were recorded in the rabbit tracheal epithelium after 4-day normobaric hypoxia [72]. In human airway epithelial cells the decrease of kinocilia number due to hypoxia was described in ALI (air-liquid interface) cell cultures derived from two COPD patients [87].

Conclusion

Hypoxia arouses significant interest in the scientific community, as seen in the increasing number of publications concerning hypoxia published in the last decades. Particularly the COVID-19 pandemic focused the interest of the scientific community not only on hypoxia in general [95] but specifically on the hypoxia of the respiratory system, as visualized in Fig. 3.

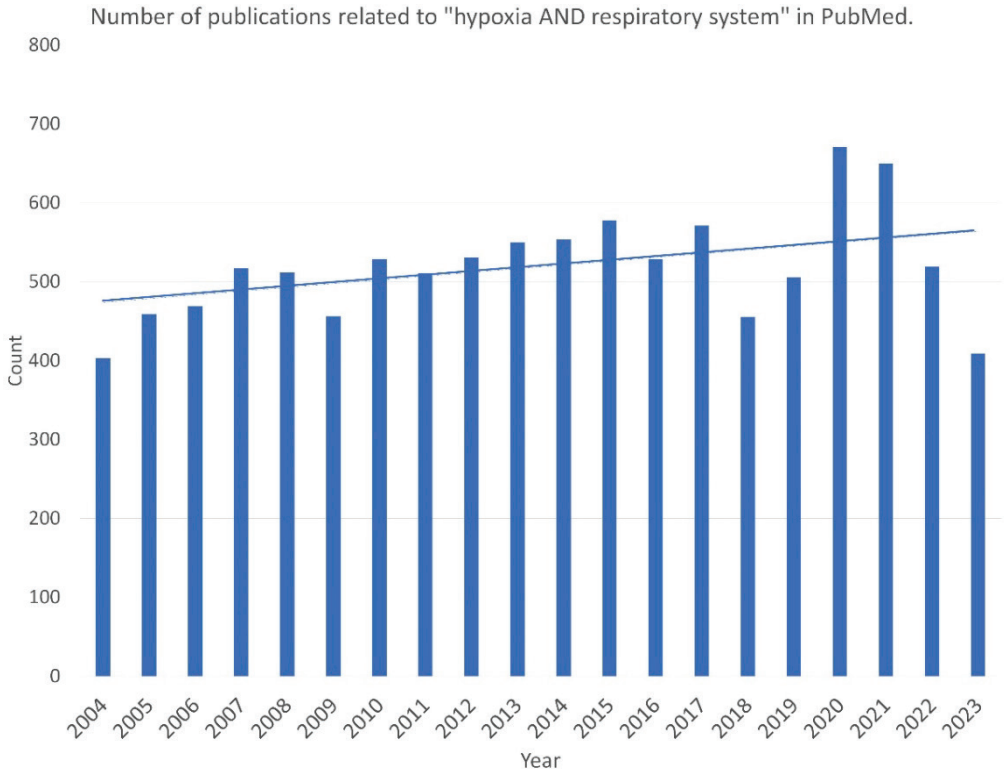

Fig. 3. Number of publications related to "hypoxia AND respiratory system" in the last two decades. Particular increase can be observed in the time of the COVID-19 pandemic.

The unique position of the airway epithelium at the interface between ambient and vascular oxygen supply makes it interesting for the comprehensive understanding of the hypoxia influence on the respiratory system. Therefore, we attempted to review the literature and summarize the current understanding of this topic. The common effects of the hypoxic conditions, either local or general, seem to be 1) secretory cell stimulation, hypersecretion and subsequent proliferation, 2) epithelial barrier functional changes, and 3) ciliogenesis impairment. All the described changes can exacerbate the cycle of impaired mucociliary clearance, infection, and inflammation leading to damage of airway epithelium and subsequent airway wall remodeling. The modulation of hypoxia regulatory mechanisms may be one of the strategies for the prevention of airway remodeling changes and the treatment of obstructive respiratory diseases or diseases with mucus hyperproduction.

Conflict of Interest

There is no conflict of interest.

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References

1. Semenza GL. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. Journal of Applied Physiology 2000;88:1474-1480. https://doi.org/10.1152/jappl.2000.88.4.1474
2. Hsia CCW, Schmitz A, Lambertz M, Perry SF, Maina JN. Evolution of air breathing: oxygen homeostasis and the transitions from water to land and sky. Compr Physiol 2013;3:849-915. https://doi.org/10.1002/cphy.c120003
3. Richalet JP. The invention of hypoxia. J Appl Physiol (1985) 2021;130:157315-82. https://doi.org/10.1152/japplphysiol.00936.2020
4. Sargon MF. Lungs and hypoxia: a review of the literature. Anatomy 2021;15:76-83. https://doi.org/10.2399/ana.21.841001
5. Ošt'ádal B, Kolář F. Myocardial Hypoxia and Ischemia. In: *Cardiac Ischemia: From Injury to Protection.* B OŠŤÁDAL, F KOLÁŘ (eds), Boston, MA, Springer US, 1999, pp 1-44. https://doi.org/10.1007/978-1-4757-3025-8_1, https://doi.org/10.1007/978-1-4757-3025-8_1
6. Genbacev O, Zhou Y, Ludlow JW, Fisher SJ. Regulation of human placental development by oxygen tension. Science 1997;277:1669-1672. https://doi.org/10.1126/science.277.5332.1669
7. Wakeland AK, Soncin F, Moretto-Zita M, Chang CW, Horii M, Pizzo D, et al. Hypoxia directs human extravillous trophoblast differentiation in a hypoxia-inducible factor-dependent manner. Am J Pathol 2017;187:767-780. https://doi.org/10.1016/j.ajpath.2016.11.018
8. Robrahn L, Jiao L, Cramer T. Barrier integrity and chronic inflammation mediated by HIF-1 impact on intestinal tumorigenesis. Cancer Letters 2020;490:186-192. https://doi.org/10.1016/j.canlet.2020.07.002
9. Taylor CT, Colgan SP. Regulation of immunity and inflammation by hypoxia in immunological niches. Nat Rev Immunol 2017;17:774-785. https://doi.org/10.1038/nri.2017.103
10. Lee P, Chandel NS, Simon MC. Cellular adaptation to hypoxia through hypoxia inducible factors and beyond. Nat Rev Mol Cell Biol 2020;21:268-283. https://doi.org/10.1038/s41580-020-0227-y
11. Nakano H, Ide H, Ogasa T, Osanai S, Imada M, Nonaka S, et al. Ambient oxygen regulates epithelial metabolism and nitric oxide production in the human nose. J Applied Physiol 2002;93:189-194. https://doi.org/10.1152/japplphysiol.00096.2002
12. Nossol C, Diesing AK, Walk N, Faber-Zuschratter H, Hartig R, Post A, et al. Air-liquid interface cultures enhance the oxygen supply and trigger the structural and functional differentiation of intestinal porcine epithelial cells (IPEC). Histochem Cell Biol 2011;136:103-115. https://doi.org/10.1007/s00418-011-0826-y
13. Mikami Y, Grubb BR, Rogers TD, Dang H, Asakura T, Kota P, et al. Chronic airway epithelial hypoxia exacerbates injury in muco-obstructive lung disease through mucus hyperconcentration. Sci Transl Med 2023;15:eabo7728. https://doi.org/10.1126/scitranslmed.abo7728

14. Ledford H, Callaway E. Biologists who decoded how cells sense oxygen win medicine Nobel. Nature 2019;574:161-2. https://doi.org/10.1038/d41586-019-02963-0
15. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A 1995;92:5510-4. https://doi.org/10.1073/pnas.92.12.5510
16. Eltzschig HK, Carmeliet P. Hypoxia and inflammation. N Engl J Med 2011;364:656-65. https://doi.org/10.1056/NEJMra0910283
17. Jorgensen K, Song D, Weinstein J, Garcia OA, Pearson LN, Inclán M, et al. High-altitude andean H194R HIF2A allele is a hypomorphic allele. Mol Biol Evolution 2023;40:msad162. https://doi.org/10.1093/molbev/msad162
18. Gossage L, Eisen T, Maher ER. VHL, the story of a tumour suppressor gene. Nat Rev Cancer 2015;15:55-64. https://doi.org/10.1038/nrc3844
19. Chen J, Khalil RA. Chapter Four - Matrix Metalloproteinases in Normal Pregnancy and Preeclampsia. In: *Progress in Molecular Biology and Translational Science*. RA KHALIL (ed.) Academic Press, 2017, 87-165. https://doi.org/10.1016/bs.pmbts.2017.04.001
20. Hubbi ME, Semenza GL. Regulation of cell proliferation by hypoxia-inducible factors. Am J Physiol Cell Physiol 2015;309:C775-82. https://doi.org/10.1152/ajpcell.00279.2015
21. Yang SL, Wu C, Xiong ZF, Fang X. Progress on hypoxia-inducible factor-3: Its structure, gene regulation and biological function (Review). Mol Med Rep 2015;12:2411-2416. https://doi.org/10.3892/mmr.2015.3689
22. Tolonen JP, Heikkilä M, Malinen M, Lee HM, Palvimo JJ, Wei GH, et al. A long hypoxia-inducible factor 3 isoform 2 is a transcription activator that regulates erythropoietin. Cell. Mol. Life Sci. 2020;77:3627-3642. https://doi.org/10.1007/s00018-019-03387-9
23. Slawski J, Jaśkiewicz M, Barton A, Kozioł S, Collawn JF, Bartoszewski R. Regulation of the HIF switch in human endothelial and cancer cells. Eur J Cell Biol 2024;103:151386. https://doi.org/10.1016/j.ejcb.2024.151386
24. Prabhakar NR, Semenza GL. Adaptive and maladaptive cardiorespiratory responses to continuous and intermittent hypoxia mediated by hypoxia-inducible factors 1 and 2. Physiol Rev 2012;92:967-1003. https://doi.org/10.1152/physrev.00030.2011
25. Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, et al. HIFα Targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. Science 2001;292:464-468. https://doi.org/10.1126/science.1059817
26. Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, et al. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 1999;399:271-275. https://doi.org/10.1038/20459
27. Aronow ME, Wiley HE, Gaudric A, Krivosic V, Gorin MB, Shields CL, et al. Von Hippel-Lindau Disease: update on pathogenesis and systemic aspects. Retina 2019;39:2243-2253. https://doi.org/10.1097/IAE.0000000000002555
28. Jung YJ, Isaacs JS, Lee S, Trepel J, Neckers L. IL-1beta-mediated up-regulation of HIF-1alpha *via* an NFkappaB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis. FASEB J 2003;17:2115-2117. https://doi.org/10.1096/fj.03-0329fje
29. Semenza GL. Hypoxia-inducible factors: mediators of cancer progression and targets for cancer therapy. Trends Pharmacol Sci 2012;33:207-214. https://doi.org/10.1016/j.tips.2012.01.005
30. Tang K, Yu Y, Zhu L, Xu P, Chen J, Ma J, et al. Hypoxia-reprogrammed tricarboxylic acid cycle promotes the growth of human breast tumorigenic cells. Oncogene 2019;38:6970-6984. https://doi.org/10.1038/s41388-019-0932-1
31. Ohh M, Yauch RL, Lonergan KM, Whaley JM, Stemmer-Rachamimov AO, Louis DN, et al. The von Hippel-Lindau tumor suppressor protein is required for proper assembly of an extracellular fibronectin matrix. Mol Cell 1998;1:959-968. https://doi.org/10.1016/S1097-2765(00)80096-9
32. Kurban G, Duplan E, Ramlal N, Hudon V, Sado Y, Ninomiya Y, et al. Collagen matrix assembly is driven by the interaction of von Hippel-Lindau tumor suppressor protein with hydroxylated collagen IV alpha 2. Oncogene 2008;27:1004-1012. https://doi.org/10.1038/sj.onc.1210709

33. Ohh M, Taber CC, Ferens FG, Tarade D. Hypoxia-inducible factor underlies von Hippel-Lindau disease stigmata. Elife 2022;11:e80774. https://doi.org/10.7554/eLife.80774
34. Thoma CR, Toso A, Gutbrodt KL, Reggi SP, Frew IJ, Schraml P, et al. VHL loss causes spindle misorientation and chromosome instability. Nat Cell Biol 2009;11:994-1001. https://doi.org/10.1038/ncb1912
35. Hergovich A, Lisztwan J, Barry R, Ballschmieter P, Krek W. Regulation of microtubule stability by the von Hippel-Lindau tumour suppressor protein pVHL. Nat Cell Biol 2003;5:64-70. https://doi.org/10.1038/ncb899
36. Cao H, Yu D, Yan X, Wang B, Yu Z, Song Y, et al. Hypoxia destroys the microstructure of microtubules and causes dysfunction of endothelial cells *via* the PI3K/Stathmin1 pathway. Cell Biosci 2019;9:20. https://doi.org/10.1186/s13578-019-0283-1
37. Schermer B, Ghenoiu C, Bartram M, Müller RU, Kotsis F, Höhne M, et al. The von Hippel-Lindau tumor suppressor protein controls ciliogenesis by orienting microtubule growth. The Journal of cell biology 2006;175:547-54. https://doi.org/10.1083/jcb.200605092
38. Hasanov E, Chen G, Chowdhury P, Weldon J, Ding Z, Jonasch E, et al. Ubiquitination and regulation of AURKA identifies a hypoxia-independent E3 ligase activity of VHL. Oncogene 2017;36:3450-63. https://doi.org/10.1038/onc.2016.495
39. Resnick A. HIF stabilization weakens primary cilia. PLOS ONE 2016;11:e0165907. https://doi.org/10.1371/journal.pone.0165907
40. Verghese E, Zhuang J, Saiti D, Ricardo SD, Deane JA. In vitro investigation of renal epithelial injury suggests that primary cilium length is regulated by hypoxia-inducible mechanisms. Cell Biol Int 2011;35:909-913. https://doi.org/10.1042/CBI20090154
41. Esteban MA, Harten SK, Tran MG, Maxwell PH. Formation of primary cilia in the renal epithelium is regulated by the von Hippel-Lindau tumor suppressor protein. J Am Soc Nephrol 2006;17:1801-1806. https://doi.org/10.1681/ASN.2006020181
42. Pfirrmann T, Gerhardt C. Life-Saver or Undertaker: The relationship between primary cilia and cell death in vertebrate embryonic development. J Dev Biol 2022;10:52. https://doi.org/10.3390/jdb10040052
43. Anvarian Z, Mykytyn K, Mukhopadhyay S, Pedersen LB, Christensen ST. Cellular signalling by primary cilia in development, organ function and disease. Nat Rev Nephrol 2019;15:199-219. https://doi.org/10.1038/s41581-019-0116-9
44. Mill P, Christensen ST, Pedersen LB. Primary cilia as dynamic and diverse signalling hubs in development and disease. Nat Rev Genet 2023;24:421-441. https://doi.org/10.1038/s41576-023-00587-9
45. Majmundar AJ, Wong WJ, Simon MC. Hypoxia inducible factors and the response to hypoxic stress. Mol Cell 2010;40:294-309. https://doi.org/10.1016/j.molcel.2010.09.022
46. Semenza GL. A compendium of proteins that interact with HIF-1α. Exp Cell Res 2017;356:128-135. https://doi.org/10.1016/j.yexcr.2017.03.041
47. Blouin CC, Pagé EL, Soucy GM, Richard DE. Hypoxic gene activation by lipopolysaccharide in macrophages: implication of hypoxia-inducible factor 1alpha. Blood 2004;103:1124-1130. https://doi.org/10.1182/blood-2003-07-2427
48. Frede S, Stockmann C, Freitag P, Fandrey J. Bacterial lipopolysaccharide induces HIF-1 activation in human monocytes *via* p44/42 MAPK and NF-kappaB. Biochem J 2006;396:517-527. https://doi.org/10.1042/BJ20051839
49. Semenza GL. HIF-1 mediates metabolic responses to intratumoral hypoxia and oncogenic mutations. J Clin Invest 2013;123:3664-71. https://doi.org/10.1172/JCI67230
50. Rohwer N, Jumpertz S, Erdem M, Egners A, Warzecha KT, Fragoulis A, et al. Non-canonical HIF-1 stabilization contributes to intestinal tumorigenesis. Oncogene 2019;38:5670-5685. https://doi.org/10.1038/s41388-019-0816-4
51. Beall CM, Cavalleri GL, Deng L, Elston RC, Gao Y, Knight J, et al. Natural selection on EPAS1 (HIF2α) associated with low hemoglobin concentration in Tibetan highlanders. Proceedings of the National Academy of Sciences 2010;107:11459-64. https://doi.org/10.1073/pnas.1002443107
52. Labie D. L'adaptation aux très hautes altitudes - Sur quels gènes une pression sélective s'est-elle exercée ? Med Sci (Paris) 2010;26:1038-9. https://doi.org/10.1051/medsci/201026121038

53. Eichstaedt CA, Pagani L, Antao T, Inchley CE, Cardona A, Mörseburg A, et al. Evidence of Early-Stage Selection on EPAS1 and GPR126 Genes in Andean High Altitude Populations. Sci Rep 2017;7:13042. https://doi.org/10.1038/s41598-017-13382-4
54. Eichstaedt C, Pagani L, Antao T, Inchley C, Cardona A, Mörseburg A, et al. New evidence of genetic adaptation to high altitude in Andean populations. Europ Respir J 2018;52:PA1274. https://erj.ersjournals.com/content/52/suppl_62/PA1274, https://doi.org/10.1183/13993003.congress-2018.PA1274
55. Song D, Navalsky BE, Guan W, Ingersoll C, Wang T, Loro E, et al. Tibetan PHD2, an allele with loss-of-function properties. Proc Natl Acad Sci U S A 2020;117:12230-8. https://doi.org/10.1073/pnas.1920546117
56. Brutsaert TD, Kiyamu M, Elias Revollendo G, Isherwood JL, Lee FS, Rivera-Ch M, et al. Association of EGLN1 gene with high aerobic capacity of Peruvian Quechua at high altitude. Proc Natl Acad Sci U S A 2019;116:24006-11. https://doi.org/10.1073/pnas.1906171116
57. Gale DP, Harten SK, Reid CDL, Tuddenham EGD, Maxwell PH. Autosomal dominant erythrocytosis and pulmonary arterial hypertension associated with an activating HIF2 alpha mutation. Blood 2008;112:919-921. https://doi.org/10.1182/blood-2008-04-153718
58. Olson N, Hristova M, Heintz NH, Lounsbury KM, van der Vliet A. Activation of hypoxia-inducible factor-1 protects airway epithelium against oxidant-induced barrier dysfunction. Am J Physiol Lung Cellular and Molecular Physiology 2011;301:L993-1002. https://doi.org/10.1152/ajplung.00250.2011
59. Bartels K, Grenz A, Eltzschig HK. Hypoxia and inflammation are two sides of the same coin. Proceedings of the National Academy of Sciences 2013;110:18351-2. https://doi.org/10.1073/pnas.1318345110
60. Palazon A, Goldrath A, Nizet V, Johnson RS. HIF Transcription factors, inflammation, and immunity. Immunity 2014;41:518-528. https://doi.org/10.1016/j.immuni.2014.09.008
61. Lee SH, Lee SH, Kim CH, Yang KS, Lee EJ, Min KH, et al. Increased expression of vascular endothelial growth factor and hypoxia inducible factor-1α in lung tissue of patients with chronic bronchitis. Clin Biochem 2014;47:552-559. https://doi.org/10.1016/j.clinbiochem.2014.01.012
62. Hammond FR, Lewis A, Elks PM. If it's not one thing, HIF's another: immunoregulation by hypoxia inducible factors in disease. FEBS 2020;287:3907-3916. https://doi.org/10.1111/febs.15476
63. McGettrick AF, O'Neill LAJ. The Role of HIF in Immunity and Inflammation. Cell Metabolism 2020;32:524-536. https://doi.org/10.1016/j.cmet.2020.08.002
64. Polke M, Seiler F, Lepper PM, Kamyschnikow A, Langer F, Monz D, et al. Hypoxia and the hypoxia-regulated transcription factor HIF-1α suppress the host defence of airway epithelial cells. Innate Immun 2017;23:373-380. https://doi.org/10.1177/1753425917698032
65. Page LK, Staples KJ, Spalluto CM, Watson A, Wilkinson TMA. Influence of hypoxia on the epithelial-pathogen interactions in the lung: implications for respiratory disease. Front Immunol 2021;12:653969. https://doi.org/10.3389/fimmu.2021.653969
66. Breeze R, Turk M. Cellular structure, function and organization in the lower respiratory tract. Environ Health Perspect 1984;55:3-24. https://doi.org/10.1289/ehp.84553
67. Davis JD, Wypych TP. Cellular and functional heterogeneity of the airway epithelium. Mucosal Immunol 2021;14:978-990. https://doi.org/10.1038/s41385-020-00370-7
68. Dudchenko O, Ordovas-Montanes J, Bingle CD. Respiratory epithelial cell types, states and fates in the era of single-cell RNA-sequencing. Biochem J 2023;480:921-939. https://doi.org/10.1042/BCJ20220572
69. Russell RJ, Boulet LP, Brightling CE, Pavord ID, Porsbjerg C, Dorscheid D, et al. The airway epithelium: an orchestrator of inflammation, a key structural barrier and a therapeutic target in severe asthma. Eur Respir J 2024;63:2301397. https://doi.org/10.1183/13993003.01397-2023
70. Cho HJ, Kim and CH. Oxygen matters: hypoxia as a pathogenic mechanism in rhinosinusitis. BMB Reports 2018;51:59-64. https://doi.org/10.5483/BMBRep.2018.51.2.014
71. Montgomery ST, Mall MA, Kicic A, Stick SM. Hypoxia and sterile inflammation in cystic fibrosis airways: mechanisms and potential therapies. Eur Respir J 2017;49:1600903. https://doi.org/10.1183/13993003.00903-2016
72. Konrádová V, Uhlík J, Vajner L, Herget J, Adášková J. Exposure to hypoxia injures tracheal epithelium (ultrastructural study). Veterinární medicína 2002;47:270-276. https://doi.org/10.17221/5834-VETMED

73. Uhlik J, Konradova V, Vajner L, Adaskova J. Normobaric hypoxia induces mild damage to epithelium of terminal bronchioles in rabbits (ultrastructural study). Veterinární medicína 2005;50:432-438. https://doi.org/10.17221/5645-VETMED
74. Konrádová V, Kanta J, Sulová J. Effect of bronchoalveolar lavage on the ultrastructure of the tracheal epithelium in rabbits. Respiration 1990;57:14-20. https://doi.org/10.1159/000195813
75. Konrádová V, Uhlík J, Vajner L, Zocová J. Reaction of the goblet cells to cholinergic stimulation. Acta Vet. Brno 1996;65:175-180. https://doi.org/10.2754/avb199665030175
76. Becci PJ, McDowell EM, Trump BF. The respiratory epithelium. II. Hamster trachea, bronchus, and bronchioles. J Natl Cancer Inst 1978;61:551-561.
77. Polosukhin VV, Cates JM, Lawson WE, Milstone AP, Matafonov AG, Massion PP, et al. Hypoxia-inducible factor-1 signalling promotes goblet cell hyperplasia in airway epithelium. J Pathol 2011;224:203-211. https://doi.org/10.1002/path.2863
78. Torres-Capelli M, Marsboom G, Li QOY, Tello D, Rodriguez FM, Alonso T, et al. Role Of Hif2α oxygen sensing pathway in bronchial epithelial club cell proliferation. Sci Rep 2016;6:25357. https://doi.org/10.1038/srep25357
79. Zhou X, Tu J, Li Q, Kolosov VP, Perelman JM. Hypoxia induces mucin expression and secretion in human bronchial epithelial cells. Translational Research 2012;160:419-427. https://doi.org/10.1016/j.trsl.2012.08.001
80. Zhou-Suckow Z, Duerr J, Hagner M, Agrawal R, Mall MA. Airway mucus, inflammation and remodeling: emerging links in the pathogenesis of chronic lung diseases. Cell Tissue Res 2017;367:537-550. https://doi.org/10.1007/s00441-016-2562-z
81. Hey J, Paulsen M, Toth R, Weichenhan D, Butz S, Schatterny J, et al. Epigenetic reprogramming of airway macrophages promotes polarization and inflammation in muco-obstructive lung disease. Nat Commun 2021;12:6520. https://doi.org/10.1038/s41467-021-26777-9
82. Shivaraju M, Chitta UK, Grange RMH, Jain IH, Capen D, Liao L, et al. Airway stem cells sense hypoxia and differentiate into protective solitary neuroendocrine cells. Science 2021;371:52-57. https://doi.org/10.1126/science.aba0629
83. Glover LE, Colgan SP. Epithelial barrier regulation by hypoxia-inducible factor. Ann Am Thorac Soc 2017;14:S233-6. https://doi.org/10.1513/AnnalsATS.201608-610MG
84. Colgan SP, Campbell EL, Kominsky DJ. Hypoxia and Mucosal Inflammation. Annu Rev Pathol 2016;11:77-100. https://doi.org/10.1146/annurev-pathol-012615-044231
85. Song HA, Kim YS, Cho HJ, Kim SI, Kang MJ, Kim JH, et al. Hypoxia modulates epithelial permeability *via* regulation of vascular endothelial growth factor in airway epithelia. Am J Respir Cell Mol Biol 2017;57:527-535. https://doi.org/10.1165/rcmb.2016-0080OC
86. Zhou W, Yu T, Hua Y, Hou Y, Ding Y, Nie H. Effects of hypoxia on respiratory diseases: perspective view of epithelial ion transport. Am J Physiol-Lung Cellular and Molecular Physiology 2022;323:L240-250. https://doi.org/10.1152/ajplung.00065.2022
87. Dale TP, Santer MD, Haris M, Zuo W, Forsyth NR. Hypoxic conditions promote a proliferative, poorly differentiated phenotype in COPD lung tissue progenitor cells in vitro. Exp Lung Res 2023;49:12-26. https://doi.org/10.1080/01902148.2022.2158404
88. Gerovac BJ, Valencia M, Baumlin N, Salathe M, Conner GE, Fregien NL. Submersion and hypoxia inhibit ciliated cell differentiation in a notch-dependent manner. Am J Respir Cell Mol Biol 2014;51:516-525. https://doi.org/10.1165/rcmb.2013-0237OC
89. Brooks ER, Wallingford JB. Multiciliated Cells. Current Biol 2014;24:R973-982. https://doi.org/10.1016/j.cub.2014.08.047
90. Li Q, Han Z, Singh N, Terré B, Fame R, Arif U, et al. Disruption of GMNC-MCIDAS multiciliogenesis program is critical in choroid plexus carcinoma development. Cell Death & Differentiation 2022;29:1-15. https://doi.org/10.1038/s41418-022-00950-z
91. Vladar EK, Mitchell BJ. It's a family act: the geminin triplets take center stage in motile ciliogenesis. The EMBO Journal 2016;35:904-906. https://doi.org/10.15252/embj.201694206
92. Rahane D, Dhingra T, Chalavady G, Datta A, Ghosh B, Rana N, et al. Hypoxia and its effect on the cellular system. Cell Biochemistry and Function 2024;42:e3940. https://doi.org/10.1002/cbf.3940

93. Ozcan SC, Kalkan BM, Cicek E, Canbaz AA, Acilan C. Prolonged overexpression of PLK4 leads to formation of centriole rosette clusters that are connected *via* canonical centrosome linker proteins. Sci Rep 2024;14:4370. https://doi.org/10.1038/s41598-024-53985-2
94. LoMastro GM, Drown CG, Maryniak AL, Jewett CE, Strong MA, Holland AJ. PLK4 drives centriole amplification and apical surface area expansion in multiciliated cells. eLife 11:e80643. https://doi.org/10.7554/eLife.80643
95. Salyha N, Oliynyk I. Hypoxia modeling techniques: A review. Heliyon 2023;9:e13238. https://doi.org/10.1016/j.heliyon.2023.e132382

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REVIEW

In memory of more than twenty years of cooperation with Prof. Jan Herget

Mechanisms Controlling the Behavior of Vascular Smooth Muscle Cells in Hypoxic Pulmonary Hypertension

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Summary

Pulmonary hypertension is a complex and heterogeneous condition with five main subtypes (groups). This review focuses on pulmonary hypertension caused by chronic hypoxia (hypoxic pulmonary hypertension, HPH, group 3). It is based mainly on our own experimental work, especially our collaboration with the group of Professor Herget, whose fifth anniversary of death we commemorate. We have found that oxidation and degradation of the extracellular matrix (ECM) *in vitro*, in either the presence or the absence of pro-inflammatory cells, activate vascular smooth muscle cell (VSMC) proliferation. Significant changes in the ECM of pulmonary arteries also occurred *in vivo* in hypoxic rats, namely a decrease in collagen VI and an increase in matrix metalloproteinase 9 (MMP-9) in the *tunica media*, which may also contribute to the growth activation of VSMCs. The proliferation of VSMCs was also enhanced in their co-culture with macrophages, most likely due to the paracrine production of growth factors in these cells. However, hypoxia itself has a dual effect: on the one hand, it can activate VSMC proliferation and hyperplasia, but on the other hand, it can also induce VSMC hypertrophy and increased expression of contractile markers in these cells. The influence of hypoxia-inducible factors, microRNAs and galectin-3 in the initiation and development of HPH, and the role of cell types other than VSMCs (endothelial cells, adventitial fibroblasts) are also discussed.

Keywords

Vasoconstriction • Remodeling • Oxidation • Degradation • Extracellular matrix • Collagen • Proteolytic enzymes • Metalloproteinases • Macrophages • Mast cells • Smooth muscle cells • Endothelial cells • Fibroblasts • Mesenchymal stem cells • Hypoxia-inducible factor • microRNA • Galectins • Hyperplasia • Hypertrophy • Therapy of hypoxic pulmonary hypertension

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Introduction

Compared to the systemic circulation, the pulmonary vasculature is a low-pressure system. Pulmonary hypertension (PH) is defined as a mean pulmo-nary artery pressure (mPAP) greater than 20 mmHg at rest, according to the ESC/ERS 2022 Guidelines for the diagnosis and treatment of pulmonary hypertension [1]. PH is a complex, heterogeneous group of diseases with different etiologies, manifestations, course, prognosis and treatment (for a review, see [2-4]). Common to all of them is not only the elevation of mPAP, but also pathological remodelling of the pulmonary vascular wall. Most often PH leads to right heart ventricle hypertrophy and eventually failure. PH markedly worsens the quality of life of the patients and their prognosis.

The current classification of PH recognizes 5 groups [1]:

Group 1

Pulmonary arterial hypertension (PAH). This is pre-capillary hypertension [4], the primary pathology of which lies primarily in the pulmonary vasculature. Its prevalence and incidence is relatively low [5]. It includes idiopathic and familiar subtypes, as well as various drug-induced and other uncommon forms (for a review, see [1,2,6,7]).

Group 2

PH associated with left heart disease, which is the most common type of pulmonary hypertension, at least in the Western world [5]. The increased blood pressure in the left atrium propagates "backwards" to the pulmonary veins. Thus, initially, post-capillary PH develops [4], but later mPAP may also increase, resulting in combined pre- and post-capillary PH (for a review, see [2,3]).

Group 3

PH associated with lung disease and/or hypoxemia. This is the second most common type of PH [5]. It is a pre-capillary form of PH [4] and occurs mainly in chronic obstructive pulmonary disease (COPD), but it has also been reported in obstructive sleep apnea syndrome and in interstitial pulmonary fibrosis (for a review, see [2,3, 8-10]). Chronic exposure to cigarette smoke can also lead to this type of PH [11,12]. Hypoxia of high altitude also elicits PH (high-altitude pulmonary hypertension, HAPH) [13]. In comparison with PAH (group 1), which is progressive and irreversible, the pulmonary vascular remodeling during hypoxic PH is milder and is largely thought to be reversible, although some patients can also develop severe PH with irreversible vascular remodeling similar to that in the group 1 [14]. In addition, group 3 PH can overlap with group 2 (29.3 % of patients with PH) [5].

Group 4

PH associated with chronic pulmonary artery obstruction (or chronic thromboembolic pulmonary hypertension, CTEPH). This is pre-capillary hypertension [4,15].

Group 5

PH with unclear and/or multifactorial mechanisms; it is either pre-capillary or combined pre- and post-capillary PH (for a review, see [2,4,16]).

In our studies performed in collaboration with Professor Jan Herget's group, we focused on hypoxic pulmonary hypertension (HPH), which belongs to the group 3. Studies by Professor Jan Herget's group focused on HPH induced by low oxygen partial pressure in the surrounding atmosphere, e.g. in an experimental hypoxic chamber simulating high altitudes [17,18], during airway obstruction [19], or in lung diseases, such as microbial and sterile inflammation [20], asthma, emphysema [21,22], or silicosis [23].

There are two mechanisms involved in the onset and development of HPH: hypoxic pulmonary vasoconstriction and hypoxic pulmonary vascular remodeling (for a review, see [8,24]). Important actors in both these stages are vascular smooth muscle cells (VSMCs). This review focuses on the role and behavior of VSMCs in both of these stages, particularly in the vascular wall remodeling.

VSMCs in hypoxic pulmonary vasoconstriction

The immediate response of pulmonary vessels to a decrease in alveolar oxygen partial pressure is vasoconstriction [13,25]. This differs from systemic circulation, where the main response to hypoxia is vasodilation, caused by the stabilization of hypoxia-inducible factor-1α (HIF-1α), i.e. its avoidance of prolyl hydroxylation [26], and concomitant upregulation of endothelial nitric oxide (NO) synthase. Hypoxic pulmonary vasoconstriction is a physiological mechanism reducing the distribution of blood to hypoventilated areas of the lungs, thereby maintaining optimal blood oxygenation [13,27,28].

Pulmonary vasoconstriction is a functional change in the pulmonary vasculature, mediated by VSMCs, and is completely reversible, at least in health. The mechanism by which VSMCs sense hypoxia has been a subject of considerable research and debate and is beyond the scope of this text; it is reviewed in detail by Archer *et al.* in this issue. Suffice it to say here that hypoxia-induced redox changes in the VSMCs diminish the open-state probability of several types of potassium channels, resulting in cell membrane depolarization. This, in turn, activates voltage-gated calcium channels, and thus the influx of Ca^{2+} ions and subsequent activation of the contractile apparatus in VSMCs (for a review, see [8,29,24]). Ca^{2+} release from the sarcoplasmic reticulum is also an important part of the mechanism, as is Ca^{2+} influx through other types of Ca^{2+}-conductive channels (see Archer *et al.* in this issue).

Although hypoxic pulmonary vasoconstriction is

intrinsic to VSMCs, it can be modulated by the endothelium, i.e., decreased by endothelium-derived vasodilators, such as NO [25] and prostacyclin, and increased by endothelium-derived vasoconstrictors, such as endothelin and thromboxane [29], and also by inhibition of NO synthase. This inhibition may be caused, at least partly, by the elevation of asymmetric dimethylarginine, an endogenous inhibitor of NO synthesis [27].

VSMCs in structural remodeling of the pulmonary vasculature

With prolonged duration or recurrence of hypoxia, morphological remodeling of pulmonary vessels, particularly peripheral, pre-alveolar vessels, follows [25,30].

The mechanism of remodeling of the vascular wall during hypoxia involves, although it may seem somewhat paradoxical, the production of reactive oxygen species (ROS). On the one hand, it is logical to understand hypoxia as a reducing state. During hypoxia, the limited availability of oxygen prevents the generation of ROS, including superoxide (O_2^-) and its conversion to hydrogen peroxide, decreases the ratio of oxidized/ reduced redox couples (e.g., NAD^+/NADH, $NADP^+$/ NADPH, FAD^{2+}/$FADH_2$), and reduces sulfhydryl groups on various molecules [24, 29, 31,32]. On the other hand, several authors, including Prof. Herget and his co-workers [33-35], have reported an increased production of ROS in pulmonary blood vessels during chronic hypoxia. This has been attributed to the activity of enzymes such as xanthine oxidase, endothelial NO synthase (eNOS) and NADPH oxidase, all of which are capable of producing superoxide, as well as inducible NO synthase (iNOS), increasing the production of NO, which readily generates peroxynitrite in the presence of superoxide. Further indirect evidence for increased ROS production in hypoxic pulmonary hypertension is that antioxidant treatment ameliorated pulmonary hypertension in rats exposed to chronic hypoxia [33-35].

Another important mechanism of the remodeling of pulmonary blood vessels is the production of proteolytic enzymes, especially by cells of the immune system, infiltrating the vascular wall during hypoxia [36-39]. The effect of both ROS and proteolytic enzymes ultimately stimulates the proliferation of VSMCs, their hyperplasia, increased matrix deposition by these cells, and thickening of the vascular wall in pulmonary hypertension (for a review, see [8, 13, 40]).

Effects of ROS and macrophages

During chronic hypoxia, ROS are produced by cells of the pulmonary vasculature, namely endothelial cells, VSMCs, adventitial fibroblasts [14,41,42], and also cells of the immune system, such as alveolar and interstitial macrophages [33,43-45] and mast cells, infiltrating large and small pulmonary arteries and occurring subpleurally and perivascularly [36,46].

ROS can have a dual effect on cells, including VSMCs. In higher concentrations, they can cause damage to the cell membrane, mitochondria, DNA or alteration of the function of various enzymes in the sense of their inhibition (protein kinase C) or activation (proteases and endonucleases). These effects can then lead to cell growth arrest and cell death. However, dying cells and their surviving neighbors can produce growth factors and other biomolecules that ultimately stimulate the proliferation of the cell population originally damaged by oxidative stress. Moreover, some authors even believe that at lower concentrations, ROS can directly stimulate cell proliferation, acting as signaling molecules. The mechanism of this effect is similar to that of growth factors. ROS can activate the receptor and non-receptor tyrosine kinases, mitogen-activated protein kinase (MAPK), transcription factor NF-κB, the expression of proto-oncogenes c-fos, and c-jun, the apoptosis-regulating Bcl-2 gene, and also autocrine production of growth factors, which plays an important role in activating the growth of the VSMC population. Already in 1996, Burdon formulated the hypothesis that normal production of ROS is necessary for normal transduction of signals regulating cell growth [47]; for a review, see [12,48,49]. Mito-chondria and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase are considered to be the major ROS producers in the cardiovascular system. In accordance with this, studies on VSMCs under normobaric hypoxia in both humans and animals have shown that the Nox4 isoform of the NADPH oxidase complex was overexpressed, which contributed to VSMC proliferation leading to pulmonary hypertension [13]. In contrast, studies by Archer and co-workers have shown that ROS produced at physiological levels during mitochondrial electron transport maintain the open state of voltage-gated K channels, which inhibits the influx of Ca^{2+} ions into cells [29,50]. This keeps the pulmonary circulation in a relaxed state and does not promote VSMC proliferation, because Ca^{2+} ions are necessary not only for

VSMC contraction but also for proliferation [49,51].

However, ROS can also stimulate VSMC proliferation indirectly by modifying their extracellular matrix (ECM). To explore this possibility, we developed a simple model of oxidative damage to the ECM *in vitro*, i.e., namely collagen irradiated with ultraviolet (UV) light. This was a model of pathological ROS production, not a physiological basal ROS production by electron leakage from mitochondria. We used either type I collagen, which is the major ECM protein of the healthy vessel wall, or type III collagen, another important type of collagen in the blood vessel wall, whose content increases in a pathologically altered vascular wall, such as in hypertension. Although our research was primarily related to PH, we initially used rat aortic smooth muscle cells as a cellular model because of their relatively easy isolation compared to pulmonary vascular cells [52,53].

We found that UV light not only oxidized collagen but also degraded it into low molecular weight fragments. The adhesion of VSMCs to this collagen was weakened, which was reflected not only by their smaller spreading area but also by their higher susceptibility to detachment from the substrate by trypsin. Even molecular markers of cell adhesion differed between cells on irradiated collagen and on non-irradiated collagen. Cells on irradiated collagen had less developed focal adhesion plaques and contained lower concentrations of β_1-integrins, which include receptors for collagen, and also lower concentrations of focal adhesion proteins talin and vinculin, contractile protein α-actin (i.e., a marker of VSMC differentiation) and cytoskeletal protein vimentin. At the same time, VSMCs proliferated faster on irradiated collagen, at least at lower population densities - at higher densities these differences disappeared. The faster proliferation of VSMCs was explained by the fact that the cells escaped the growth control provided by cell-matrix contact. In addition, VSMCs contained increased concentrations of immunoglobulin adhesion molecules such as intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), which bind the cells of the immune system [52,53]. A similar pro-inflammatory phenotype of VSMCs, expressing ICAM-1 and producing various cytokines and chemokines, e.g. interleukins 1β and 6 (IL1β and IL6), monocyte chemoattractant protein-1 (MCP-1), further stimulating VSMC proliferation, has also been described in the pulmonary arteries of human patients suffering from HPH as well as in animal models of this disease (for a review, see [54,55]).

Our research therefore also focused on immune cells and their influence on the ECM and on the adhesion and proliferation of VSMCs. As the first of these immune cells, we focused on macrophages, as these cells are known to play an important role in the origin and development of vascular diseases, and are an important source of ROS. For example, already in 1996, it was shown by Professor Herget's group that alveolar macrophages harvested from the lungs of rats exposed to hypoxia produced more ROS than macrophages from normoxic animals [56]. In addition, macrophages can digest the ECM with their proteolytic enzymes and may even proliferate in the pathologically altered vascular wall (for a review, see [14,57]). In our experiments, we therefore grew rat aortic VSMCs either in cocultures with rat alveolar macrophages or on a collagen substrate pre-modified with these macrophages activated by TiO_2 dust.

We found that macrophages themselves did not proliferate in co-cultures but activated proliferation of VSMCs. Because this increased proliferative activity of VSMCs became apparent only after several days of coculture, we reasoned that it was induced by paracrine production of cytokines, chemokines and growth factors by macrophages (for a review, see [45,58,59]), rather than being an immediate effect of short-living oxygen radicals produced in these cells. It is known that macrophages exhibit remarkable plasticity in response to the environment and can be polarized into either pro-inflammatory M1 cells, which produce high levels of reactive oxygen and nitrogen species and pro-inflammatory cytokines, such as interferon-gamma and tumor necrosis factor-alpha (TNF-α), or into anti-inflammatory M2 cells, which promote tissue repair by producing immunomodulatory substances, such as interleukin 10 (IL10), and growth factors, such as transforming growth factor-beta (TGF-β) and vascular endothelial growth factor (VEGF) [45,60]. A recent study by Professor Herget's followers has shown that alveolar macrophages exposed to hypoxia were able to produce superoxide only *in vivo* but not *in vitro*, although they were able under both conditions to polarize to pro-proliferative M2 macrophages [44].

On collagen modified by activated macrophages, similar to UV light-modified collagen, the VSMCs adhered more weakly, were prone to spontaneous detachment, and showed signs of damage (e.g., vacuolization). The remaining undamaged VSMCs, however, proliferated rapidly and soon compensated for

these cell losses [48]. The initial unfavorable response of VSMCs may be due to an adverse effect of activated macrophages on the collagen substrate. The wall of the failing right ventricle in PH contained macrophages with activated nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) inflammasome, which induced mitochondrial damage, impaired growth and apoptosis of cardiomyocytes [60], and similar cell-damaging substances could also be retained in our macrophage-modified collagen used as a growth substrate for VSMCs.

In the context of ROS, it is also worth mentioning the dual role of NO in the pathogenesis of PH. The effect of NO on VSMCs depends on the amount of NO, the amount of ROS, and the ratio of NO to ROS. At relatively low concentrations, NO exerts vasodilatory and antiproliferative effects on VSMCs through a cyclic guanosine monophosphate (cGMP)-dependent pathway [25,61,62]. However, at high concentrations, NO readily reacts with oxygen and especially with superoxide to form highly reactive substances such as peroxynitrite [25]. At the same time, the production of peroxynitrite is also increased at higher ROS concentrations [12]. Through these mechanisms, NO changes its initial beneficial effect of relieving PH into an effect that further exacerbates PH by contributing to tissue injury [12,13,25,30,33].

Effect of proteolytic enzymes and mast cells

Cells of the immune system that infiltrate the vascular wall during hypoxia also produce proteolytic enzymes, such as chymases, tryptases and metallo-proteinases (MMPs). These proteolytic enzymes degrade the ECM of pulmonary vessels, especially collagen [33,63,64], and thus are another important factor that induces VSMC proliferation by releasing these cells from growth inhibition by the physiological ECM (for a review, see [52,53,65]). VSMC proliferation then leads to thick-ening of the pulmonary vessel walls, increasing their rigidity and peripheral resistance, and thus leads to long-term fixation of PH. The thickening and the rigidity of pulmonary vessels are further promoted by the increased synthesis of ECM molecules, particularly collagen, by the multiplied VSMCs [14,57].

Among the cells of the immune system, in addition to macrophages, we have paid special attention to mast cells. These cells have been relatively little considered in the pathogenesis of HPH, and in the pathogenesis of vascular diseases in general. They have even been referred to as "forgotten cells" in the literature, although in addition to VSMCs, macrophages and fibroblasts, they are another important cell type present in a pathologically altered blood vessel wall (for a review, see [39,46]). These cells also play an important role in various fibrotic diseases, such as renal, pulmonary, hepatic and cardiac fibrosis and the formation of hypertrophic scars (for a review, see [66]).

In our studies on the effect of mast cells on VSMC proliferation, we chose RBL-2H3 cells, i.e. a rat basophilic leukemia (mastocytoma) cell line, as a model of mast cells infiltrating the walls of pulmonary blood vessels. We first compared the production of proteolytic enzymes in these cells and in cell types present in the vascular wall, such as endothelial cells, VSMCs and fibroblasts. We found that the production of chymases, tryptases and metalloproteinases was several times higher in RBL-2H3 cells than in vascular wall cell types. Moreover, the production of these enzymes was usually higher in RBL-2H3 cells cultured in a hypoxic atmosphere (3 % O_2 + 5 % CO_2) than in a normoxic atmosphere (21 % O_2 + 5 % CO_2), whereas the production of proteolytic enzymes in vascular wall cell types was not affected by hypoxia or was even reduced. However, immunofluorescence staining of MMP-13 revealed that endothelial cells and VSMCs cultured under hypoxic conditions contained more numerous and more brightly stained granules with this enzyme than the cells cultured in normoxia [37,38]. In a follow-up study, we then monitored the growth of VSMCs on type I collagen pre-exposed to RBL-2H3 cells cultivated for 48 hours in normoxia or hypoxia. On collagen pre-modified by normoxic RBL-2H3 cells, the proliferation activity of VSMCs was similar to that on unmodified collagen. However, on collagen pre-modified by hypoxic RBL-2H3 cells, the cell population doubling time was significantly shorter, and the final cell population density was significantly higher than on unmodified collagen. This behavior of VSMCs was explained by the degradation of collagen by proteases released from RBL-2H3 cells, which was more pronounced in hypoxic RBL-2H3 cells [39].

Increased collagenolysis is known to stimulate the migration and proliferation of mesenchymal cells, including VSMCs, and is an important contributor to vascular remodeling in HPH. Consistent with this, the metalloproteinase inhibitor Batimastat significantly reduced experimental HPH in rats, while reducing the muscularization of peripheral lung vessels and right ventricular hypertrophy [67]. Similarly, disodium cromoglycate (DSCG), an inhibitor of mast cell

degranulation, decreased the amount of collagen cleavage fragments in pre-alveolar vessels, inhibited muscularization in peripheral pulmonary arteries, and reduced the development of PH [68]. Interestingly, this effect was only observed when DSCG was applied in the early phase of hypoxia exposure. By contrast, when DSCG was administered to rats with already developed chronic HPH, it delayed the regression of HPH upon return to normoxia by preventing the cleavage of the increased amount of collagen in the blood vessel walls [69]. Another mechanism by which mast cells, including RBL-2H3 cells, can stimulate the growth of VSMCs, is through their production of various chemokines and cytokines, such as TNF-α, interleukins, leukotrienes and MCP-1 (for a review, see [58]).

We further focused on the adhesion and growth of VSMCs on collagen modified by primary mast cells isolated from rat lungs. Type I collagen was deposited in polystyrene culture wells and was exposed for 1 or 3 days to mast cells seeded into the inserts above the collagen and cultured in a normoxic or hypoxic atmosphere. The collagen substrate was then rinsed with phosphate-buffered saline (PBS) and was seeded with rat aortic VSMCs (approx. 2150 cells/cm^2). The growth curves of VSMCs in Fig. 1 show that collagen pre-modified by mast cells (MCs) promoted the growth of VSMCs more than unmodified collagen. This difference was more apparent on collagen modified by MCs for 3 days than on collagen modified by MCs for only 1 day. On collagen modified by MCs for 1 day, the VSMCs reached a higher population density on day 5 after seeding (Fig. 1 A, B), while on collagen modified for 3 days, a higher cell population density was already reached on day 3 after seeding, and this difference was more striking on collagen modified with hypoxic MCs (Fig. 1 C, D). Overall, the final population densities of VSMCs tended to be higher on collagen modified by hypoxic MCs than modified by normoxic MCs.

However, a disadvantage of primary cultures of mast cells from rat lungs (by bronchoalveolar lavage) was the frequent presence of bacterial contamination. We therefore performed further studies on extracts from normoxic and hypoxic mast cells, containing proteases. The mast cell extracts were prepared using a solution containing 1 part PBS, 1 part 280 mM sorbitol, 1 mM Ca^{2+} and 0.5 mM Mg^{2+}. Collagen type I, deposited on the bottoms of polystyrene culture wells, was exposed to these extracts for 20 hours in a cell incubator, and was then rinsed with PBS and deionized H_2O, dried and seeded with rat aortic VSMCs (approx. 2150 cells/cm^2). The cells were cultured either in standard Dulbecco's Modified Eagle's Minimum Essential Medium (DMEM) with 10 % of fetal bovine serum (FBS) or in a chemically defined serum-free medium (BD Biocoat, Cat. No. 355160), supplemented with epidermal growth factor (EGF), fibroblast growth factor (FGF) and insulin, already used in our previous study [39]. The reason was to minimize the adsorption of serum proteins, such as vitronectin, fibronectin and albumin, which influence cell adhesion and spreading and can mask the effect of the modified collagen on VSMCs.

The advantage of using a serum-free medium was evident on day 1 after cell seeding. As shown in Fig. 2A, the number of cells on day 1 after seeding tended to be highest on collagen modified with hypoxic mast cell extract. This was more evident in the serum-free medium than in the standard serum-supplemented medium. In the serum-supplemented medium, the size of the cell spreading area was similar in VSMCs on all collagen layers. However, in the serum-free medium, the cell spreading area was smaller in VSMCs on collagen modified with extracts from mast cells, whether normoxic or hypoxic (Fig. 2B).

In the following days, the number of VSMCs increased faster in cells on unmodified collagen, and on day 7 it became significantly higher than on collagen modified with normoxic or hypoxic mast cell extracts (Fig. 2C). However, in a serum-free medium (where the adsorption of molecules with a potential masking effect was minimized), the highest cell numbers were achieved on collagen modified with hypoxic mast cell extract (Fig. 2D). In this case, there is also an interesting connection between the relatively small adhesion area of the cells and the increased growth of their population. This is in line with the generally accepted fact that cell proliferation is highest at the intermediate level of cell-matrix adhesion [70].

Among all proteolytic enzymes produced by mast cells, we finally turned most attention to MMP-13. This enzyme is an interstitial collagenase, the principal enzyme responsible for the initiation of collagen breakdown in the rat species during the development of HPH [36]. We therefore studied the adhesion, growth and viability of VSMCs in cultures on collagen I exposed to MMP-13. Collagen I was adsorbed on polystyrene culture dishes, digested with MMP-13, seeded with rat aortic VSMC (approx. 2500 cells/cm^2) and incubated in DMEM with 10 % of FBS for 1 to 7 days.

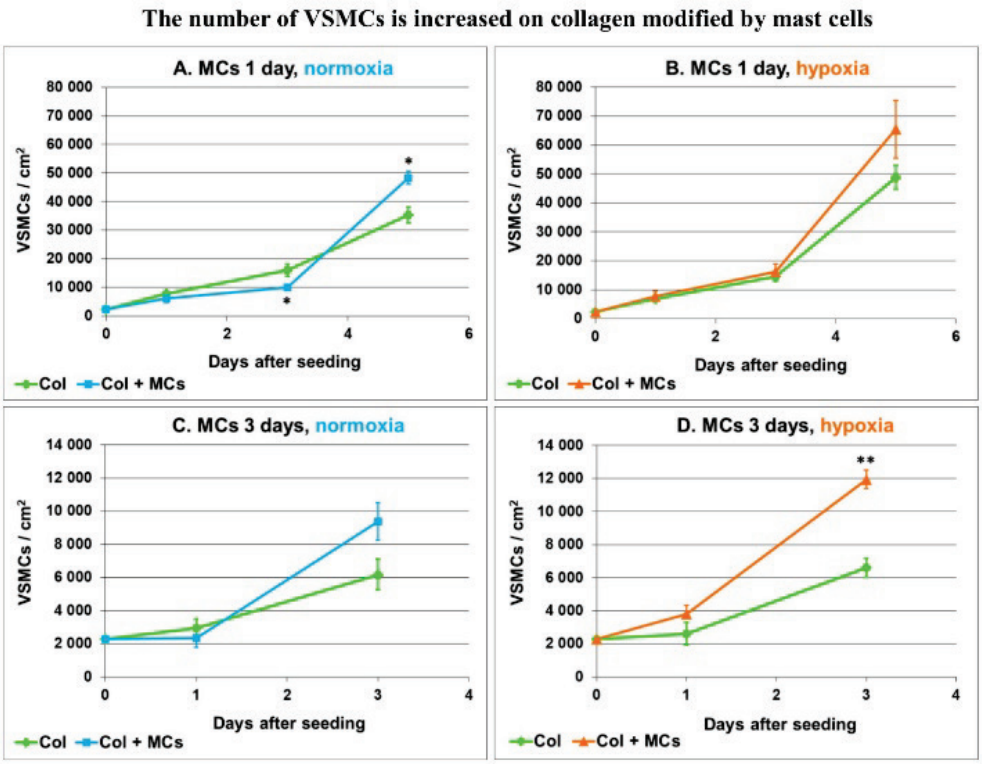

Fig 1. Growth curves of rat aortic VSMCs seeded on unmodified type I collagen (Col) and Col pre-incubated with primary rat lung mast cells (Col + MCs) for 1 day (**A, B**) or for 3 days (**C, D**) under normoxic (**A, C**) or hypoxic (**B, D**) conditions. Mean ± SEM from 18 samples for each experimental group, Student t-test for unpaired data. Statistical significance: *p≤0.05 and **p≤0.01 in comparison with Col.

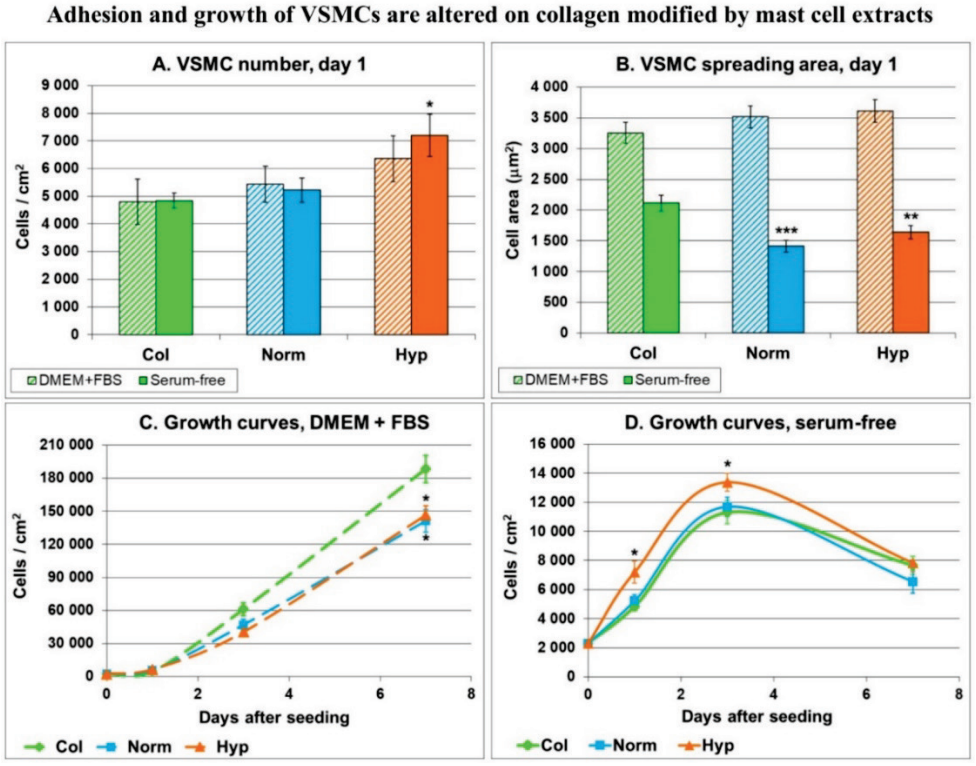

Fig. 2. The number (**A**) and the spreading area (**B**) of rat aortic VSMCs on day 1 after seeding on unmodified type I collagen (Col) or with Col exposed to extracts from mast cells cultured under normoxia (Norm) or hypoxia (Hyp). Mean ± SEM from 9-18 samples (A) or from 34-82 cells (B) for each experimental group. Growth curves of these VSMCs from day 1 to day 7 (**C, D**). The cells were cultured either in a standard serum-supplemented medium (DMEM + FBS) or in a serum-free medium. Student t-test for unpaired data. Statistical significance: **p≤0.01 and ***p≤0.001 in comparison with Col.

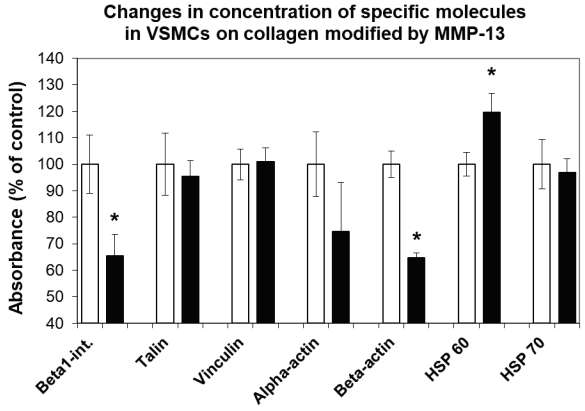

Fig. 3. Changes in the concentration of adhesion molecules (β_1-integrins, talin, vinculin), cytoskeletal proteins (α-actin and β-actin) and heat-shock proteins 60 and 70 (HSP 60, HSP 70) in rat aortic VSMCs cultured on unmodified collagen I (white columns) and on MMP-13-degraded collagen I (black columns). Measured by ELISA per mg of protein on day 3 after seeding. The absorbances of cell samples from the degraded collagen are expressed as percentages of the values obtained from control cells on unmodified collagen. Mean ± SEM from 2-4 experiments, Student t-test for unpaired data, *p<0.05 compared to control values.

We found that the VSMCs on MMP-13-treated collagen adhered in about 1.5 times lower initial numbers than the cells on unmodified collagen (2200 ± 200 *vs.* 1490 ± 650 cells/cm^2; p<0.01). In addition, these cells were usually less spread, i.e. they contacted the modified collagen by a smaller cell adhesion area. Their shape was often round or spindle-like, whereas the cells on unmodified collagen were mainly polygonal. The concentration of β_1-integrin adhesion receptors and β-actin in cells on MMP-13-treated collagen was lower by about 35 % (Fig. 3). The concentrations of focal adhesion proteins talin and vinculin, and a contractile protein α-actin, were unchanged, but the clustering of the first two proteins into focal adhesion plaques, as well as the assembly of α- and β-actin-containing microfilaments, were lower (Fig. 4). The cells on MMP-13-treated collagen contained more heat-shock protein 60 (by 20 ± 7 %), and were more prone to cell death, as indicated by a more than 3 times higher number of trypan blue-stained cells (Fig. 5A). As a result, the growth curves showed that the cells on MMP-13-modified collagen proliferated more slowly than the cells on the control unmodified collagen.

However, the VSMCs on the modified collagen proliferated for a longer period of time, whereas on unmodified collagen the cells reached their maximum population density earlier and entered the stationary phase (Fig. 5B). These results suggested that the cells on MMP-13-degraded collagen escaped the ECM-mediated growth control more easily and increased their turnover [53,65]. Increased turnover of VSMCs, i.e. coexistence of proliferation and apoptosis, has been shown repeatedly in the pulmonary vascular cells of rats with HPH (for a review, see [8]).

Changes in the ECM of pulmonary arteries in vivo

Our further studies on changes in the ECM of pulmonary arteries during hypoxia and their potential influence on the behavior of VSMCs were performed in rats *in vivo*. The rats were exposed to hypoxia (10 % O_2) in a normobaric hypoxic chamber for four days. The rats were then euthanized, and pulmonary arteries (approximately 250–400 μm in diameter, 3rd–5th order, classified as conduit arteries) were subjected to immunohistochemical, proteomic, and real-time PCR analyses, focused particularly on non-fibrillar collagens of type IV and VI [71]. Type IV collagen is located in the basal lamina of cells, including VSMCs, and it helps maintain VSMCs in a differentiated state. Plating VSMCs on type IV collagen induced an increase in contractile proteins, namely α-actin and myosin heavy chain, in these cells. Moreover, various types of stem cells seeded on type IV collagen spontaneously differentiated towards smooth muscle cell phenotype (for a review, see [72]). Type VI collagen is located in the basement membrane and in the interstitial space between cells. It has been reported to induce the differentiation of fibroblasts into myofibroblasts, manifested by their expression of smooth muscle α-actin (for a review, see [72]). Type VI collagen consists of three chains (α1, α2, and α3). The α3(VI) chain is approximately three times longer than the α1(VI) and α2(VI) chains and, unlike the other chains, contains a Kunitz-like terminal domain (C5), with a sequence very similar to that of Kunitz-type A proteinase inhibitors (first described by Bonaldo *et al.* 1989 [73]). This C5 terminal collagen α3(VI) domain with 58 residues is present as a propeptide in the newly formed type VI collagen microfibrils (composed of α1, α2 and α3 chains). However, immediately after the microfibrils are secreted into the ECM, this C5 propeptide is cleaved off and is no longer present in the mature collagen VI fibrils [74]. This C5 domain, after cleavage from the α3(VI) main chain, represents a biologically active peptide, endotrophin [75-77]. Endotrophin appears to be one of the key players in the signaling effects mediated by collagen VI, including its pro-fibrotic nature and chemoattractant properties for macrophages [77]. Endotrophin was first identified by Park and Scherer (2012) as a pathological signal that promotes breast cancer growth [78], and is

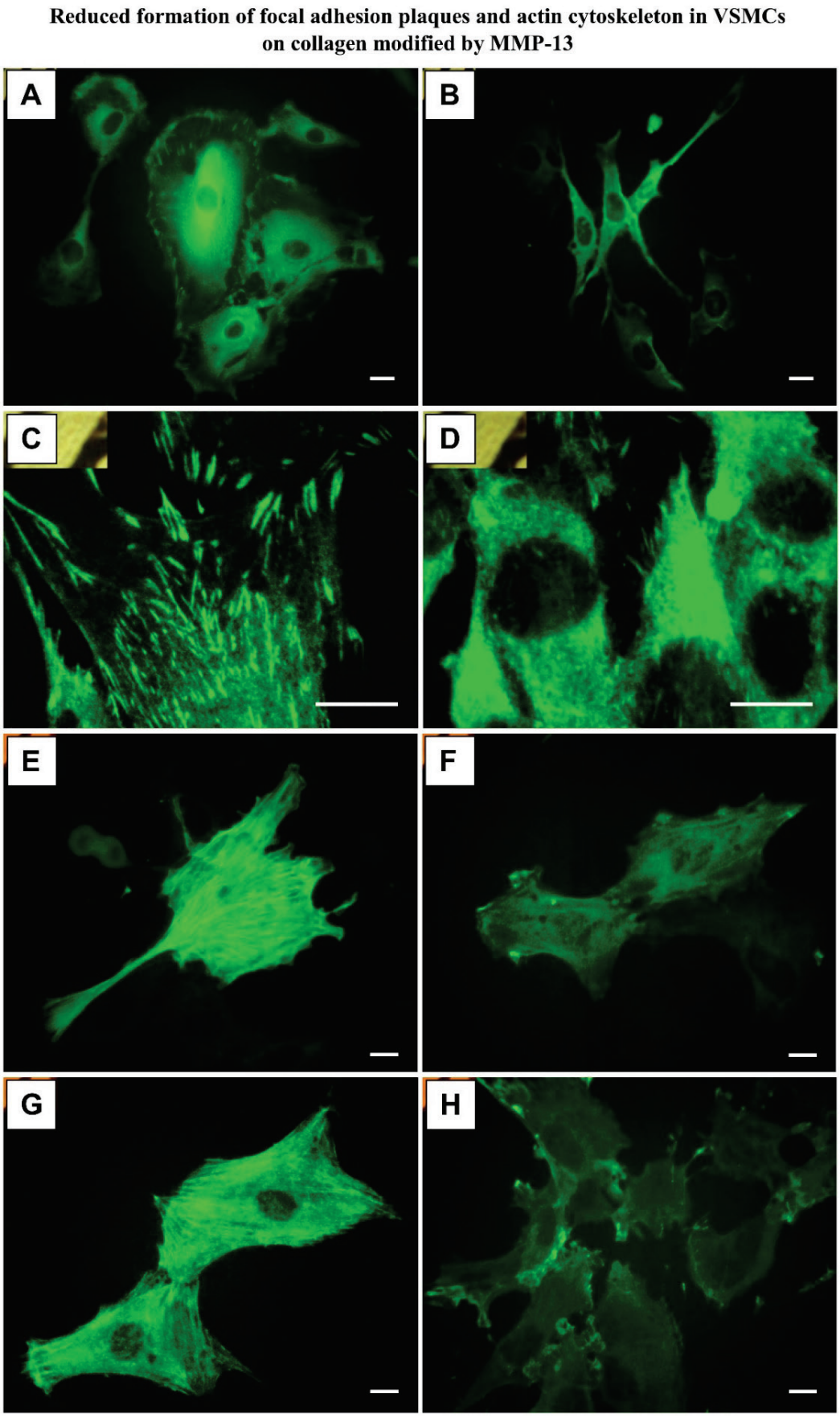

Fig. 4. Immunofluorescence staining of vinculin (**A, B**), talin (**C, D**), alpha-actin (**E, F**) and beta-actin (**G, H**) in 3-day-old cultures of rat aortic VSMCs grown on unmodified collagen I (**A, C, E, G**) or on collagen I digested with MMP-13 (**B, D, F, H**). Zeiss Axioplan epifluorescence microscope, scale bar = 10 μm.

an important marker and driver of fibroinflammatory diseases (for a review, see [79]).

We found that the expression of type IV collagen in the *tunica media* of arteries of hypoxia-exposed rats was not changed at protein and mRNA levels. However, type VI collagen was significantly reduced in the *tunica media* of hypoxic rats at protein level (Fig. 6), while its expression at mRNA level increased. This phenomenon was described for the first time in our study [71]. At the same time, we detected a significant increase in MMP-9 in the *tunica media*, while the expression of MMP-2 at both protein and mRNA levels was decreased in the *tunica media*. We concluded that the loss of collagen VI and increasing concentration of endotrophin could be important factors inducing the phenotypic modulation of VSMCs, i.e., loss of their contractile filaments (e.g., α-actin-containing) and activating their migration and proliferation, which leads to remodeling of the pulmonary arteries during hypoxic pulmonary hypertension. At the same time, collagen VI was retained in the *tunica adventitia*, which could promote the differentiation of adventitial fibroblasts to myofibroblasts [71].

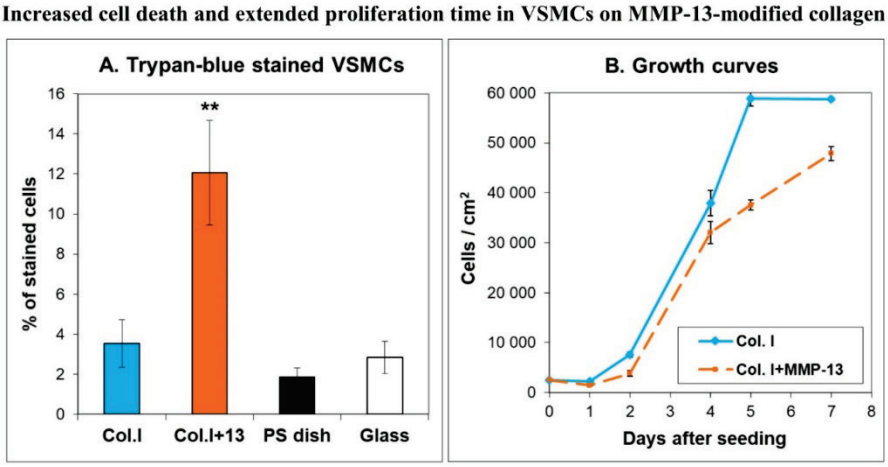

Fig. 5. A) Percentage of trypan blue-stained cells in 2-day-old cultures of rat aortic VSMCs on unmodified collagen I (Col. I), on collagen I digested by MMP-13 (Col. I + 13), on polystyrene tissue culture dishes (PS dish), and on glass coverslips (Glass). Mean ± SEM from 15 measurements. Student t-test for unpaired data, **p<0.01 compared to control values on Col. I. **B**) Growth curves of VSMCs on Col. I and Col. I+MMP-13. Mean ± SEM from 20 measurements (days 1 to 4) or from 4 measurements (days 5 to 7).

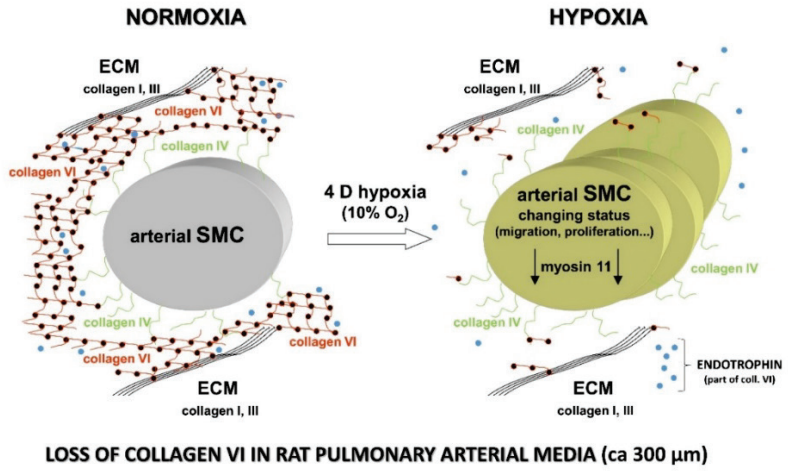

Fig. 6. A significant decrease in the content of collagen VI (red lines with black dots: collagen VI forms this type of "beady" fibrils) in the tunica media of pulmonary arteries (about 300 µm in diameter) during hypoxia. At the same time, the amount of type IV collagen (green on the surface of the VSMCs) remained unchanged. Fibrillar collagens (type I and III) are drawn in black, and collagen VI anchors them to collagen IV. A biologically active peptide, endotrophin, cleaved from the α3(VI) is drawn in blue. The status of some VSMCs changes during the development of HPH (decreasing concentration of the protein myosin 11, a marker of the differentiated status of VSMCs). Modified from [71].

A recent comprehensive and systematic proteomic study by the group of K.R. Stenmark shows significant changes in the matrisome (i.e. the ensemble of genes encoding ECM and ECM-associated proteins) in calf pulmonary vessels in response to hypoxia. Major changes included a strong immune response and wound repair signature characterized by increased levels of complement components (mainly membrane attack complex C5 to C9 components), coagulation cascade proteins (fibrinogen and fibrin), and provisional matrix glycoproteins (tenascin C, fibronectin). In addition, the authors observed an upregulation of ECM-modifying enzymes (proteases and protease inhibitors, enzymes involved in collagen biosynthesis and stabilization), growth factors (TGF-β3, insulin-like growth factor-2, IGF-2), and core ECM proteins involved in vascular stiffening, such as collagens (fibrillar, e. g. types I and III, and non-fibrillar, e.g. types IV and VIII), fibronectin, as well as the glycoproteins vitronectin, which promotes cell adhesion spreading and migration, and periostin, which is associated with epithelial-mesenchymal transition [80].

The role of hypoxia-inducible factors in vascular remodeling

In addition to the effects of the changes in ROS and ECM investigated in our previous studies, other important players in the stimulation of VSMC proliferation and vascular remodeling during HPH are hypoxia-inducible factors (HIFs). HIFs are oxygen-sensitive transcription factors governing the metabolic response of cells to low oxygen levels. There are three basic members of the human HIF family, namely HIF-1, HIF-2 and HIF-3. HIFs are heterodimers composed of oxygen-sensitive α subunit (HIF-1α, HIF-2α, HIF-3α) and oxygen-insensitive β subunit (HIF-1β, HIF-2β, HIF-3β) [8]. HIF-1α is primarily expressed in VSMCs, HIF-2α is predominantly found in endothelial cells (although its role in VSMCs should not be underestimated), and HIF-3a is predominantly expressed in pulmonary fibroblasts [51]. The interplay between all members of the HIF family then leads to the onset and development of HPH.

HIFs are responsible for the activation or inhibition of more than 2 % of human genes participating in the cellular adaptive response in order to maintain oxygen homeostasis [8,40,51,81]. Erythropoietin was the first of the genes for which the HIF regulation was described (by HIF-1α), and in this way, pharmacological interference with the HIF system was investigated as a possible therapy for anemia. Professor Herget's followers focused on roxadustat, a prolyl hydroxylase inhibitor and HIF stabilizer [82]. It is known that HIFs promote VSMC contraction by inhibiting K^+ channels, which leads to the influx of Ca^{2+} ions [8,51]. The application of roxadustat can therefore be associated with a risk of increased pulmonary vascular resistance and vasoconstrictor reactivity. Fortunately, this risk was not confirmed when roxadustat was administered to rats for 14 days. However, this risk should be taken into account, since HIFs and their stabilizers or activators can support blood vessel remodeling [82]. Calcium ions entering the VSMCs can stimulate their proliferation, especially in combination with the shift from oxidative phosphorylation to oxygen-independent glycolysis, which likens the behavior of VSMCs to that of tumor cells [8,49,51,55,59]. In this context, it is interesting to note that gene therapy for oxygen-sensitive Kv1.5 channels, whose expression was downregulated by HIF activation, reduced pulmonary hypertension in chronically hypoxic rats [83].

HIFs also up-regulate growth factors/receptor tyrosine kinases, activate protein kinase B (AKT) and extracellular signal-regulated kinase (ERK), and mamma-lian target of rapamycin (mTOR), i.e. factors that promote cell proliferation and survival, and suppress growth inhibitory factors such as phosphatase and tensin homolog (PTEN), p53 and the Hippo signaling pathway [8,51].

In endothelial cells, hypoxia and HIF-1α upregulate genes for growth factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) [95], which stimulate the migration and proliferation of VSMCs [40]. Last but not least, HIF-1α increases the expression of CD146, which is a co-receptor of VEGF receptor 2 (VEGFR2) and PDGF receptor-β (PDGFR-β), and further enhances the expression of HIF-1α [8,51,84].

Ultimately, HIFs in VSMCs activate the migration and proliferation, stimulate a switch from the quiescent contractile phenotype to a synthetic and proliferative phenotype, inhibit VSMC apoptosis, and also induce osteogenic differentiation of VSMCs, which results in blood vessel calcification [8,51,55]. VSMCs also acquire a pro-inflammatory phenotype, characterized by the expression of cell adhesion molecules of the immunoglobulin and selectin families, and by the production of various chemokines, cytokines and growth factors, attracting cells of the immune system (e.g.

monocytes, macrophages, lymphocytes) and promoting their proliferation [8,14,54,55,57].

It can be summarized that HIF-1 plays a major role in driving VSMC proliferation, while HIF-2 plays a major role in inflammatory cell recruitment *via* activation of endothelial cells and VSMCs to a pro-inflammatory phenotype. HIF-2α is therefore considered to play a major role in the initiation of HPH, whereas HIF-1α may play a major role in the progression and perpetuation of the disease [85,86]. Chronic PH is then characterized by the accumulation of persistently activated cell types in the pulmonary vessels, exhibiting aberrant expression of genes involved in proliferation, apoptosis resistance, inflammation and ECM remodeling [55].

The role of miRNA in vascular remodeling

Other important factors in the onset and development of HPH, including vascular remodeling, are microRNAs (miRNAs). These molecules are single-stranded small noncoding RNAs (18–22 nt in length) that can directly degrade or repress the translation of their target mRNAs, thus negatively regulating gene expression at the posttranscriptional level [58,87]. As it is estimated that at least 30 % of genes in the human genome are directly regulated by miRNAs, a growing number of studies are revealing that miRNAs play a unique and pivotal role in the progression of HPH through the phenotypic switch of VSMCs from a contractile to a synthetic phenotype. The non-proliferative differentiated contractile phenotype is characterized by the expression of myocardin, α-actin, SM22α (early markers of VSMC differentiation), h-caldesmon and calponin-1 (intermediate markers of VSMC differentiation) and desmin, meta-vinculin, SM1 and the SM2 isoforms of myosin heavy chain and smoothelin (late markers of VSMC differentiation). During the transition to the synthetic phenotype, these markers are gradually lost, in order from late to early, so that some level of the early markers, e.g. α-actin, is retained by cells in the synthetic phenotype (for a review, see [54,57]). In addition, some isoforms of differentiation markers are replaced by others, e.g. α-actin by β-actin or SM1 and SM2 isoforms of myosin heavy chain by myosin heavy chain embryonic (SMemb). Other markers of synthetic VSMCs include the presence of tropomyosin 4, increased synthesis of ECM proteins and glycoproteins, such as collagen and osteopontin-8, and particularly increased migration and proliferation activity [87].

Table 1. MicroRNAs which promote or inhibit the switch from contractile to synthetic phenotype and proliferation of VSMCs in HPH.

Dependence/effect	Pro-proliferative	Anti-proliferative	Reference
Hypoxia-dependent	miRNA-9	miRNA-17~92 cluster	[87]
	miRNA-20a	miRNA-30c	
	miRNA-23a	miRNA-124	
	miRNA-214	miRNA-140	
		miRNA-206	
		miRNA-449	
	miRNA-17	miRNA-26b-5p	[58]
	miRNA-18a-5p	miRNA-140-5p	
	miRNA-19a	miRNA-150	
	miRNA-92b-3	miRNA-223	
	miRNA-143	miRNA-760	
	miRNA-145		
	miRNA-155-5p		
	miRNA-214		
	miRNA-1260b		
Growth factor-dependent	miRNA-221	miRNA-21	[87]
	miRNA-15b	miRNA-132	
	miRNA-96		
	miRNA-24		

The transition from the contractile to the synthetic phenotype and *vice versa* is regulated by specific miRNAs that ultimately either stimulate VSMC proliferation or have an antiproliferative effect (Table 1). Modulation of their expression could therefore be used in the prevention and therapy of HPH.

Cell types other than VSMC in vascular remodeling

During HPH, a metabolically reprogrammed, proliferative and pro-inflammatory phenotype is acquired not only by VSMCs but also by other cell types present in the blood vessel wall. These cells include intimal endothelial cells and adventitial fibroblasts, which can be considered as "sentinel cells" separating VSMCs from the blood and the surrounding vascular environment, and which are activated first in response to various adverse factors [4,14,49,59]. Endothelial cells can produce endothelin-1, a potent vasoconstrictor, which stimulates the expression of HIF-1α in VSMCs, promotes the proliferation of VSMCs and fibroblasts, and facilitates the production of ECM by these cells [11,51]. Moreover, endothelial cells can undergo a so-called endothelial-to-mesenchymal transition and acquire a VSMC-like phenotype, characterized by the presence of smooth muscle α-actin [85].

A similar transformation can be observed in adventitial fibroblasts, which transform into myo-fibroblasts positive for smooth muscle α-actin, and resemble VSMCs [12,14,40,54]. These fibroblasts are capable of proliferating, stimulating the proliferation of VSMCs, and particularly recruiting monocytes and lymphocytes (e.g., T-cells) and activating them into cells with the pro-inflammatory and pro-remodeling phenotype [14,88,89]. Last but not least, VSMC-like cells capable of proliferation and paracrine secretion of growth factors can arise from progenitor cells, either resident in the vascular wall or circulating in the blood after their release from the bone marrow [12,54]. Importantly, a recent study by Hu *et al*. (2023) [90] showed that human and bovine pulmonary vascular fibroblasts from patients or animals with PH exhibited even greater expression of cytokines, chemokines and growth factors than VSMCs and endothelial cells from the same vessels, and that HIF inhibition alone was not sufficient to reverse the persistently activated phenotype of these fibroblasts.

Effect of hypoxia on the growth of VSMCs in vitro

The pulmonary arteries used to study ECM changes *in vivo* (part "Changes in the ECM of pulmonary arteries *in vivo*" above) were also used to isolate VSMCs and culture them *in vitro* for growth studies. The arteries (approx. 250–400 μm in diameter, 3^{rd}–5^{th} order) were dissected from the lungs of normoxic and hypoxic adult male Wistar rats under a microscope, were visually cleared of *tunica adventitia*, and were minced into fragments (0.5 mm^3 or less). These fragments were digested by collagenase, and VSMCs were isolated from them by an explantation method [52,91,92]. The identity of the VSMCs was verified by smooth muscle α-actin detection at the protein level [71]. The cells were then cultured in a humidified air atmosphere either under normoxic conditions (21 % O_2 and 5 % CO_2) or under hypoxic conditions (2.5 % O_2 and 5 % CO_2) in a high-glucose DMEM supplemented with 10 % of FBS and gentamicin (40 μg/ml) [92]. The growth of four experimental groups of cells was compared:

(a) VSMCs from hypoxic rats cultured in a hypoxic atmosphere ("hyp in hyp"),

(b) VSMCs from hypoxic rats cultured in a normoxic atmosphere ("hyp in norm"),

(c) VSMCs from normoxic rats cultured in a hypoxic atmosphere, ("norm in hyp")

(d) VSMCs from normoxic rats cultured in a normoxic atmosphere ("norm in norm").

We found that the proliferation activity, measured by the increase in cell number from day 1 to day 7 after seeding, tended to be highest in group (a) and lowest in group (d) (Fig. 7). Although these differences were not statistically significant, this result is consistent with our earlier findings of increased VSMC growth on collagen modified by hypoxic mast cells (Figs 1, 2). This result is also in agreement with previous studies by other authors, who described an increased proliferative activity of pulmonary arterial VSMCs in HPH in humans and in various experimental models (for a review, see [8,54]). It is also evident that culturing cells from hypoxic donors in normoxia tended to attenuate the proliferative activity of VSMCs, whereas culturing cells from normoxic donors in hypoxia enhanced this activity, although not to the level of the activity of hypoxic cells cultured in hypoxia (Fig. 7).

Effect of hypoxia on markers of VSMC differentiation in vitro

Interesting results were obtained when the expression of genes for markers of VSMC differentiation at the mRNA level was studied by real-time qPCR in cultures of VSMC isolated from pulmonary arteries of normoxic and hypoxic rats. These markers included smooth muscle *α*-actin, calponin-1 and myosin heavy

chain, i.e. early, intermediate and late markers of VSMC differentiation, respectively (for a review, see [54,57,87]). We expected a loss in the mRNA expression of these markers as a sign of phenotypic modulation of VSMCs during HPH. However, the expression of *α*-actin was unchanged in hypoxic VSMCs, and the expression of calponin-1 and particularly myosin heavy chain was even increased (Fig. 8). At the same time, the expression of type I collagen was unchanged, but the cells from hypoxic rats showed an increased expression of galectin-3. This β-galactosyl-binding protein, considered to be implicated in the pathogenesis of HPH, can have a dual effect on VSMCs. On the one hand, an increased expression of Gal-3 in VSMCs was reported to be associated with increased proliferation activity of these cells and their lower tendency to apoptosis [93]. Moreover, in VSMCs, Gal-3 induced the expression of osteopontin, a marker of phenotypic modulation of VSMCs towards synthetic phenotype, associated with VSMC proliferation and vascular calcification [94]. On the other hand, Gal-3 was reported to stimulate cell differentiation towards contractile VSMC phenotype, e.g. by inducing the expression of α-actin in endothelial cells [95] and by increasing the expression of α-actin and calponin in VSMCs [92,94]. This is probably related to the pro-fibrotic effect of Gal-3, as α-actin and calponin can also be considered as markers of fibrosis [92,96].

Hypoxia may also have a dual effect on the differentiation status of cells. On the one hand, as already explained, it promotes the dedifferentiation of initially contractile VSMCs into a synthetic phenotype, but on the other hand, it is used to differentiate stem cells into different cell types, such as endothelial cells [97,98], chondrocytes [99], osteoblasts [100], cardiomyocytes (for a review, see [101]), and also smooth muscle cells. For example, in a recent study by Lin *et al*. (2020) [102], human subcutaneous adipose tissue-derived stem cells (ADSCs), cultured in an induction medium consisting of low-glucose DMEM supplemented with 1 % of FBS, TGF-β1 (5 ng/ml) and bone morphogenetic protein-4 (BMP-4; 2.5 ng/ml) and subjected to hypoxia (1 % of O_2), increased the expression of *α*-actin, SM22*α*, calponin and myosin heavy chain, i.e. markers of VSMC differentiation, which was mediated by N6-adenosine methyltransferases (Mettl3). At the same time, however, hypoxia and Mettl3 induced paracrine production of VEGF, TGF-β, hepatocyte growth factor (HGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), basic fibroblast growth factor (bFGF), and stromal cell-derived factor-1 (SDF-1) in ADSCs, i.e. factors promoting not only the differentiation but also the proliferation of ADSCs.

Therefore, the described dual effects of hypoxia, i.e., two opposing tendencies of hypoxia to stimulate either growth or differentiation of VSMCs, may have coincided in our studies on HPH. As expected, we observed an increased tendency of hypoxic VSMCs to proliferate, i.e., to increase in number, although this tendency did not reach

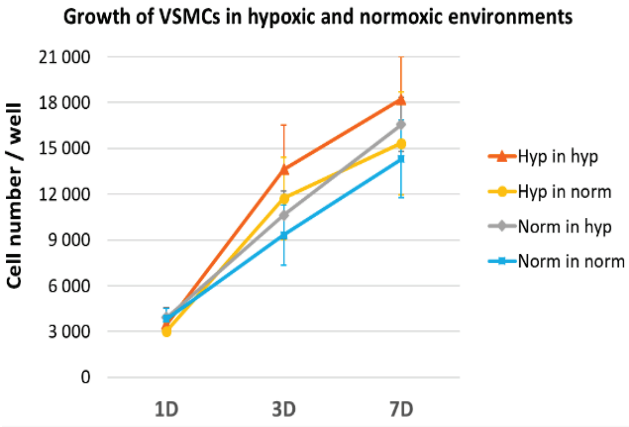

Fig. 7. VSMCs showed a tendency to increase their growth activity under hypoxic conditions. When VSMCs were isolated from pulmonary arteries of hypoxic rats and cultivated for 3 and 7 days (3D, 7D) in a hypoxic atmosphere (Hyp in hyp), they reached on average higher numbers than when cultured in a normoxic atmosphere (Hyp in norm), and higher numbers than VSMCs from normoxic rats grown in a hypoxic atmosphere (Norm in hyp). The lowest average cell numbers were observed for VSMCs from normoxic rats grown in a normoxic atmosphere (Norm in norm). The number of initially adhered cells on 1 day (1D) after seeding was similar in all tested groups. Mean ± SEM, n = 5. One Way ANOVA, Student-Newman-Keuls Method, $p \leq 0.05$. No statistically significant difference was detected.

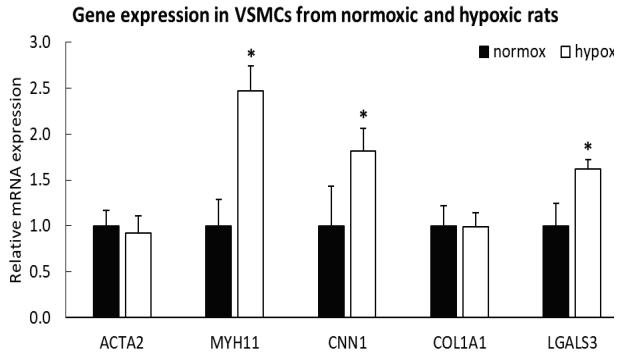

Fig. 8. Expression of genes encoding α-actin (ACTA2), myosin heavy chain (MYH11), calponin-1 (CNN1), type I collagen (COL1A1) and galectin-3 (LGALS3) in VSMCs isolated from pulmonary arteries of normoxic and hypoxic rats (passage 2) and cultivated for 6 days in a normoxic atmosphere and in a hypoxic atmosphere, respectively. Mean + SD, n = 5. Student's t-test, $p \leq 0.05$. * significant difference compared to normoxic cells.

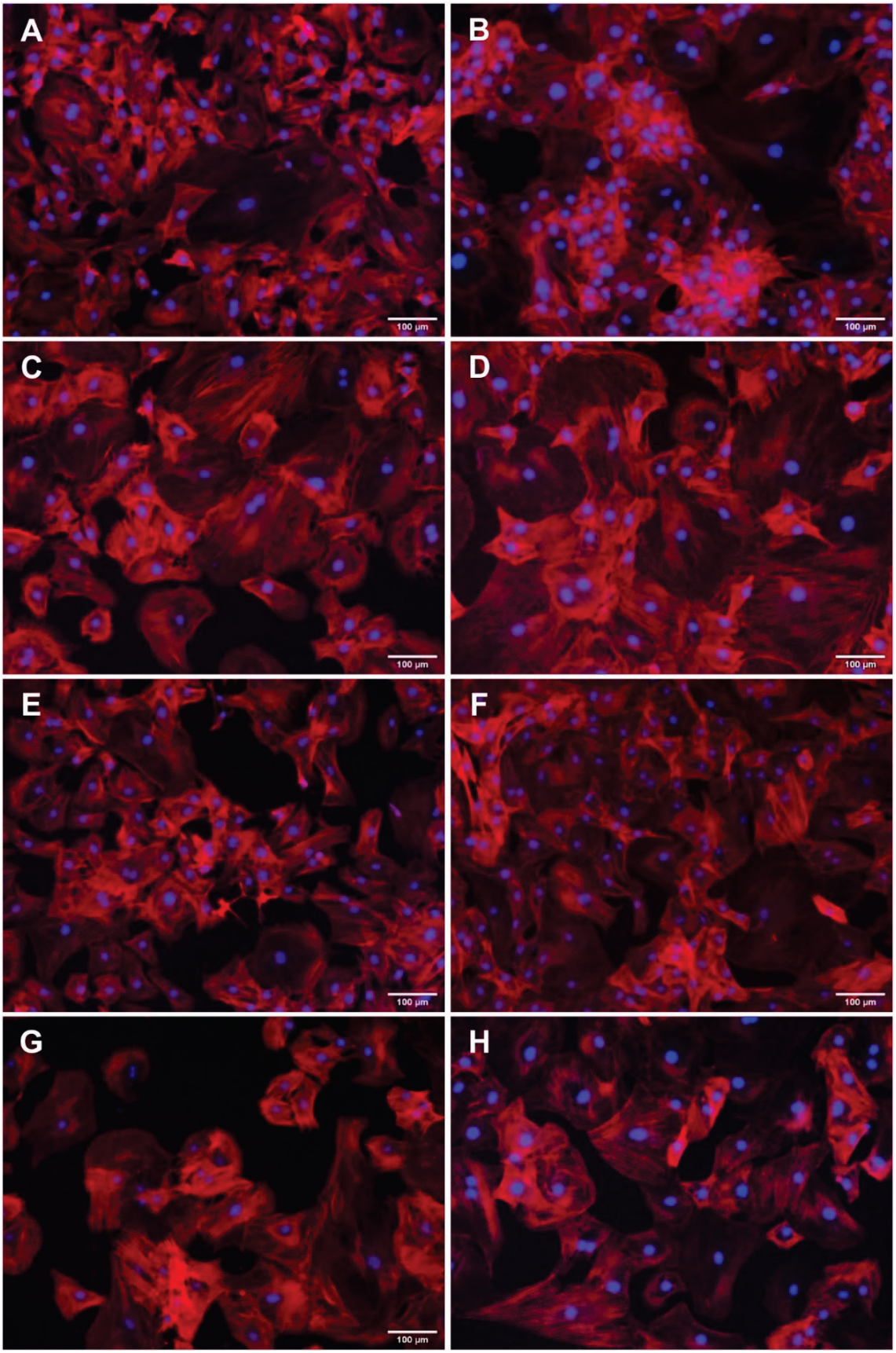

Fig. 9. Morphology of VSMCs isolated from pulmonary arteries of normoxic (**A, B, E, F**) and hypoxic (**C, D, G, H**) rats and cultivated for 1 day (**A, C, E, G**) and for 4 days (**B, D, F, G**) in DMEM with 10 % of FBS (**A-D**) or with 0.5 FBS (**E-H**) in a normoxic atmosphere (**A, B, E, F**) and in a hypoxic atmosphere (**C, D, G, H**). F-actin in the cells was stained with phalloidin conjugated with TRITC, cell nuclei were counterstained with Hoechst 33342. Olympus IX 71 microscope, DP 70 digital camera. Scale bar represents 100 µm.

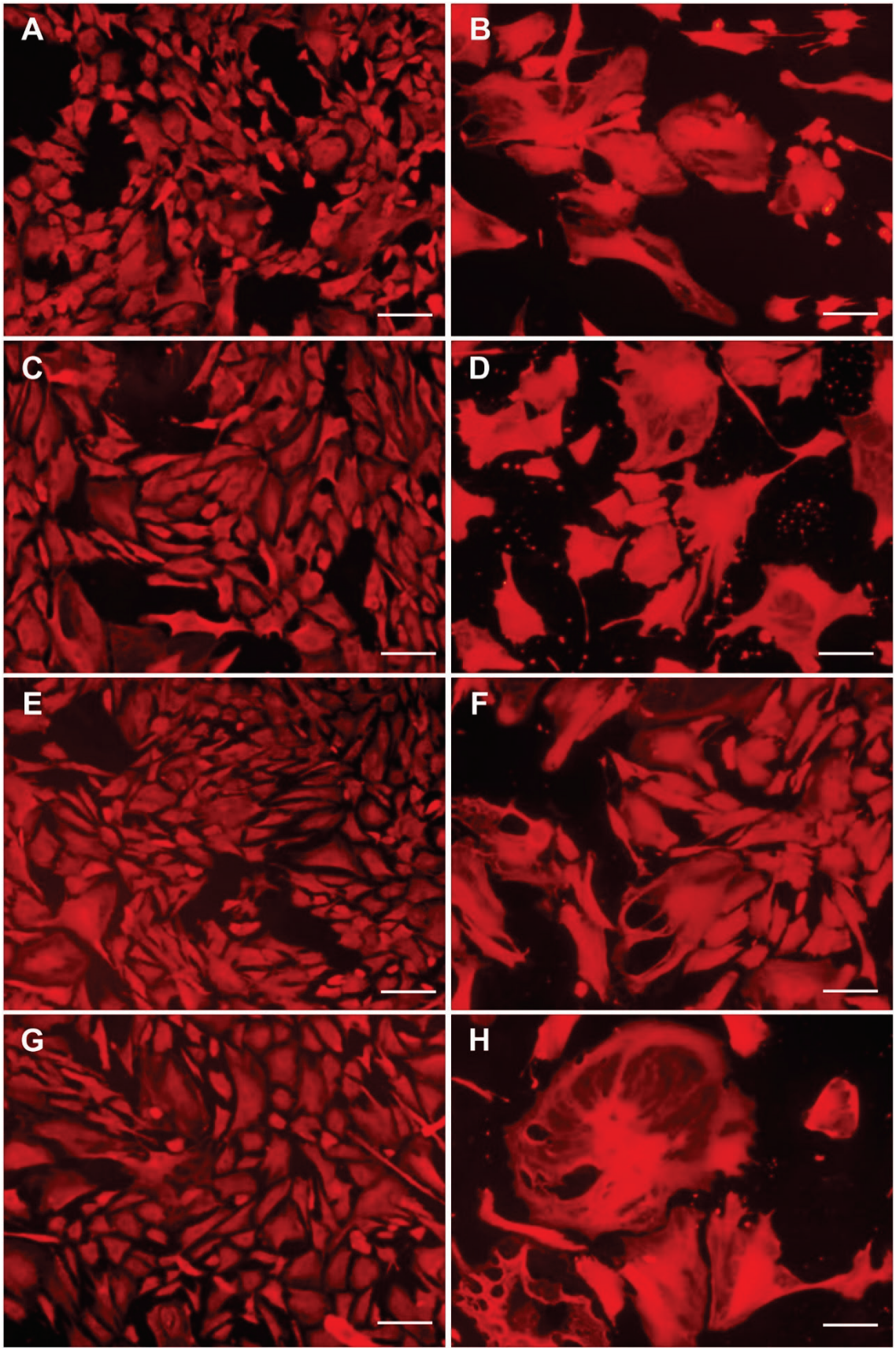

Fig. 10. Morphology of VSMCs isolated from pulmonary arteries of normoxic (**A, C, E, G**) and hypoxic (**B, D, F, H**) rats and cultivated in DMEM with 10 % of FBS for 4 days in pure polystyrene wells (**A, B**), in wells coated with type I collagen (**C, D**), with type IV collagen (**E, F**) or with type VI collagen (**G, H**). Cells stained with Texas Red C_2 maleimide. Olympus IX 51 microscope, DP 70 digital camera, obj. 10x. Scale bar represents 100 μm.

statistical significance. At the same time, we also observed an increased expression of differentiation markers in VSMCs. This led us to the idea that in addition to hyperplasia, hypertrophy of VSMCs could also occur in our experimental setup.

The question of VSMC hypertrophy in HPH

First, it is important to note that even under basal physiological conditions, pulmonary (and also systemic) VSMCs are a highly heterogeneous population, ranging from less differentiated cells prone to proliferative and synthetic behavior to highly differentiated quiescent cells with contractile function [103]. The representation of individual subpopulations of VSMCs in the pulmonary vascular bed varies transversely and longitudinally in the *tunica media* of pulmonary arteries. For example, the middle *tunica media* of the bovine main pulmonary artery contained highly differentiated VSMCs (expressing both α-actin and SM myosin), while the sub-endothelial and outer media also contained less differentiated VSMCs expressing only α-actin [104]. At the same time, the content of highly differentiated cells increased in the proximal-to-distal axis, so that the distal pulmonary arteries contained a relatively homogeneous population of differentiated contractile VSMCs [105]. Obviously, these morphologically different subpopulations of VSMCs also exhibited different growth responses to hypoxic exposure. For example, hypoxia is thought to activate proliferation only in a specific less differentiated subpopulation of VSMCs, rather than directly causing phenotypic modulation and proliferation of originally differentiated contractile VSMCs [103,104]. Another important point is that the growth activation of VSMCs involves not only an increase in their number, but also an increase in their volume and protein content [105]. Moreover, the growth responses of VSMCs can change over time - the proliferative activity of VSMCs was higher in the early stages than in the late stages of HPH [54].

For studies of VSMC morphology, we used the same VSMCs as for the ECM and proliferation studies, i.e., described in the first paragraphs of parts *"*Changes in the ECM of pulmonary arteries *in vivo"* and "Effect of hypoxia on the growth of VSMCs *in vitro"*. As shown in Fig. 9, the population of VSMCs isolated from the pulmonary arteries of normoxic rats and cultured under normoxic conditions was heterogeneous, i.e. containing cells adhering to the substrate with a spreading area of varying size and shape and with a more or less developed filamentous actin (F-actin) cytoskeleton (Fig. 9 A, B). This is consistent with the previously described heterogeneity of VSMCs isolated by an explantation method from bovine pulmonary arteries, containing small rhomboid cells and larger spindle-shaped or cobblestone-like epitheloid cells, which also differed in immunofluorescence staining for SM alpha-actin and SM myosin [104].

However, in cultures of VSMCs isolated from pulmonary arteries of hypoxic rats, more cells were spread over a larger area on the culture substrate than in the case of VSMCs from normoxic arteries, which may be indicative of their larger volume (Fig. 9 C, D). Furthermore, this larger cell spreading area did not depend on the composition of the culture medium or the cell adhesion substrate. This larger area of hypoxic VSMCs was found both in the standard culture medium with 10 % FBS (Fig. 9 A-D) and in the serum-deprived medium with only 0.5 % FBS (Fig. 9 E-H), and not only on the culture polystyrene (Fig. 9) but also on various ECM proteins present in the vessel wall, such as collagen I, IV, VI, and fibronectin (Fig. 10). In addition, the larger spreading area of hypoxic VSMCs was evident not only when these cells were cultured under hypoxia (Fig. 9), but also when they were cultured under normoxia ("hyp in norm"; Fig. 10). These results suggest that larger cells were not generated or selected by the culture conditions, but were already primarily present in the pulmonary arteries of hypoxic rats. Increased volume and proteo-synthesis in VSMCs in response to hypoxia were found in the outer media of the main pulmonary artery and in distal pulmonary arteries (diameter from 1500 μm to 100 μm) [105], i.e. in VSMCs from similar regions to those used in our experiments.

An increased volume of VSMCs of intrapulmonary arteries was also observed in a study by Shimoda *et al*. (2001) [106] in mice exposed for 21 days to normobaric hypoxia in a hypoxic chamber with 10 ± 0.5 % O_2. This increased volume of VSMCs explained, at least partly, the reduction in K_V current density in these cells. However, normal HIF-1α levels were a prerequisite for this VSMC hypertrophy, because hypoxic mice with a null allele at the *Hif1a* locus, i.e. *Hif1a(+/-)* mice, lacked this hypertrophic response of VSMCs, as well as the reduction in K_V current induced by chronic hypoxia.

Hypertrophy and strengthening of the contractile apparatus of VSMCs may result from their increased mechanical stress in hypertension. In the vessels of the systemic circulation, this hypertrophy may also be

associated with polyploidy of the cells. This polyploidy is the result of incomplete growth stimulation, which leads to DNA synthesis and mitosis of cells that are not followed by karyokinesis of the cells, or at least their cytokinesis, which also leads to the formation of binucleated cells. This process is also referred to as endoreduplication [107]. VSMCs with duplicated or multiplied chromosomal equipment are then considered to be more efficient in the synthesis of contractile and ECM proteins, and thus better able to withstand an increased mechanical load. In this sense, hypertrophy and polyploidization of cells are considered a kind of differentiation of VSMCs [91]; for a review, see [57]. Another mechanism of possible polyploidy in pulmonary hypertension could be cell fusion initiated by circulating cells that contribute to tissue repair [108]. However, the presence of polyploidy in pulmonary vessels is rather unlikely, as shown by studies based on flow cytometry [109] or based on fluorescent *in situ* hybridization (FISH; [108]). Consistent with this, VSMCs of the outer media of the bovine main pulmonary artery and distal pulmonary arteries, which increased their volume and photosynthesis in response to hypoxia, exhibited relatively low DNA synthesis [105].

However, cell hypertrophy can also be a sign of cell senescence. A recent study by Born *et al.* (2023) [110] showed that HPH was associated with the accumulation of senescent VSMC, and also endothelial cells, mainly at sites of vascular hypertrophy. This accumulation coincided with increases in the DNA damage markers gamma H2A histone family member X (γ-H2AX) and tumor suppressor p53 binding protein 1 (53BP1). Senescent VSMCs stimulated the migration and growth of neighboring cells through the secretion of paracrine factors, whereas senescent endothelial cells released pro-inflammatory factors attracting cells of the immune system. Nevertheless, the elimination of senescent cells by senolytic therapies aggravated PH, which resulted mainly from the removal of senescent endothelial cells and further activation of VSMC proliferation and loss of lung capillaries [110]. Senescent cells with reduced proliferative capacity have been demonstrated in pulmonary arteries during HPH in studies by the group of Stenmark *et al*. Their occurrence depended on the duration and the severity of hypoxia, the duration of hypertension, and the localization in the pulmonary circulation, where they varied in the length and thickness of the vessels (for a review, see [54]).

The work of Prof. Herget's group and collaborating scientists from the Institute of Physiology of the Czech Academy of Sciences in the field of pulmonary hypertension is schematically shown in Fig. 11.

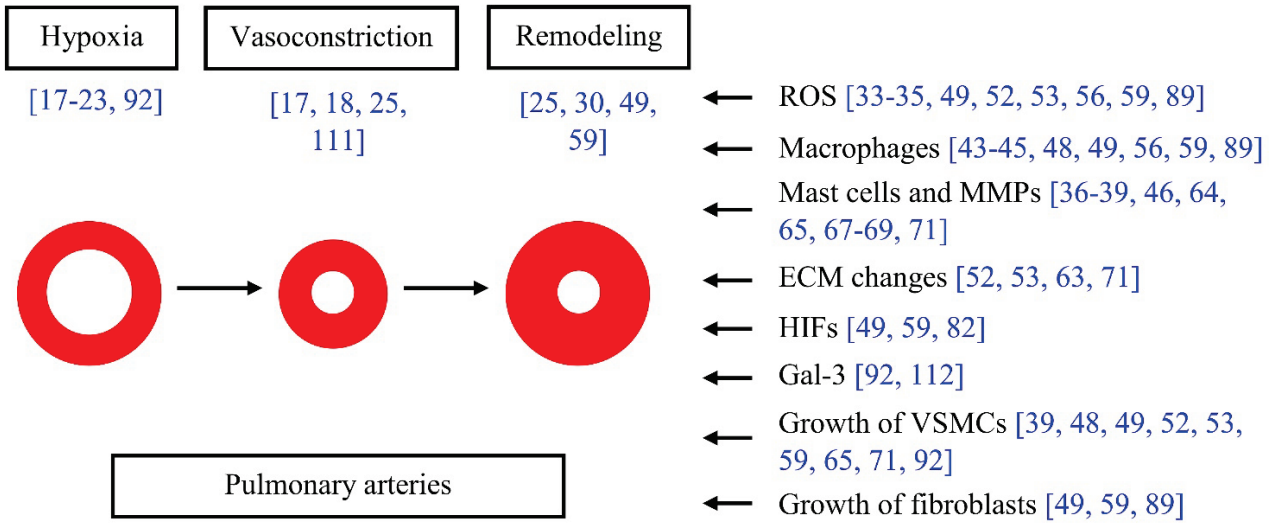

Fig. 11. Work of Professor Jan Herget´s group and scientists from the Institute of Physiology of the Czech Academy of Sciences. Resulting publications in brackets.

Treatment options for HPH

Despite decades of research on HPH mechanisms, the practical outcome – effective therapy – still remains limited. As extensive recent reviews on this topic are available, we supplement our review focusing

on pulmonary vascular remodeling in hypoxia only with a brief, non-exhaustive overview of the main concepts.

While several options to slow down or even stop the progression of PAH (group 1 PH) are available, they are not necessarily useful and could even be counterproductive in HPH [113]. The main reason is that these drugs (targeting NO/cGMP axis, prostacyclin, or endothelin) [114-116] not only reduce the morphological remodeling of the pulmonary vascular wall, but also inhibit hypoxic pulmonary vasoconstriction. As this physiological mechanism is important for optimization of lung ventilation/perfusion ratio and thus arterial blood oxygenation, its inhibition easily results in further worsening of oxygenation (already compromised in patients with HPH). Currently, the therapy of patients with HPH therefore primarily targets the underlying disease, e.g. COPD with bronchodilators or interstitial lung diseases with anti-inflammatory drugs and antifibrotics (for a review, see [9,113]).

The problem of the inhibition of hypoxic pulmonary vasoconstriction may, in principle, be overcome by using inhaled instead of systemically administered agents. That way, the agent reaches poorly ventilated regions of the lung much less easily that the well ventilated areas. Any vasodilator (and anti-proliferative) effect is thus meager in the poorly ventilated parts and the worsening of the oxygenation is mostly prevented.

A good example of such a substance is inhaled NO. It must be administered in low concentrations (low ppm at most) to prevent lateral diffusion from the ventilated to the non-ventilated alveoli. This also limits the potential toxic effects of NO [117,118]. Inhaled NO has the added advantage of being selective only for pulmonary vessels. After diffusion from the alveoli, it is very rapidly scavenged by hemoglobin in erythrocytes and therefore does not enter the systemic vasculature in large enough quantities to cause vasodilation. However, NO has the major drawback of being severely toxic in higher concentrations, which hinders its usefulness for long-term outpatient treatment.

Prostacyclin analogues can also be administered by inhalation, and they (e.g. treprostinil) were shown to be useful in patients with PH associated with interstitial lung disease, i.e. PH belonging to group 3 [119-121].

Other promising therapeutic interventions against vascular remodeling in HPH could include silencing or enhancing the specific miRNAs mentioned above (Table 1; [58,87]), and also silencing the expression of HIFs by small interfering RNAs (siRNAs) [85,122] antisense oligonucleotides or small molecule inhibitors [86].

Statins, competitive inhibitors of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductases, are another promising group of drugs for the therapy of HPH. Although they were primarily developed to reduce cardiovascular risks by lowering the level of blood cholesterol, they display a number of health-positive effects unrelated to their primary function (so-called pleiotropic effects). They proved beneficial in several animal models of HPH and some human studies. Moreover, they may amplify the effects of other drugs targeting HPH [8,111].

Conclusion and further perspectives

This review focuses on the history of more than twenty years of collaboration between a group of scientists at the Institute of Physiology of the Czech Academy of Sciences, and Professor Jan Herget's group at the 2nd Faculty of Medicine of Charles University, and was written to mark the five-year anniversary of Jan's death. The collaboration concerned HPH, and in particular the changes in pulmonary vascular wall components. With prolonged, continuous or intermittent hypoxia, vascular wall remodeling can occur when migration, proliferation and phenotypic modulation of VSMCs and other cell types present in the vascular wall, such as endothelial cells and adventitial fibroblasts, are activated. In this activation, an important role is played by reactive oxygen and nitrogen species, by the presence of pro-inflammatory cells (macrophages, mast cells), by degradation of the extracellular matrix by proteases and qualitative changes in its composition, by the production of hypoxia-inducible factors, by changes in the expression of specific micro RNAs, and also by the expression of galectins (galectin-3).

Our results show that hypoxia has a dual effect on the growth of VSMCs. On the one hand, it promotes their proliferation and hyperplasia; on the other hand, it also promotes the hypertrophy of VSMCs and the expression of contractile proteins in these cells, such as calponin-1 and myosin heavy chain, which seems to be related to the pro-fibrotic effect of hypoxia. This dual effect of hypoxia on VSMC growth in terms of hyperplasia and hypertrophy needs to be further investigated.

Abbreviations

53BP1, tumor suppressor p53 binding protein 1; ACTA2, actin alpha 2, an actin protein also referred to as alpha-actin, alpha-actin-2, aortic smooth muscle or alpha-smooth muscle actin (α-SMA, SMactin, alpha-SM-actin, ASMA); ADSC(s), adipose tissue-derived stem cell(s); AKT, alpha serine/threonine-protein kinase, protein kinase B; ANOVA, Analysis of Variance; Bcl-2, B cell lymphoma-2; bFGF, basic fibroblast growth factor (also known as FGF-2); BMP-4, bone morphogenetic protein-4; CD146, cluster of differentiation 146, also known as the melanoma cell adhesion molecule (MCAM) or cell surface glycoprotein MUC18; cGMP, cyclic guanosine monophosphate; *CNN1*, gene encoding calponin-1; Col, collagen; *COL1A1*, gene encoding collagen type I alpha 1; COPD, chronic obstructive pulmonary disease; CTEPH, chronic thromboembolic pulmonary hypertension; DMEM, Dulbecco's Modified Eagle's Minimum Essential Medium; DNA, deoxyribonucleic acid; DSCG, disodium cromoglycate; ECM, extracellular matrix; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; ERS, European Respiratory Society; ESC, European Society of Cardiology; F-actin, filamentous actin; $FAD^{2+}/FADH_2$, oxidized and reduced form of flavin adenine dinucleotide; FBS, fetal bovine serum; FGF, fibroblast growth factor; FISH, fluorescent *in situ* hybridization; Gal-3, galectin-3; γ-H2AX, gamma H2A histone family member X; GM-CSF, granulocyte-macrophage colony-stimulating factor; HAPH, high-altitude pulmonary hypertension; HGF, hepatocyte growth factor; HIF(s), hypoxia-inducible factor(s); HPH, hypoxic pulmonary hypertension; HSP, heat-shock protein; Hyp, hypoxia; Hypox, hypoxia; ICAM-1, intercellular cell adhesion molecule-1; IGF, insulin-like growth factor; IL, interleukin; iNOS, inducible nitric oxide synthase; *LGALS3*, gene encoding human galectin-3; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; MC(s), mast cells; Mettl3, methyltransferase 3 (N6-Adenosine-Methyltransferase Complex Catalytic Subunit); miRNA(s), micro ribonucleic acid(s); MMP(s), metalloproteinase(s); mTOR, mammalian target of rapamycin; mPAP, mean pulmonary artery pressure; mRNA, messenger ribonucleic acid; *MYH11*, Myosin Heavy Chain 11 (a gene encoding myosin-11 protein, also referred to as AAT4, FAA4, SMHC, SMMHC, myosin heavy chain 11, smooth muscle myosin heavy chain 11, VSCM2, SMMS-1); NAD^+/NADH, oxidized/reduced form of nicotinamide adenine dinucleotide; $NADP^+$/NADPH, oxidized/reduced form of nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NLRP3, nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3; NO; nitric oxide; Norm; normoxia; Normox, normoxia; nt, nucleotide; *p*, probability under the null hypothesis of obtaining a real-valued test statistic at least as extreme as the one obtained; p53, Tumor protein P53, cellular tumor antigen p53 (UniProt name), or transformation-related protein 53 (TRP53), a regulatory protein that is often mutated in human cancers; PAH, pulmonary arterial hypertension; PBS, phosphate-uffered saline; PCR, polymerase chain reaction; PDGF, platelet-derived growth factor; PDGFR-β; platelet-derived growth factor receptor-β; PH, pulmonary hypertension; PS, polystyrene; PTEN, phosphatase and tensin homolog; qPCR, quantitative polymerase chain reaction; RBL-2H3, a subline of rat basophilic leukemia cells; RNAs; ribonucleic acid(s); ROS, reactive oxygen species; SEM, Standard Error of the Mean; SD, Standard Deviation; SDF-1, stromal cell-derived factor-1; SM1, SM2, smooth muscle myosin of SM1 and SM2 isoforms; SM22α, Smooth Muscle 22α, a marker of adult smooth muscle; SMC(s), smooth muscle cell(s); siRNA(s), small interfering RNA(s); SMemb, smooth muscle myosin heavy chain embryonic; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor-alpha; TRITC, tetramethylrhodamine isothiocyanate; UV, ultra-violet; VCAM-1, and vascular cell adhesion olecule-1; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2; *vs*., *versus*; VSMC(s), vascular smooth muscle cell(s).

Conflict of Interest

There is no conflict of interest.

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References

1. Humbert M, Kovacs G, Hoeper MM, Badagliacca R, Berger RMF, Brida M, Carlsen J, Coats AJS, Escribano-Subias P, Ferrari P, Ferreira DS, Ghofrani HA, Giannakoulas G, Kiely DG, Mayer E, Meszaros G, Nagavci B, Olsson KM, Pepke-Zaba J, Quint JK, Rådegran G, Simonneau G, Sitbon O, Tonia T, Toshner M, Vachiery JL, Noordegraaf AV, Delcroix M, Rosenkranz S, Grp EESD. 2022 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension. Eur Respir J 2023;61: 2200879. https://doi.org/10.1183/13993003.00879-2022
2. Vonk Noordegraaf A, Groeneveldt JA, Bogaard HJ. Pulmonary hypertension. Eur Respir Rev 2016;25:4-11. https://doi.org/10.1183/16000617.0096-2015
3. Hoeper MM, Ghofrani HA, Grünig E, Klose H, Olschewski H, Rosenkranz S. Pulmonary Hypertension. Dtsch Arztebl Int 2017;114:73-84. https://doi.org/10.3238/arztebl.2016.0073
4. Thompson AAR, Lawrie A. Targeting Vascular Remodeling to Treat Pulmonary Arterial Hypertension. Trends Mol Med 2017;23:31-45. https://doi.org/10.1016/j.molmed.2016.11.005
5. Wijeratne DT, Lajkosz K, Brogly SB, Lougheed MD, Jiang L, Housin A, Barber D, Johnson A, Doliszny KM, Archer SL. Increasing Incidence and Prevalence of World Health Organization Groups 1 to 4 Pulmonary Hypertension A Population-Based Cohort Study in Ontario, Canada. Circ-Cardiovasc Qual 2018;11:e003973. https://doi.org/10.1161/CIRCOUTCOMES.117.003973
6. Thenappan T, Ormiston ML, Ryan JJ, Archer SL. Pulmonary arterial hypertension: pathogenesis and clinical management. BMJ 2018;360:j5492. https://doi.org/10.1136/bmj.j5492
7. Simonneau G, Montani D, Celermajer DS, Denton CP, Gatzoulis MA, Krowka M, Williams PG, Souza R. Haemodynamic definitions and updated clinical classification of pulmonary hypertension. Eur Respir J 2019;53:1801913. https://doi.org/10.1183/13993003.01913-2018
8. Chai T, Qiu C, Xian Z, Lu Y, Zeng Y, Li J. A narrative review of research advances in hypoxic pulmonary hypertension. Ann Transl Med 2022;10:230. https://doi.org/10.21037/atm-22-259
9. Singh N, Dorfmuller P, Shlobin OA, Ventetuolo CE. Group 3 Pulmonary Hypertension: From Bench to Bedside. Circ Res 2022;130:1404-1422. https://doi.org/10.1161/CIRCRESAHA.121.319970
10. Maimaitiaili N, Zeng YX, Ju PA, Zhakeer G, Guangxi E, Yao HY, Shi YF, Zhai M, Zhuang JH, Peng WH, Zhuoga D, Yu Q. NLRC3 deficiency promotes hypoxia-induced pulmonary hypertension development via IKK/NF-?B p65/HIF-1a pathway. Exp Cell Res 2023;431:113755. https://doi.org/10.1016/j.yexcr.2023.113755
11. Zhang YP, Xu CB. The roles of endothelin and its receptors in cigarette smoke-associated pulmonary hypertension with chronic lung disease. Pathol Res Pract 2020;216:153083. https://doi.org/10.1016/j.prp.2020.153083
12. Karnati S, Seimetz M, Kleefeldt F, Sonawane A, Madhusudhan T, Bachhuka A, Kosanovic D, Weissmann N, Krüger K, Ergün S. Chronic Obstructive Pulmonary Disease and the Cardiovascular System: Vascular Repair and Regeneration as a Therapeutic Target. Front Cardiovasc Med 2021;8:649512. https://doi.org/10.3389/fcvm.2021.649512
13. Siques P, Pena E, Brito J, El Alam S. Oxidative stress, kinase activation, and inflammatory pathways involved in effects on smooth muscle cells during pulmonary artery hypertension under hypobaric hypoxia exposure. Front Physiol 2021;12:690341. https://doi.org/10.3389/fphys.2021.690341
14. Pugliese SC, Poth JM, Fini MA, Olschewski A, El Kasmi KC, Stenmark KR. The role of inflammation in hypoxic pulmonary hypertension: from cellular mechanisms to clinical phenotypes. Am J Physiol Lung Cell Mol Physiol 2015;308:L229-L252. https://doi.org/10.1152/ajplung.00238.2014

15. Humbert M, Guignabert C, Bonnet S, Dorfmüller P, Klinger JR, Nicolls MR, Olschewski AJ, Pullamsetti SS, Schermuly RT, Stenmark KR, Rabinovitch M. Pathology and pathobiology of pulmonary hypertension: state of the art and research perspectives. Eur Respir J 2019;53:1801887. https://doi.org/10.1183/13993003.01887-2018
16. Mandras SA, Mehta HS, Vaidya A. Pulmonary Hypertension: A Brief Guide for Clinicians. Mayo Clin Proc 2020;95:1978-1988. https://doi.org/10.1016/j.mayocp.2020.04.039
17. Suggett AJ, Herget J. Effect of alpha-methyldopa on the pulmonary vascular changes induced by chronic hypoxia in rats. Clin Sci Mol Med 1977;53:397-400. https://doi.org/10.1042/cs0530397
18. Herget J, Kuklík V. Perinatal lung injury extends in adults the site of hypoxic pulmonary vasoconstriction upstream. Physiol Res 1995;44:25-30.
19. Herget J, Paleček F, Vízek M, Holusa R. Causes of Experimental Pulmonary-Hypertension in Rats. Physiol Bohemoslov 1976;25:411-418.
20. Herget J, Paleček F, Preclík P, Čermáková M, Vízek M, Petrovická M. Pulmonary-Hypertension Induced by Repeated Pulmonary Inflammation in the Rat. J Appl Physiol Respir Environ Exerc Physiol 1981;51:755-761. https://doi.org/10.1152/jappl.1981.51.3.755
21. Herget J, Holusa R, Palecek F. Pulmonary-Hypertension in Rats with Experimental Emphysema. Physiol Bohemoslov 1974;23:55-65.
22. Herget J, Paleček F, Čermáková M, Vízek M. Pulmonary-Hypertension in Rats with Papain Emphysema. Respiration 1979;38:204-212. https://doi.org/10.1159/000194082
23. Herget J, Kuncová M, Havránková J, Paleček F. Pulmonary-Hypertension in Silicotic Rats. Arch Environ Health 1979;34:320-324. https://doi.org/10.1080/00039896.1979.10667424
24. Weir EK, López-Barneo J, Buckler KJ, Archer SL. Acute oxygen-sensing mechanisms. N Engl J Med 2005;353:2042-2055. https://doi.org/10.1056/NEJMra050002
25. Hampl V, Herget J. Role of nitric oxide in the pathogenesis of chronic pulmonary hypertension. Physiol Rev 2000;80:1337-1372. https://doi.org/10.1152/physrev.2000.80.4.1337
26. Osada-Oka M, Ikeda T, Imaoka S, Akiba S, Sato T. VEGF-enhanced proliferation under hypoxia by an autocrine mechanism in human vascular smooth muscle cells. J Atheroscler Thromb 2008;15:26-33. https://doi.org/10.5551/jat.E533
27. Böger R, Hannemann J. Dual role of the L-arginine-ADMA-NO pathway in systemic hypoxic vasodilation and pulmonary hypoxic vasoconstriction. Pulm Circ 2020;10:2045894020918850. https://doi.org/10.1177/2045894020918850
28. Slobod D, Damia A, Leali M, Spinelli E, Mauri T. Pathophysiology and clinical meaning of ventilation-perfusion mismatch in the acute respiratory distress syndrome. Biology (Basel) 2022;12:67. https://doi.org/10.3390/biology12010067
29. Dunham-Snary KJ, Wu DC, Sykes EA, Thakrar A, Parlow LRG, Mewburn JD, Parlow JL, Archer SL. Hypoxic pulmonary vasoconstriction from molecular mechanisms to medicine. Chest 2017;151:181-192. https://doi.org/10.1016/j.chest.2016.09.001
30. Herget J, Bíbová J, Novotná J. [Mechanisms of remodeling of pulmonary blood vessels in chronic hypoxia]. Cesk Fysiol 1999;48:179-184.
31. Archer SL, Will JA, Weir EK. Redox status in the control of pulmonary vascular tone. Herz 1986;11:127-141.
32. Archer SL, Huang J, Henry T, Peterson D, Weir EK. A Redox-Based O2 Sensor in Rat Pulmonary Vasculature. Circ Res 1993;73:1100-1112. https://doi.org/10.1161/01.RES.73.6.1100
33. Herget J, Wilhelm J, Novotná J, Eckhardt A, Vytášek R, Mrázková L, Ošťádal M. A possible role of the oxidant tissue injury in the development of hypoxic pulmonary hypertension. Physiol Res 2000;49:493-501.
34. Lachmanová V, Hnilicková O, Povýsilová V, Hampl V, Herget J. N-acetylcysteine inhibits hypoxic pulmonary hypertension most effectively in the initial phase of chronic hypoxia. Life Sci 2005;77:175-182. https://doi.org/10.1016/j.lfs.2004.11.027
35. Hodyc D, Johnson E, Skoumalová A, Tkaczyk J, Maxová H, Vízek M, Herget J. Reactive oxygen species production in the early and later stage of chronic ventilatory hypoxia. Physiol Res 2012;61:145-151. https://doi.org/10.33549/physiolres.932206

36. Vajner L, Vytášek R, Lachmanová V, Uhlík J, Konrádová V, Novotná J, Hampl V, Herget J. Acute and chronic hypoxia as well as 7-day recovery from chronic hypoxia affects the distribution of pulmonary mast cells and their MMP-13 expression in rats. Int J Exp Pathol 2006;87:383-391. https://doi.org/10.1111/j.1365-2613.2006.00493.x
37. Maxová H, Novotná J, Vajner L, Tomášová H, Vytášek R, Vízek M, Bačáková L, Valoušková V, Eliášová T, Herget J. In Vitro Hypoxia Increases Production of Matrix Metalloproteinases and Tryptase in Isolated Rat Lung Mast Cells. Physiol Res 2008;57:903-910. https://doi.org/10.33549/physiolres.931278
38. Maxová H, Bačáková L, Lisá V, Novotná J, Tomášová H, Vízek M, Herget J. Production of proteolytic enzymes in mast cells, fibroblasts, vascular smooth muscle and endothelial cells cultivated under normoxic or hypoxic conditions. Physiol Res 2010;59:711-719. https://doi.org/10.33549/physiolres.931909
39. Maxová H, Bačáková L, Eckhardt A, Mikšík I, Lisá V, Novotná J, Herget J. Growth of vascular smooth muscle cells on Collagen I exposed to RBL-2H3 mastocytoma cells. Cell Physiol Biochem 2010;25:615-622. https://doi.org/10.1159/000315080
40. Gallardo-Vara E, Ntokou A, Dave JM, Jovin DG, Saddouk FZ, Greif DM. Vascular pathobiology of pulmonary hypertension. J Heart Lung Transplant 2023;42:544-552. https://doi.org/10.1016/j.healun.2022.12.012
41. Stenmark KR, Bouchey D, Nemenoff R, Dempsey EC, Das M. Hypoxia-induced pulmonary vascular remodeling: contribution of the adventitial fibroblasts. Physiol Res 2000;49:503-517.
42. Nozik-Grayck E, Stenmark KR. Role of reactive oxygen species in chronic hypoxia-induced pulmonary hypertension and vascular remodeling. Adv Exp Med Biol 2007;618:101-112. https://doi.org/10.1007/978-0-387-75434-5_8
43. Zaloudíková M, Herget J, Vízek M. The contractile response of isolated small pulmonary arteries induced by activated macrophages. Physiol Res 2014;63:267-270. https://doi.org/10.33549/physiolres.932698
44. Žaloudíková M, Vytášek R, Rašková M, Vízek M, Uhlík J, Hampl V. The effect of exposure to hypoxia on superoxide formation by alveolar macrophages is indirect. Life Sci 2019;236:116864. https://doi.org/10.1016/j.lfs.2019.116864
45. Žaloudíková M. Mechanisms and Effects of Macrophage Polarization and Its Specifics in Pulmonary Environment. Physiol Res 2023;72:S137-S156. https://doi.org/10.33549/physiolres.935058
46. Maxová H, Herget J, Vízek M. Lung mast cells and hypoxic pulmonary hypertension. Physiol Res 2012;61:1-11. https://doi.org/10.33549/physiolres.932221
47. Burdon RH. Control of cell proliferation by reactive oxygen species. Biochem Soc Trans 1996;24:1028-1032. https://doi.org/10.1042/bst0241028, https://doi.org/10.1042/bst024521sc
48. Bačáková L, Herget J, Wilhelm J. Influence of macrophages and macrophage-modified collagen I on the adhesion and proliferation of vascular smooth muscle cells in culture. Physiol Res 1999;48:341-351.
49. Plecitá-Hlavatá L, D'Alessandro A, El Kasmi K, Li M, Zhang H, Ježek P, Stenmark KR. Metabolic Reprogramming and Redox Signaling in Pulmonary Hypertension. Adv Exp Med Biol 2017;967:241-260. https://doi.org/10.1007/978-3-319-63245-2_14
50. Archer SL, Nelson DP, Weir EK. Simultaneous measurement of O_2 radicals and pulmonary vascular reactivity in rat lung. J Appl Physiol (1985) 1989;67:1903-1911. https://doi.org/10.1152/jappl.1989.67.5.1903
51. Hu YQ, Zhao YC, Li P, Lu H, Li H, Ge JB. Hypoxia and panvascular diseases: exploring the role of hypoxia-inducible factors in vascular smooth muscle cells under panvascular pathologies. Sci Bull (Beijing) 2023;68:1954-1974. https://doi.org/10.1016/j.scib.2023.07.032
52. Bačáková L, Wilhelm J, Herget J, Novotná J, Eckhart A. Oxidized collagen stimulates proliferation of vascular smooth muscle cells. Exp Mol Pathol 1997;64:185-194. https://doi.org/10.1006/exmp.1997.2219
53. Bačáková L, Lisá V, Kubínová L, Wilhelm J, Novotná J, Eckhart A, Herget J. Ultraviolet light-irradiated collagen III modulates expression of cytoskeletal and surface adhesion molecules in rat aortic smooth muscle cells in vitro. Virchows Arch 2002;440:50-62. https://doi.org/10.1007/s004280100463
54. Stenmark KR, Frid MG, Graham BB, Tuder RM. Dynamic and diverse changes in the functional properties of vascular smooth muscle cells in pulmonary hypertension. Cardiovasc Res 2018;114:551-564. https://doi.org/10.1093/cvr/cvy004
55. Hu CJ, Zhang H, Laux A, Pullamsetti SS, Stenmark KR. Mechanisms contributing to persistently activated cell phenotypes in pulmonary hypertension. J Physiol 2019;597:1103-1119. https://doi.org/10.1113/JP275857

56. Wilhelm J, Sojková J, Herget J. Production of hydrogen peroxide by alveolar macrophages from rats exposed to subacute and chronic hypoxia. Physiol Res 1996;45:185-191.
57. Bacakova L, Travnickova M, Filova E, Matějka R, Stepanovska J, Musilkova J, Zarubova J, Molitor M. *The Role of Vascular Smooth Muscle Cells in the Physiology and Pathophysiology of Blood Vessels.* SAKUMA K (ed.) In:. Muscle Cell and Tissue. London, United Kingdom: IntechOpen; 2018. p. 229-256. https://doi.org/10.5772/intechopen.77115
58. Huang XJ, Akguen EE, Mehmood K, Zhang H, Tang ZX, Li Y. Mechanism of hypoxia-mediated smooth muscle cell proliferation leading to vascular remodeling. Biomed Res Int 2022;2022:3959845. https://doi.org/10.1155/2022/3959845
59. D'Alessandro A, El Kasmi KC, Plecitá-Hlavatá L, Ježek P, Li M, Zhang H, Gupte SA, Stenmark KR. Hallmarks of Pulmonary Hypertension: Mesenchymal and Inflammatory Cell Metabolic Reprogramming. Antioxid Redox Signal 2018;28:230-250. https://doi.org/10.1089/ars.2017.7217
60. Al-Qazazi R, Lima PDA, Prisco SZ, Potus F, Dasgupta A, Chen KH, Tian L, Bentley RET, Mewburn J, Martin AY, Wu DC, Jones O, Maurice DH, Bonnet S, Provencher S, Prins KW, Archer SL. Macrophage-NLRP3 activation promotes right ventricle failure in pulmonary arterial hypertension. Am J Respir Crit Care Med 2022;206:608-624. https://doi.org/10.1164/rccm.202110-2274OC
61. Napoli C, Paolisso G, Casamassimi A, Al-Omran M, Barbieri M, Sommese L, Infante T, Ignarro LJ. Effects of nitric oxide on cell proliferation novel insights. J Am Coll Cardiol 2013;62:89-95. https://doi.org/10.1016/j.jacc.2013.03.070
62. Hildebrand S, Ibrahim M, Schlitzer A, Maegdefessel L, Röll W, Pfeifer A. PDGF regulates guanylate cyclase expression and cGMP signaling in vascular smooth muscle. Commun Biol 2022;5:197. https://doi.org/10.1038/s42003-022-03244-9, https://doi.org/10.1038/s42003-022-03140-2
63. Novotná J, Herget J. Exposure to chronic hypoxia induces qualitative changes of collagen in the walls of peripheral pulmonary arteries. Life Sci 1998;62:1-12. https://doi.org/10.1016/S0024-3205(97)01032-1
64. Novotná J, Herget J. Possible role of matrix metalloproteinases in reconstruction of peripheral pulmonary arteries induced by hypoxia. Physiol Res 2002;51:323-334. https://doi.org/10.33549/physiolres.930238
65. Bačáková L, Herget J, Novotná J, Eckhart A, Lisá V. Adhesion, growth and stress adaptation of vascular smooth muscle cells in cultures on collagen I degraded by matrix metalloproteinase - 13. Ateroskleróza: metabolizmus, klinika a liečba 2002;6:155-161.
66. Roberts ISD, Brenchley PEC. Mast cells: the forgotten cells of renal fibrosis. J Clin Pathol 2000;53:858-862. https://doi.org/10.1136/jcp.53.11.858
67. Herget J, Novotná J, Bíbová J, Povýsilová V, Vanková M, Hampl V. Metalloproteinase inhibition by Batimastat attenuates pulmonary hypertension in chronically hypoxic rats. Am J Physiol-Lung C 2003;285:L199-L208. https://doi.org/10.1152/ajplung.00167.2002
68. Banasová A, Maxová H, Hampl V, Vízek M, Povysilová V, Novotná J, Vajnerová O, Hnilicková O, Herget J. Prevention of mast cell degranulation by disodium cromoglycate attenuates the development of hypoxic pulmonary hypertension in rats exposed to chronic hypoxia. Respiration 2008;76:102-107. https://doi.org/10.1159/000121410
69. Maxová H, Vasilková M, Novotná J, Vajnerová O, Banasová A, Vízek M, Herget J. Prevention of mast cell degranulation by disodium cromoglycate delayed the regression of hypoxic pulmonary hypertension in rats. Respiration 2010;80:335-339. https://doi.org/10.1159/000312403
70. Bacakova L, Filova E, Parizek M, Ruml T, Svorcik V. Modulation of cell adhesion, proliferation and differentiation on materials designed for body implants. Biotechnol Adv 2011;29:739-767. https://doi.org/10.1016/j.biotechadv.2011.06.004
71. Žaloudíková M, Eckhardt A, Vytášek R, Uhlík J, Novotný T, Bačáková L, Musílková J, Hampl V. Decreased collagen VI in the tunica media of pulmonary vessels during exposure to hypoxia: a novel step in pulmonary arterial remodeling. Pulm Circ 2019;9:2045894019860747. https://doi.org/10.1177/2045894019860747
72. Shamhart PE, Meszaros JG. Non-fibrillar collagens: Key mediators of post-infarction cardiac remodeling? J Mol Cell Cardiol 2010;48:530-537. https://doi.org/10.1016/j.yjmcc.2009.06.017

73. Bonaldo P, Colombatti A. The carboxyl terminus of the chicken Alpha-3 chain of collagen-vi is a unique mosaic structure with glycoprotein Ib-Like, Fibronectin Type-Iii, and kunitz modules. J Biol Chem 1989;264:20235-20239. https://doi.org/10.1016/S0021-9258(19)47052-X
74. Aigner T, Hambach L, Söder S, Schlötzer-Schrehardt U, Pöschl E. The C5 domain of Col6A3 is cleaved off from the Col6 fibrils immediately after secretion. Biochem Biophys Res Commun 2002;290:743-748. https://doi.org/10.1006/bbrc.2001.6227
75. Cescon M, Gattazzo F, Chen PW, Bonaldo P. Collagen VI at a glance. J Cell Sci 2015;128:3525-3531. https://doi.org/10.1242/jcs.169748
76. Wang JY, Pan WS. The biological role of the collagen Alpha-3 (VI) Chain and its cleaved c5 domain fragment endotrophin in cancer. Oncotargets Ther 2020;13:5779-5793. https://doi.org/10.2147/OTT.S256654
77. Sun K, Park J, Kim M, Scherer PE. Endotrophin, a multifaceted player in metabolic dysregulation and cancer progression, is a predictive biomarker for the response to PPARγ agonist treatment. Diabetologia 2017;60:24-29. https://doi.org/10.1007/s00125-016-4130-1
78. Park J, Scherer PE. Adipocyte-derived endotrophin promotes malignant tumor progression. J Clin Invest 2012;122:4243-4256. https://doi.org/10.1172/JCI63930
79. Henriksen K, Genovese F, Reese-Petersen A, Audoly LP, Sun K, Karsdal MA, Scherer PE. Endotrophin, a Key Marker and Driver for Fibroinflammatory Disease. Endocr Rev 2024;45:361-378. https://doi.org/10.1210/endrev/bnad036
80. Williams J, Maroney SP, Schmitt LR, Brown RD, Krafsur G, Frid MG, McCabe MC, Iheagwam FN, Gandjeva A, Williams KJ, Luyendyk JP, Saviola AJ, Tuder RM, Stenmark K, Hansen KC. A bovine model of hypoxia-induced pulmonary hypertension reveals a gradient of immune and matrisome response with a complement signature found in circulation. Am J Pathol, accepted.
81. Manalo DJ, Rowan A, Lavoie T, Natarajan L, Kelly BD, Ye SQ, Garcia JGN, Semenza GL. Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. Blood 2005;105:659-669. https://doi.org/10.1182/blood-2004-07-2958
82. Novák T, Žaloudíková M, Smolková P, Kaftanová B, Edlmanová J, Krása K, Hampl V. Hypoxia-inducible factors activator, roxadustat, increases pulmonary vascular resistance in rats. Physiol Res 2023;72:S587-S592. https://doi.org/10.33549/physiolres.935220
83. Pozeg ZI, Michelakis ED, McMurtry MS, Thébaud B, Wu XC, Dyck JRB, Hashimoto K, Wang SH, Harry G, Sultanian R, Koshal A, Archer SL. In vivo gene transfer of the O-sensitive potassium channel Kv1.5 reduces pulmonary hypertension and restores hypoxic pulmonary vasoconstriction chronically hypoxic rats. Circulation 2003;107:2037-2044. https://doi.org/10.1161/01.CIR.0000062688.76508.B3
84. Luo YT, Teng X, Zhang LL, Chen JN, Liu Z, Chen XH, Zhao S, Yang S, Feng J, Yan XY. CD146-HIF-1α hypoxic reprogramming drives vascular remodeling and pulmonary arterial hypertension. Nat Commun 2019;10:3551. https://doi.org/10.1038/s41467-019-12107-7, https://doi.org/10.1038/s41467-019-11500-6
85. Pullamsetti SS, Mamazhakypov A, Weissmann N, Seeger W, Savai R. Hypoxia-inducible factor signaling in pulmonary hypertension. J Clin Invest 2020;130:5638-5651. https://doi.org/10.1172/JCI137558
86. Hu CJ, Poth JM, Zhang H, Flockton A, Laux A, Kumar S, McKeon B, Mouradian G, Li M, Riddle S, Pugliese SC, Brown RD, Wallace EM, Graham BB, Frid MG, Stenmark KR. Suppression of HIF2 signalling attenuates the initiation of hypoxia-induced pulmonary hypertension. Eur Respir J 2019;54:1900378. https://doi.org/10.1183/13993003.00378-2019
87. Zhang WF, Tao ZY, Xu F, Diao Q, Li J, Zhou L, Miao YX, Xie SS, Wan JJ, Xu RL. An overview of miRNAs involved in PASMC phenotypic switching in pulmonary hypertension. Biomed Res Int 2021;2021:5765029. https://doi.org/10.1155/2021/5765029
88. Kumar S, Frid MG, Zhang H, Li M, Riddle S, Brown RD, Yadav SC, Roy MK, Dzieciatkowska ME, D'Alessandro A, Hansen KC, Stenmark KR. Complement-containing small extracellular vesicles from adventitial fibroblasts induce proinflammatory and metabolic reprogramming in macrophages. JCI Insight 2021;6:e148382. https://doi.org/10.1172/jci.insight.148382

89. Plecitá-Hlavatá L, Brázdová A, Křivonosková M, Hu CJ, Phang T, Tauber J, Li M, Zhang H, Hoetzenecker K, Crnkovic S, Kwapiszewska G, Stenmark KR. Microenvironmental regulation of T-cells in pulmonary hypertension. Front Immunol 2023;14:1223122. https://doi.org/10.3389/fimmu.2023.1223122
90. Hu CJ, Laux A, Gandjeva A, Wang LY, Li M, Brown RD, Riddle S, Kheyfets VO, Tuder RM, Zhang H, Stenmark KR. The effect of hypoxia-inducible factor inhibition on the phenotype of fibroblasts in human and bovine pulmonary hypertension. Am J Respir Cell Mol Biol 2023;69:73-86. https://doi.org/10.1165/rcmb.2022-0114OC
91. Bačáková L, Pellicciari C, Bottone MG, Lisá V, Mareš V. A sex-related difference in the hypertrophic versus hyperplastic response of vascular smooth muscle cells to repeated passaging in culture. Histol Histopathol 2001;16:675-684. https://doi.org/10.14670/HH-16.675
92. Sedlář A, Vrbata D, Pokorná K, Holzerová K, Červený J, Kočková O, Hlaváčková M, Doubková M, Musílková J, Křen V, Kolář F, Bačáková L, Bojarová P. Glycopolymer Inhibitors of Galectin-3 Suppress the Markers of Tissue Remodeling in Pulmonary Hypertension. J Med Chem 2024;67:9214-9226. https://doi.org/10.1021/acs.jmedchem.4c00341
93. Barman SA, Li XY, Haigh S, Kondrikov D, Mahboubi K, Bordan Z, Stepp DW, Zhou JL, Wang YS, Weintraub DN, Traber EE, Snider L, Jonigk D, Sullivan ENE, Crislip RY, Butcher JT, Thompson J, Su YC, Chen F, Fulton DJR. Galectin-3 is expressed in vascular smooth muscle cells and promotes pulmonary hypertension through changes in proliferation, apoptosis, and fibrosis. Am J Physiol Lung Cell Mol Physiol 2019;316:L784-L797. https://doi.org/10.1152/ajplung.00186.2018
94. Tian L, Chen K, Cao J, Han Z, Wang Y, Gao L, Fan Y, Wang C. Galectin-3 induces the phenotype transformation of human vascular smooth muscle cells via the canonical Wnt signaling. Mol Med Rep 2017;15:3840-3846. https://doi.org/10.3892/mmr.2017.6429
95. Li TZM, Zha LH, Luo H, Li SQ, Zhao L, He JN, Li XH, Qi QQ, Liu YW, Yu ZX. Galectin-3 mediates endothelial-to-mesenchymal transition in pulmonary arterial hypertension. Aging Dis 2019;10:731-745. https://doi.org/10.14336/AD.2018.1001
96. Choo YY, Sakai T, Komatsu S, Ikebe R, Jeffers A, Singh KP, Idell S, Tucker TA, Ikebe M. Calponin 1 contributes to myofibroblast differentiation of human pleural mesothelial cells. Am J Physiol Lung Cell Mol Physiol 2022;322:L348-L364. https://doi.org/10.1152/ajplung.00289.2021
97. Bekhite MM, Finkensieper A, Rebhan J, Huse S, Schultze-Mosgau S, Figulla HR, Sauer H, Wartenberg M. Hypoxia, leptin, and vascular endothelial growth factor stimulate vascular endothelial cell differentiation of human adipose tissue-derived stem cells. Stem Cells Dev 2014;23:333-351. https://doi.org/10.1089/scd.2013.0268
98. Podkalicka P, Stepniewski J, Mucha O, Kachamakova-Trojanowska N, Dulak J, Loboda A. Hypoxia as a driving force of pluripotent stem cell reprogramming and differentiation to endothelial cells. Biomolecules 2020;10:1614. https://doi.org/10.3390/biom10121614
99. Shimomura S, Inoue H, Arai Y, Nakagawa S, Fujii Y, Kishida T, Shin-Ya M, Ichimaru S, Tsuchida S, Mazda O, Kubo T. Hypoxia promotes differentiation of pure cartilage from human induced pluripotent stem cells. Mol Med Rep 2022;26:229. https://doi.org/10.3892/mmr.2022.12745
100. Yu X, Wan QL, Ye XL, Cheng Y, Pathak JL, Li ZB. Cellular hypoxia promotes osteogenic differentiation of mesenchymal stem cells and bone defect healing via STAT3 signaling. Cell Mol Biol Lett 2019;24:64. https://doi.org/10.1186/s11658-019-0191-8
101. Chen W, Zhuo Y, Duan D, Lu M. Effects of hypoxia on differentiation of mesenchymal stem cells. Curr Stem Cell Res Ther 2020;15:332-339. https://doi.org/10.2174/1574888X14666190823144928
102. Lin JY, Zhu QQ, Huang JY, Cai RF, Kuang YP. Hypoxia Promotes Vascular Smooth Muscle Cell (VSMC) Differentiation of Adipose-Derived Stem Cell (ADSC) by Regulating Mettl3 and Paracrine Factors. Stem Cells Int 2020;2020:2830565. https://doi.org/10.1155/2020/2830565
103. Frid MG, Dempsey EC, Durmowicz AG, Stenmark KR. Smooth muscle cell heterogeneity in pulmonary and systemic vessels - Importance in vascular disease. Arterioscler Thromb Vasc Biol 1997;17:1203-1209. https://doi.org/10.1161/01.ATV.17.7.1203

104. Frid MG, Aldashev AA, Dempsey EC, Stenmark KR. Smooth muscle cells isolated from discrete compartments of the mature vascular media exhibit unique phenotypes and distinct growth capabilities. Circ Res 1997;81:940-952. https://doi.org/10.1161/01.RES.81.6.940
105. Stiebellehner L, Frid MG, Reeves JT, Low RB, Gnanasekharan M, Stenmark KR. Bovine distal pulmonary arterial media is composed of a uniform population of well-differentiated smooth muscle cells with low proliferative capabilities. Am J Physiol Lung Cell Mol Physiol 2003;285:L819-L828. https://doi.org/10.1152/ajplung.00062.2003
106. Shimoda LA, Manalo DJ, Sham JSK, Semenza GL, Sylvester JT. Partial HIF-1α deficiency impairs pulmonary arterial myocyte electrophysiological responses to hypoxia. Am J Physiol Lung Cell Mol Physiol 2001;281:L202-L208. https://doi.org/10.1152/ajplung.2001.281.1.L202
107. Gui Y, Yin H, He JY, Yang SH, Walsh MP, Zheng XL. Endoreduplication of human smooth muscle cells induced by 2-methoxyestradiol: a role for cyclin-dependent kinase 2. Am J Physiol Lung Cell Mol Physiol 2007;292:H1313-H1320. https://doi.org/10.1152/ajpheart.00867.2006
108. Majka SM, Skokan M, Wheeler L, Harral J, Gladson S, Burnham E, Loyd JE, Stenmark KR, Varella-Garcia M, West J. Evidence for cell fusion is absent in vascular lesions associated with pulmonary arterial hypertension. Am J Physiol Lung Cell Mol Physiol 2008;295:L1028-L1039. https://doi.org/10.1152/ajplung.90449.2008
109. Orton EC, LaRue SM, Ensley B, Stenmark K. Bromodeoxyuridine labeling and DNA content of pulmonary arterial medial cells from hypoxia-exposed and nonexposed healthy calves. Am J Vet Res 1992;53:1925-1930. https://doi.org/10.2460/ajvr.1992.53.10.1925
110. Born E, Lipskaia L, Breau M, Houssaini A, Beaulieu D, Marcos E, Pierre R, Do Cruzeiro M, Lefevre M, Derumeaux G, Bulavin DV, Delcroix M, Quarck R, Reen V, Gil J, Bernard D, Flaman JM, Adnot S, Abid S. Eliminating Senescent Cells Can Promote Pulmonary Hypertension Development and Progression. Circulation 2023;147:650-666. https://doi.org/10.1161/CIRCULATIONAHA.122.058794
111. Krása K, Vajnerová O, Ďurišová J, Minaříkova M, Miková D, Srbová M, Chalupský K, Kaftanová B, Hampl V. Simvastatin and dehydroepiandrosterone sulfate effects against hypoxic pulmonary hypertension are not additive. Physiol Res 2022;71:801-810. https://doi.org/10.33549/physiolres.934913
112. Sedlář A, Trávníčková M, Bojarová P, Vlachová M, Slámová K, Křen V, Bačáková L. Interaction between galectin-3 and integrins mediates cell-matrix adhesion in endothelial cells and mesenchymal Stem Cells. Int J Mol Sci 2021;22:5144. https://doi.org/10.3390/ijms22105144
113. Mocumbi A, Humbert M, Saxena A, Jing ZC, Sliwa K, Thienemann F, Archer SL, Stewart S. Pulmonary hypertension. Nat Rev Dis Primers 2024;10:1. https://doi.org/10.1038/s41572-023-00486-7, https://doi.org/10.1038/s41572-024-00493-2
114. Nathan SD, Barbera JA, Gaine SP, Harari S, Martinez FJ, Olschewski H, Olsson KM, Peacock AJ, Pepke-Zaba J, Provencher S, Weissmann N, Seeger W. Pulmonary hypertension in chronic lung disease and hypoxia. Eur Respir J 2019;53:1801914. https://doi.org/10.1183/13993003.01914-2018
115. Otani N, Tomoe T, Kawabe A, Sugiyama T, Horie Y, Sugimura H, Yasu T, Nakamoto T. Recent Advances in the Treatment of Pulmonary Arterial Hypertension. Pharmaceuticals (Basel) 2022;15:1277. https://doi.org/10.3390/ph15101277
116. Rubin LJ. Endothelin receptor antagonists for the treatment of pulmonary artery hypertension. Life Sci 2012;91:517-521. https://doi.org/10.1016/j.lfs.2012.07.033
117. Redaelli S, Magliocca A, Malhotra R, Ristagno G, Citerio G, Bellani G, Berra L, Rezoagli E. Nitric oxide: Clinical applications in critically ill patients. Nitric Oxide-Biol Ch 2022;121:20-33. https://doi.org/10.1016/j.niox.2022.01.007
118. Rawat M, Lakshminrusimha S, Vento M. Pulmonary hypertension and oxidative stress: Where is the link? Semin Fetal Neonatal Med 2022;27:101347. https://doi.org/10.1016/j.siny.2022.101347
119. Waxman A, Restrepo-Jaramillo R, Thenappan T, Ravichandran A, Engel P, Bajwa A, Allen R, Feldman J, Argula R, Smith P, Rollins K, Deng CQ, Peterson L, Bell H, Tapson V, Nathan SD. Inhaled Treprostinil in Pulmonary Hypertension Due to Interstitial Lung Disease. N Engl J Med 2021;384:325-334. https://doi.org/10.1056/NEJMoa2008470

120. Behr J. Inhaled Treprostinil in Pulmonary Hypertension in the context of interstitial lung disease: a success, finally. Am J Respir Crit Care Med 2022;205:144-146. https://doi.org/10.1164/rccm.202110-2444ED
121. Piccari L, Wort SJ. Use of inhaled treprostinil in patients with interstitial lung disease and pulmonary hypertension: to boldly go where no other pulmonary vasodilator has gone before? Thorax 2024;79:295-296. https://doi.org/10.1136/thorax-2023-221167
122. Wan JJ, Yi J, Wang FY, Zhang C, Dai AG. Expression and regulation of HIF-1a in hypoxic pulmonary hypertension: Focus on pathological mechanism and pharmacological treatment. Int J Med Sci 2024;21:45-60. https://doi.org/10.7150/ijms.88216

REVIEW

Pediatric Chronic Heart Failure: Age-Specific Considerations of Medical Therapy

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Summary

Chronic heart failure (CHF) is a rare entity in children but carries a burden of high mortality and morbidity. Medical treatment of pediatric CHF is largely based on guidelines for the adult population. In contrast to adults, evidence for the efficacy of medications in treating CHF in children is sparse. This may be due to the difficulty of conducting high-powered studies in children or to true differences in the mechanisms of CHF pathophysiology. Recent observations suggest that CHF in children differs from adults at the molecular and cellular levels. Different pathways are involved, leading to less fibrosis and hypertrophy than in adults, with potential implications for therapy. The main pathophysiological goals of medical treatment of pediatric CHF due to systemic left ventricular dysfunction are discussed in this review. These include preload and afterload optimization, diminishing cardiomyocyte apoptosis and necrosis as well as interstitial fibrosis, and optimizing myocardial oxygen consumption. The pediatric myocardium should be provided with optimal conditions to achieve its regenerative potential. The cornerstones of medical CHF therapy are angiotensin converting enzyme inhibitors (ACEI), beta blockers and mineralocorticoid receptor antagonists. There are potential benefits of tissue ACEI and β1-selective beta blockers in children. Angiotensin receptor blockers are an alternative to ACEI and their slightly different mechanism of action may confer certain advantages and disadvantages. Diuretics are employed to achieve a euvolemic state. Digoxin is used more frequently in children than in adults. Promising new drugs already routinely used in adults include angiotensin receptor-neprilysin inhibitors and sodium-glucose contransporter 2 inhibitors.

Key words

Pediatric heart failure • Heart failure with reduced ejection fraction (HFrEF) • ACE inhibitor • Beta blocker • Digoxin

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Introduction

Chronic heart failure (CHF) is a rare entity in children but carries a burden of high mortality and morbidity [1]. Guidelines for the medical management of CHF in children have been developed [2,3] but due to the lack of large prospective multicenter randomized controlled trials in the pediatric population, the therapy is largely based on extrapolation of results from such trials conducted in adults. It is uncertain whether the inability to prove the efficacy of HF medications in children is due to actual differences between the adult and pediatric populations or due to the challenges in designing a robust pediatric CHF study [4].

The high-level evidence coming from adult trials cannot be simply transferred to children. The pediatric CHF population is a significantly different and more heterogenous one, regarding both the spectrum of diagnoses and age-dependent pharmacokinetics and pharmacodynamics [4].

The aim of this review is to highlight the specific features of the pediatric population concerning the medications used to treat CHF. Many comprehensive reviews focused on pediatric HF including aspects such as etiology, presentation, diagnostics and treatment have been published recently [5–8]. This review's focus is pediatric CHF resulting from systemic left ventricular systolic dysfunction. Specific types of congenital heart

disease such as the failing univentricular circulation are beyond the scope of this article.

Cellular and molecular mechanisms involved in pediatric failing myocardium

During development, numerous changes take place in the physiology of myocardial contraction, both on the cellular and molecular levels. Thus, the contraction and metabolism of the immature myocardium is markedly different from that of the adult. Underdeveloped mitochondria and abundant intracellular glycogen granules in the immature cardiomyocyte result in glucose being the predominant energy source, in contrast to fatty acids in adult cardiomyocytes. Similarly, the sarcoplasmic reticulum isn't fully developed, making the immature myocardium much more dependent on extracellular calcium due to insufficient intracellular stores [9]. Changes in the expression of sarcomeric proteins also occur during development, resulting in marked alterations of functional properties, both pre- and postnatally [10,11]. In the case of myocardial pathology in pediatric CHF, age-related differences are compounded by a number of additional disorders, which together may influence the response of pediatric patients to specific medications [12,13].

Cardiac growth, differentiation, proliferation and consecutively regenerative and repair mechanisms are inversely related to the patient's age. Replication of cardiomyocytes occurs mainly during embryonic and fetal development and deceases rapidly after birth. Human infants still show some evidence of minor cardiomyocyte proliferation, which decreases during childhood to nondetectable levels in adults [14]. Typically, further cardiac enlargement occurs only by cell hypertrophy. Factors involved in the cessation of proliferation after birth include cardiomyocyte polyploidization, transition from hypoxia to hyperoxia, acquisition of endothermy, changes in intercellular interactions, hormonal regulation, and subsequent downregulation of various cell-cycle factors and upregulation of cell-cycle inhibitors [15,16]. The increase in cardiac afterload resulting from changes of circulation after birth also very likely plays a role [17].

The adaptive growth response of the cardiac muscle to increased workload changes significantly with the transition from proliferation to hypertrophy. Results from animal studies suggest that the adaptation of the neonatal heart to increased pressure load is based on transient hyperplasia followed by hypertrophy of ventricular cardiomyocytes [16]. Preserved myocardial capillarization and the absence of fibrosis are unique features of cardiomegaly induced early after birth – in contrast to the adult myocardium where fibrosis is typical [16]. Although the switch in the myocardial response to load occurs early after birth, possible residual mechanisms of hyperplasia, presumably corresponding to the rate of cardiomyocyte proliferation, should be considered in infant heart failure. Cell-based therapies aiming to utilize the regenerative mechanisms are emerging [18].

The differences in the mechanisms involved in CHF in children versus adults that are still not fully understood. Notable distinctions have been shown on the levels of hypertrophy, myocardial fibrosis and gene expression profiles. The finding that adverse remodeling does not drive disease progression in pediatric patients with dilated cardiomyopathy could be an explanation why standard adult CHF medications don't work so well in children [19,20]. These drugs primarily target mechanisms of pathological remodeling (hypertrophy and fibrosis) that appear to be much less involved in the pathophysiology of heart failure in children than in adults.

The pathways dysregulated in the pediatric heart are distinct from those regulated in the adult failing heart (Table 1). Pediatric failing heart is maintained in an undifferentiated state unlike the adult heart that shows activation of the innate immune system, fatty acid and oxidative metabolism [19]. Dysregulation of both matrix metalloproteinases responsible for breakdown of extra-cellular matrix and their tissue inhibitors of leads to fibrosis in adults, whereas increased expression of MMP-2 in pediatric hearts could be a compensatory reaction to increased profibrotic stimuli and could account for the reduced fibrotic phenotype in children [20]. Differences between certain microRNAs expression have been suggested to play a role in better tolerability and outcomes with posphodiesterase-3 inhibition (treatment by milrinone) in children than in adults [21]. It would seem that pediatric age-specific mechanisms need to be focused on. However, which of the differences presented might be appropriate therapeutic targets remains to be clarified.

Striking differences in adrenoreceptor regulation have also been pointed out. Probably most significant is the fact that both β1- and β2-ARs are down-regulated in children with CHF, whereas β2-AR expression is preserved in adults [22]. This has potential therapeutic implications already, as discussed in the section on beta blockers.

Table 1. Distinct features of adult and pediatric heart failure. Data derived mainly from dilated cardiomyopathy [19–22].

Adults	Children
No cardiomyocyte proliferation	Some cardiomyocyte proliferation and highest regeneration potential in children < 1 year
Adverse remodeling	
Cardiomyocyte hypertrophy	Minimal cardiomyocyte hypertrophy
Myocardial fibrosis (interstitial and perivascular)	Minimal fibrosis (both interstitial and perivascular)
↔ coronary microvascular density	↑ coronary microvascular density
Molecular changes and gene expression	
↑ oxidative reduction, inflammation, fatty acid metabolism	↑ cell adhesion, ion and transmembrane transport, visual perception
Fibrosis-related: ↓ MMP-9, TIMP-3	Fibrosis-related: ↑ MMP-2, TIMP-2
↓ regulation of β1-AR	↓ regulation of both β1-AR and β2-AR
↓ regulation of connexin43	↑ regulation of connexin43
↑ phosphatase expression	↔ phosphatase expression
↓ phosphorylation of phospholamban	↔ phosphorylation of phospholamban
MicroRNAs involved: let-7, miR-1, miR-133a/b, miR-100, miR-195, miR-199, miR-214, miR-222, miR-23a/b, miR-29a/b, miR-30 family, miR-320	MicroRNAs involved: miR-130b, miR-204, miR-331-3p, miR-188-5p, miR-1281, miR-572, miR-765, miR-223, miR-125a-3p and miR-1268

β1-AR, β1-adrenoreceptor; β2-AR, β2-adrenoreceptor; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases.

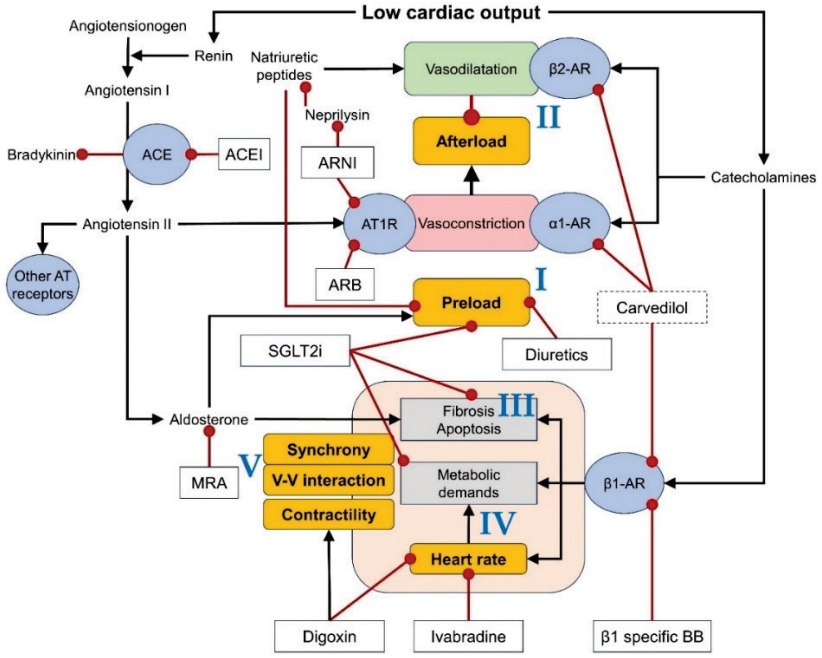

Fig. 1. Pathophysiology of pediatric chronic heart failure due to systemic left ventricular dysfunction and main targets for therapy:
I – Preload optimization, achieving normovolemia; **II** – Afterload reduction, ideally while preserving β2-adrenoreceptor function; **III** – Diminishing cardiomyocyte apoptosis and necrosis, and myocardial interstitial fibrosis; **IV** – Heart rate reduction to improve myocardial metabolic demands; **V** – Maintaining or re-establishing synchrony and taking into account the ventriculo-ventricular interaction (non-pharmacologic therapies). Determinants of cardiac output are shown in orange.
ACE, angiotensin converting enzyme; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; ARNI, angiotensin receptor – neprilysin inhibitor; AT1R, angiotensin II receptor type 1; α1-AR, α1-adrenoreceptor; BB, beta blocker; β1-AR, β1-adrenoreceptor; β2-AR, β2-adrenoreceptor; MRA, mineralocorticoid receptor antagonist; SGL2i, sodium-glucose contransporter 2 inhibitor; V-V interaction, ventriculo-ventricular interaction.

Pathophysiology and therapeutic strategy in pediatric chronic heart failure

Cardiac output is determined by preload, afterload, myocardial contractility and synchrony, heart rate, and ventriculo-ventricular interaction [23]. Heart failure can be caused by pathologies of one or more of these factors. Correction of the underlying condition should always be the primary objective in children. However, if the cause of CHF cannot be eliminated, medical treatment is indicated to mitigate or reverse the pathophysiological consequences of CHF, increase the quality of life and prolong survival.

Low cardiac output leads to decreased renal perfusion and activation of the sympathetic nervous system and the renin-angiotensin-aldosterone system. This is beneficial in an acute setting, but chronic stimulation ultimately leads to progressive impairment of cardiac function. Catecholamine release causes vasoconstriction, decreases kidney perfusion and induces the production of renin, and conversion of angiotensin I to angiotensin II. Angiotensin II further exacerbates vasoconstriction and additionally stimulates aldosterone secretion by constriction renal afferent arterioles, increasing sodium and water reabsorption. High afterload due to vasoconstriction results in loss of functional cardiomyocytes and pathological myocardial remodeling, which further exacerbates cardiac dysfunction and ultimately leads to decompensation. Catecholamines contribute to myocardial apoptosis mainly through the activation of β1-adrenoreceptors (β1-AR), also negatively affecting myocardial metabolism and increasing oxygen and energy consumption [24]. Natriuretic peptides partially mitigate these negative consequences, attenuating cardiac remodeling, apoptosis, hypertrophy, and fibrosis, as well as decreasing renin and aldosterone production [25]. The main pathophysiological mechanisms of CHF and the points of pharmacological interventions are summarized in Figure 1.

The general goals of pediatric CHF therapy should be: I) preload optimization by achieving normovolemia (reducing congestion but avoiding intravascular volume depletion); II) afterload reduction without jeopardizing the coronary perfusion; III) diminishing cardiomyocyte apoptosis and necrosis as well as interstitial fibrosis; IV) optimizing myocardial oxygen consumption (particularly by heart rate reduction); V) re-establishing myocardial synchrony as well as ventriculo-ventricular interaction (non-pharmacological therapies), and VI) allowing time to establish repair mechanisms [23,26].

Heart rate reduction to the lowest effective level should be one of the main goals of CHF therapy [23]. Heart rate is inversely related to average life span in most organisms and negative effects of elevated heart rate include increased ventricular work, myocardial oxygen consumption, endothelial stress, arterial stiffness, and decreased myocardial oxygen supply [27]. Association of elevated resting heart rate with increased mortality has been shown both in adults [28] and children [29] with cardiovascular disease. Similarly, heart rate reduction was associated with improved outcomes in both populations [30,31]. Reducing the heart rate per se is probably more important than the specific drug used to achieve it (beta blockers, ivabradine, digoxin).

Reversible pulmonary artery banding in young children with dilated cardiomyopathy and preserved right ventricular function is also a novel strategy to improve ventriculo-ventricular interaction and left ventricular function [32,33]. This procedure utilizes the fact that the ability of each ventricle to eject blood is largely dependent on the function of the other ventricle and the position of the interventricular septum. The main effects or pulmonary artery banding in this setting include: 1) increase in right ventricular wall stress enhancing contractility due to the Anrep effect, 2) leftward shift of the interventricular septum that contributes to restoration of an ellipsoidal shape of the left ventricle, and 3) reduction in left ventricular preload and end-diastolic pressure, with subsequent optimization of the Frank–Starling curve and left ventricular diastolic and systolic function. Additionally, myocardial sensing apparatus might translate the mechanical stress stimulus into the activation of regulatory pathways of cardiomyocyte proliferation and regeneration [34]. The method shows encouraging results and may be an effective alternative to mechanical support or transplantation in selected infants. However, the selection of optimal candidates, timing and delicate setting of banding tightness remains a challenge. The banding may later be dilated using a catheter procedure depending on other findings.

Chronic heart failure medications

According to the guidelines for adults, every patient with heart failure and reduced ejection fraction should be treated with a combination of 4 drugs: 1) a drug

Table 2. Drugs commonly used to treat pediatric chronic heart failure.

Drug	Usual starting dose	Usual target dose	Dosing interval
Angiotensin converting enzyme inhibitors (ACEI)			
Captopril*	0.3 mg/kg/day	0.5 mg/kg/day in newborns 1-2 mg/kg/day in older children	Three times daily
Enalapril	0.1 mg/kg/day	0.2 – 0.4 mg/kg/day	Twice daily
Lisinopril	0.05 mg/kg/day	0.2 – 0.4 mg/kg/day	Once daily
Ramipril*	0.05 mg/kg/day	0.1 – 0.2 mg/kg/day	Once or twice daily
Angiotensin receptor blockers (ARB)			
Losartan	0.5 mg/kg/day	1 – 2 mg/kg/day	Once daily
Angiotensin receptor-neprilysin inhibitor (ARNI)			
Sacubitril-valsartan	1.6 – 3.2 mg/kg/day†	4.6 – 6.2 mg/kg/day†	Twice daily
Mineralocorticoid receptor antagonists (MRA)			
Spironolactone	1 – 2 mg/kg/day	1 – 3 mg/kg/day	One to three times daily
Beta blockers (BB)			
β-1 selective			
Metoprolol	0.2 – 0.4 mg/kg/day	1 – 2 mg/kg/day	Once or twice daily
Bisoprolol*	0.05 mg/kg/day	0.2 – 0.3 mg/kg/day	Once daily
Nonselective with α-blocking effect			
Carvedilol*	0.1 mg/kg/day	0.8 – 1 mg/kg/day ‡	Twice daily
Diuretics			
Furosemide	1 – 4 mg/kg/day	taper after other medications are set if tolerated	One to three times daily
Hydrochlorothiazide	1 – 3 mg/kg/day	taper after other medications are set if tolerated	Once or twice daily
Cardiac glycosides			
Digoxin	0.005 – 0.01 mg/kg/day §	Target trough level 0.5 – 0.9 ng/ml	Once or twice daily
Pacemaker current (I_f) inhibitor			
Ivabradine	0.1 mg/kg/day	0.1 – 0.3 mg/kg/day	Twice daily
Sodium-Glucose Co-transporter 2 (SGLT2) inhibitors			
Dapagliflozin	0.1 – 0.2 mg/kg/day (max 10mg)	0.1 – 0.2 mg/kg/day (max 10mg)	Once daily

* preferred agents (explained in the text in greater detail), † the total dose of sacubitril and valsartan summed, ‡ target dose may be up to 3 mg/kg/day in infants [69], § newborns may require lower doses and infants higher doses

interfering with the renin-angiotensin system (an angiotensin converting enzyme inhibitor – ACEI, an angiotensin receptor blocker – ARB, or an angiotensin-neprilysin inhibitor – ARNI), 2) a beta blocker (BB), 3) a mineralocorticoid receptor antagonist (MRA) and 4) a sodium-glucose cotransporter 2 inhibitor (SGLT2i) [35-37]. Diuretics are indicated to achieve euvolemic state as needed and relieve symptoms. Other medications with weaker evidence used in specific indications include ivabradine, digoxin, and an oral soluble guanylate cyclase stimulator vericiguat [35,36]. Mechanisms of action are summarized in Figure 1. Commonly used specific drugs, including their dosing, are summarized in Table 2.

The perspective on these drug classes in children is discussed in the following sections. The suggestions are based on the medications' impact on the pathophysiology of heart failure rather than evidence-based outcomes which are scarce. For each class, we discuss which specific drugs might be especially advantageous for the pediatric population.

Medications influencing the renin-angiotensin-aldosterone system

Angiotensin converting enzyme inhibitors (ACEI)

Angiotensin converting enzyme (ACE) converts angiotensin I to angiotensin II. Angiotensin II has potent vasoconstrictor effects and also stimulates growth factors that regulate endothelial, cardiomyocyte, and fibroblast growth, development, and function, leading to myocardial hypertrophy and fibrosis [38]. ACE also inactivates bradykinin, a peptide with vasodilatory and natriuretic effects. ACE inhibition diminishes the deleterious effects of angiotensin II and also promotes the vasodilatory and vasoprotective effects of bradykinin.

Recommendations for the use of ACEI for children with left ventricular dysfunction are unequivocal [3]. The safety profile of ACEI in children is favorable, and probably similar to that seen in adults. Although they are usually well tolerated, side effects include hypotension, renal dysfunction, hyperkalemia, cough and angioedema [38] and seem to be largely independent of a specific ACEI. Because of the risk of renal dysfunction is higher in patients with lower cardiac output, ACEI should be started at a low dose and up-titrated. The pathophysiology of ACEI-induced cough is not completely clear, but it is usually attributed to an increased concentration of bradykinin [39].

In most pediatric trials, captopril and enalapril have been studied [38]. Captopril and enalapril are still currently the most widely used ACEI, although there is significant variability between centers [40]. Short-acting serum ACEI may be preferred in neonates, whose glomerular filtration rate is lower and the risk of renal dysfunction higher. However, a question arises whether another ACEI would be preferable in older children [23].

ACEI can be classified into tissue-affinity or serum-affinity groups, defined mainly by their lipophilicity. It has been hypothesized that that tissue ACEI might have a higher cardioprotective effect because of better tissue penetration [26,41]. However, there are studies that do not support such hypothesis [42,43]. Still, this factor may be taken into account when deciding for a specific ACEI. Fosinopril and quinapril are the ACEIs with highest tissue affinity, followed by Ramipril. Enalapril, captopril and lisinopril are at the opposite end of the spectrum [42,44].

The duration of action of different ACEI and the resulting dosing interval is another deciding factor. The recommended dosing interval for patients with CHF is usually three times daily for captopril, twice daily for enalapril, and once daily for lisinopril. Ramipril can be given once or twice daily [35,37]. It is worth noting that trough–peak ratios are quite similar for enalapril, lisinopril and ramipril [44]. Although there are some suggestions that twice-daily administration of long-acting ACEI might have advantages [45], no robust evidence supports this [46]. Especially in infants, once-daily dosing might decrease the risk of renal side effects. Experience with long-acting ACEI in children is based mainly on the hypertensive population, published results showing good tolerance of lisinopril from infant age [47] and ramipril from the age of 2 years [48].

To summarize, the optimal ACEI for children should be long-acting to allow once-daily dosing and should have high tissue activity [26]. In patients beyond newborn age, ramipril might be the drug to meet these requirements.

Angiotensin receptor blockers (ARB)

Inhibition of AT1 receptors to angiotensin II is a mechanism employed by angiotensin receptor blockers (ABR). Placing the blockade further downstream in the renin-angiotensin-aldosterone system has similar effects (as outlined in Figure 1), however, there are two major differences [49].

By keeping the ACE active, bradykinin

accumulation does not occur and the side effects of angioedema or cough are usually avoided. However, the positive effects specific to ACE inhibition are also lost. Besides the beneficial vascular effects of increased bradykinin concentration, these include increased nitric oxide bioavailability and decrease in von Willenbrand factor [49]. Greater anti-inflammatory effects of ACEI versus ARB have been pointed out [50].

The second difference relates to the concentration of angiotensin II. Chronic blockade of AT1 receptors by ARB induces an increase in plasma angiotensin II and results in overstimulation of its other available receptors (AT2 and AT4 receptors). AT1 receptors mediate the primary undesirable effects of angiotensin II. The role of AT2 receptors has been understood as a mechanism to counterbalance the pathological processes (preventing inflammation, apoptosis, fibrosis; and promoting NO-dependent vasodilation), their stimulation in the setting of ARB treatment potentially reaching or even exceeding the additional favorable effects of ACEI [51]. However, animal experiments focusing on them have produced conflicting results [49]. Therefore, the fact whether the effects of AT2 and AT4 receptors are rather beneficial or deleterious in heart failure is a matter of debate. Little is known about developmental changes in the types of angiotensin II receptors, except that AT2 receptors are abundant during the fetal period and decline rapidly after birth [51].

In adults with CHF and reduced ejection fraction, ARB have been reported to be similarly effective as ACEI [52]. However, as opposed to ACEI, no study has been able to show a reduction in all-cause mortality associated with ARB [37]. Direct comparisons between the two groups are missing and there are concerns about whether ARB achieve as high cardiovascular protection as ACEI [49].

The combination of ACEI and ARB therapy has also been investigated in adults with CHF. The addition of ARB to ACEI therapy has been shown to provide some benefits, but there are concerns about adverse effects especially regarding the combination with other high-evidence CHF medications [53,54]. Thus, the combination of ACEI and ARB is not typically used.

Little is known about differences between adults and children in this regard. The effects of both ACEI and ARB have their own distinct specifics, and whether one group has a clear benefit over the other remains to be clarified both in adults and children.

At present, ARB are usually reserved for children who do not tolerate ACEI, as in adults. They are generally considered effective and safe alternatives alternative to ACEI and were mostly tested in children above 1 year of age [55]. Losartan is the most commonly used ARB in children and is administered once daily.

Angiotensin receptor-neprilysin inhibitor (ARNI)

Sacubitril/valsartan is a new drug with a unique mechanism of action, inhibiting neprilysin in addition to being an AT1 receptor blocker. Neprilysin is an enzyme whose main role is the breakdown of natriuretic peptides. Its inhibition therefore results in an increase in the concentration of natriuretic peptides, enhancing their cardiovascular beneficial effects, particularly vasodilation and natriuresis [56].

The superiority of sacubitril/valsartan to ACEI was demonstrated in adults with CHF and reduced ejection fraction [57]. A similar study was designed in children but the results have not been published yet [58]. Sacubitril/valsartan was approved for children with CHF from 1 year of age based on partial data from this study and the results from the adult population.

Mineralocorticoid receptor antagonists (MRA)

Mineralocorticoid receptor antagonists block the renin-angiotensin-aldosterone system on the level of aldosterone receptors in renal distal tubules. In addition to preventing the long-term deleterious effects of aldo-sterone on the heart, they act as potassium-sparing diuretics, increasing sodium excretion and potassium reabsorption. However, they have only modest diuretic effect, are generally well tolerated, and have little or no chronic effect on blood pressure [8].

It has been shown that aldosterone levels are only transiently depressed by the use of ACEI in patients with CHF [59]. The additive beneficial effect of MRA on survival was clearly shown in adults with CHF and reduced ejection fraction who were already treated by ACEI [60]. Hence the recommendations to add a MRA to ACEI treatment for both adults and children [3]. There is pediatric experience with both spironolactone [61] and eplerenone [62].

Beta blockers (BB)

Beta blockers antagonize the deleterious effects of chronic sympathetic myocardial activation and can reverse pathologic left ventricular remodeling. Recom-

mendations for the treatment of heart failure with reduced ejection fraction by BB in adults [35,37] are based on the results of many large trials that have shown reductions in mortality and morbidity as well as symptom relief. Metoprolol, bisoprolol and carvedilol have been tested [63–65]. In children, only studies with carvedilol have been performed, retrospective studies suggesting that carvedilol improves ejection fraction and symptoms [66]. However, the only prospective randomized trial with carvedilol in children failed to demonstrate these benefits [67].

β1-adrenoreceptors (β1-AR) have been traditionally understood as the "cardiotoxic" subtype whereas the β2-adrenoreceptors (β2-AR) as "cardio-protective" [68]. This perspective is based on the fact that most of the undesirable effects of catecholamines on the heart (increase in heart rate, energy consumption, arrhythmogenesis, pro-apoptotic effects) are mediated by β1-AR, while β2-AR mediate beneficial cardiovascular effects (vasodilation and subsequent reduction of blood pressure, anti-apoptotic signaling). Although this relationship is not as black and white and a more complex cross-talk takes place between receptors' effects, most evidence suggests that β1-AR are more closely coupled to cardiotoxic pathways [68]. Differences regarding the response of beta receptors to CHF have been shown between adults and children. These differences include the down-regulation of both β1-AR and β2-AR in children, whereas β2-AR expression is maintained in adults. Stimulation of α1-AR is clearly undesirable as they cause vasoconstriction and subsequently increase afterload.

Carvedilol is a non-selective BB (antagonizing both β1-AR and β2-AR) with an additional α-blocking effect. Alpha-blockade confers the additional advantageous effect of reducing afterload. An unequivocal benefit in CHF treatment has been demonstrated in adults, as with β1-selective BB (metoprolol and bisoprolol). There may be numerous reasons why the same effect has not been demonstrated in children. One possible explanation relates to the design of the study – factors such as low power, heterogeneous population, high placebo effect, and a high proportion of very young children in whom much higher doses are required to achieve the same exposure to carvedilol as in adults [69]. However, another explanation is that in children the positive effects (α-blockade) may not outweigh the potential negative effects (β2-blockade). Thus, some authors have suggested a selective β1-blocker should be more beneficial in children than carvedilol [23,26]. Studies performed in a mouse model of cardiomyopathy would suggest the same. Selective β1-blockade was effective both in young and adult mice, whereas nonselective beta blockade was only effective in adult mice [70].

There is experience with both carvedilol and bisoprolol in pediatrics. The need for an inverse age-dependent increase in carvedilol doses, especially because of faster elimination in infants, has been pointed out previously [69,71]. Bisoprolol is not as widely used, but good tolerability has been described even in infants [26,72].

The importance of selective targeting of individual adrenergic receptors in children with CHF remains to be seen. At present, it is not clear whether any one BB is preferable to another. The choice of a particular drug may depend on individual patient factors. A failing heart should certainly not be burdened by high blood pressure, and a patient prone to hypertension will certainly benefit from the additional afterload reduction provided by carvedilol. In contrast, a patient with poor tolerance to CHF therapy and a tendency to hypotension may benefit more from selective β1-blockade. Taking into account the differences in β-AR expression in children and adults, preference should rather be given to β1-selective beta blockers, especially in patients with dilated cardiomyopathy on whom the research has been mainly focused.

Diuretics

Loop diuretics inhibit the $Na^+/K^+/2Cl^-$ cotransporter in the loop of Henle, increasing the renal excretion of sodium chloride, potassium, and water. They should be used in acute HF to achieve a euvolemic state. However, long-term treatment with diuretics can be detrimental because of further stimulation of the neurohumoral axis by intravascular volume depletion [26]. Indications for treatment of pediatric CHF with diuretics should therefore be strict and efforts should be made to discontinue diuretic therapy in those patients in whom it is possible. Especially in the context of other modern drugs that promote diuresis and natriuresis (such as ARNI and SGLT2i), the need for chronic diuretic treatment is diminishing.

Digoxin

Digoxin is the most relevant drug from the group of cardiac glycosides. Its mechanism of action is the inhibition of Na^+/K^+ ATPase, which is involved in the exchange of intracellular sodium for extracellular potassium during the repolarization phase of the cardiac cycle. This results in an increase in intracellular calcium stores by a sodium-calcium exchange mechanism and subsequent increase in cardiac contractility. Another effect is the slowing of sinus rhythm and prolonging of atrioventricular nodal conduction [73]. The combination of inotropic and bradycardic action is unique for digoxin compared to all other sympathomimetic inotropes that cause tachycardia [73].

Digoxin has a very narrow therapeutic range with a risk of intoxication. The target level is 0.5 – 0.9 ng/ml [3,35], with the risk of adverse effects increasing at levels above 1.2 ng/ml [74]. Digoxin has numerous drug interactions, with its levels increasing, for example, with concomitant use of carvedilol or amiodarone. The toxicity presents with inappetence, nausea, and arrhythmias – in children usually sinus bradycardia or atrioventricular blockade, tachyarrhythmias are rare in contrast to adults [73].

In children, moreover, the pharmacokinetics of digoxin depend on age. Therefore, age-dependent dosing is necessary in addition to monitoring of plasma concentrations. Digoxin is excreted by the kidneys. Neonates require lower doses due to renal functional immaturity [73,75]. Infants, on the other hand, require increased doses due to their larger distribution area and higher clearance of digoxin [75].

Digoxin played an essential role in CHF treatment for centuries. Recently, however, it has been sidelined by more modern drugs, which have been clearly shown to reduce mortality in large studies in adults. This has not been demonstrated with digoxin, with some studies even suggesting increased mortality and risk of arrhythmias associated with it [76,77]. There are many factors to consider, such as the greater morbidity of patients treated with digoxin at baseline or the substantial concentration dependence of the beneficial and adverse effects of digoxin. Although the benefits of digoxin in adults are unclear, a meta-analysis suggests that digoxin reduces the risk of hospitalization and improves symptoms [78]. In children with CHF, there are only few small studies in patients with left to right shunts showing conflicting results [73]. However, there are reports emerging that digoxin decreases interstage mortality in patients with congenital heart disease with single ventricle physiology [79,80]. Hence, it is possible that certain specific groups of patients will benefit from digoxin treatment more than others.

To summarize, digoxin should be used cautiously at low levels (serum concentrations of 0.5 – 0.9 ng/mL), with careful use in patients with renal dysfunction, or in combination with medications that can alter digoxin levels [3,6]. Current guidelines recommend digoxin as a second-line drug reserved for children with CHF who remain symptomatic despite being treated by the higher-evidence medications [3].

Available data suggest that digoxin is still very widely used in children in current clinical practice [73,81]. We believe as well that digoxin still has a role in the treatment of pediatric CHF. Re-emphasizing the importance of heart rate reduction, digoxin's unique effect combines it with an enhancement of cardiac contraction. For certain patients at least, this effect can be highly beneficial.

Ivabradine

Ivabradine reduces heart rate via inhibiting the funny channels in the sinoatrial node, reducing the inward funny current (I_f) and slowing phase-4 repolarization of the pacemaker cells [82]. The importance of heart rate reduction has already been discussed above. The advantage of ivabradine is its heart rate-reducing effect is reasonably specific, having almost no other cardiovascular effects.

Ivabradine is a second-line drug in pediatric CHF therapy used to further reduce heart rate when it remains elevated despite BB treatment. The safety has been validated in children with CHF [83]. Ivabradine effectively reduced heart rate and increased ejection fraction of the left ventricle in this study.

Sodium-Glucose Co-transporter 2 inhibitors (SGLT2i)

Gliflozins act as inhibitors of the sodium-glucose co-transporter 2 (SGLT2) in the proximal tubule of the kidney. Their primary role has been as antidiabetic agents as they increase glucose excretion and thus improve glycemic control. A reduction of heart failure-related events has been observed in patients treated by SGLT2i and a clear survival benefit has

been later confirmed in patients with CHF, regardless of their ejection fraction and regardless of the presence of diabetes [84,85].

Although the exact mechanism of improving CHF by SGLT2 inhibition is uncertain, there are numerous proposed beneficial effects of SGLT2i. Diuresis and natriuresis are associated with glucose excretion. However, no concomitant activation of the renin-angiotensin-aldosterone system occurs, a unique feature that is clearly desirable in CHF [86]. Other beneficial effects on the heart are both direct and indirect. Direct mechanisms include anti-apoptotic effects, reduced oxidative stress and inflammation, improved ion handling and improved cardiac energy metabolism. Indirect effects include the decrease in ventricular filling pressures, decrease in afterload, improved vascular function, erythropoiesis, and improved renal function [87].

First clinical experience in children with CHF is emerging. SGLT2i appear to be well-tolerated, in a small study no patients experienced symptomatic hypoglycemia or hypovolemia and there were no clinically significant changes in blood chemistries or vital signs after initiation of dapagliflozin [88]. An improvement in ejection fraction was observed. However, urinary tract infection is a possible complication, especially in small children.

Given the numerous beneficial systemic effects of SGLT2i as well as direct effects on myocardial metabolism, it can be expected that at least some of these effects will be pronounced in children. We believe it is reasonable to consider treatment with a SGLT2i in children with severe CHF, with the threshold for the use of this medication decreasing with the patient's age.

Other new drugs

Omecamtiv mecarbil is a selective activator of cardiac myosin. It augments the speed of ATP hydrolysis, increasing the number of myosin heads binding to actin and thus increasing the contractile force. The duration of systole is increased but calcium cycling and energy consumption remains unchanged [89]. In adults with HF with reduced ejection fraction, benefits have been reported, particularly in patients with severely impaired ejection fraction [90]. However, The US Food and Drug Administration (FDA) has declined to approve omecamtiv mecarbil for treatment of adults with chronic heart failure with reduced ejection fraction recently, citing a lack of evidence on efficacy. No data is available in children.

Vericiguat is a soluble guanylate cyclase stimulator. Its mechanism of action involves the enhancement of the cGMP pathway stimulation and direct increase of endogenous nitric oxide. Benefits have been demonstrated in symptomatic adults with HF with reduced ejection fraction [91]. No data are currently available in children but there is a dedicated clinical trial in children with CHF in progress.

Initiation and escalation of pediatric CHF treatment

A stepwise approach to treat pediatric patients with CHF has been suggested both in the guidelines [2,3] and more recent reviews [7]. A suggested approach is summarized in Figure 2.

The therapy is usually initiated with an ACEI, and a MRA is added either upfront or depending on the next development. Target doses of these drugs can be reached relatively quickly. Alternatives to ACEI are either ARB or ARNI. ARNI instead of ACEI can be used in patients with insufficient response to ACEI treatment or upfront based on clinical decision (heart failure severity). Beta blockers are usually reserved for patients with more severe findings or symptoms, should be started at low dose, up-titrated more slowly and carefully and not always well-tolerated [7]. The selectivity of various BB to adrenergic receptors and advantages of selective β1-blockade should be taken into account.

The importance of heart rate reduction should be stressed and digoxin or ivabradine can be added if insufficient heart rate reduction is achieved with BB.

SGLT2 inhibitors proved to be very useful drugs with numerous beneficial effects in all types of CHF in adults and can be initiated at the target dose without the need of up-titration. They may be considered in older children.

When the target doses are reached, diuretic therapy should be reduced or discontinued, if possible, to avoid undesirable hypovolemia.

Conclusion

Medical treatment of pediatric chronic heart failure is largely based on guidelines for the adult population. In contrast to adults, evidence for the efficacy of medications in treating CHF in

children is very sparse. This may be due to the difficulty of conducting high-powered studies in children or to true differences in the mechanisms of CHF pathophysiology in children. Recent observations suggest that CHF in children differs from adults at the molecular and cellular levels. In order to improve the outcomes of pediatric CHF, it may be necessary to focus on certain pathways and pathophysiological mechanisms specific to children.

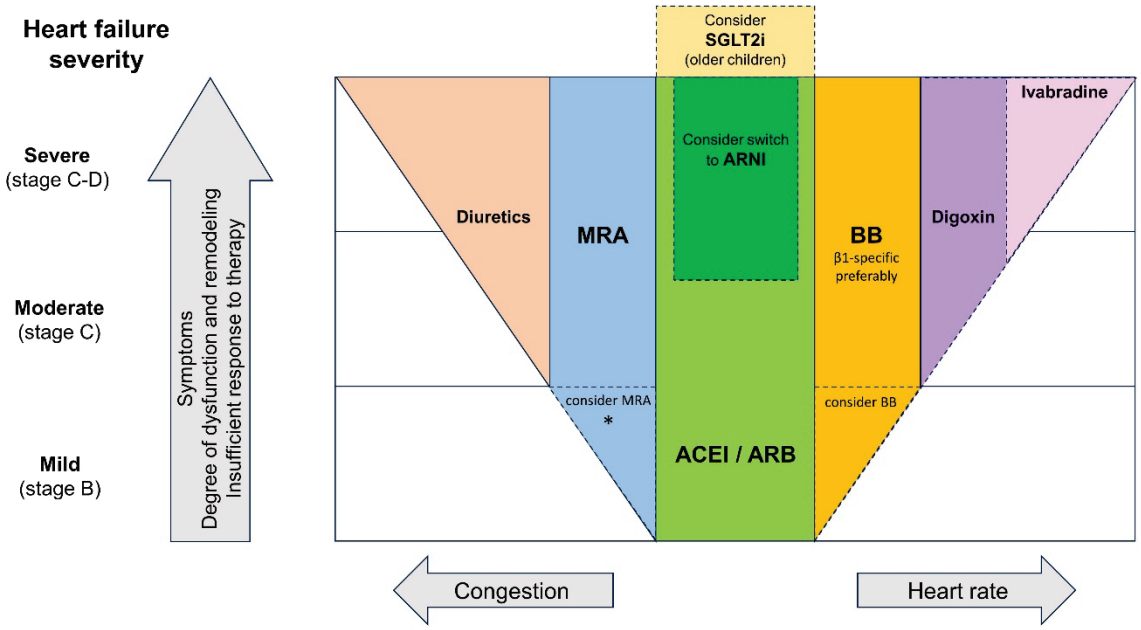

Fig. 2. Stepwise approach to treatment of pediatric chronic heart failure due to systemic left ventricular dysfunction. Created mainly based on [2,7,23,26]. Heart failure staging according to [35].
ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; ARNI, angiotensin receptor – neprilysin inhibitor; BB, beta blocker; MRA, mineralocorticoid receptor antagonist; SGL2i, sodium-glucose contransporter 2 inhibitor, * especially in cases of fibrosis

A stepwise approach to introducing medication is usually used. The cornerstones of pediatric CHF treatment are ACE inhibitors, beta blockers and mineralocorticoid receptor antagonists. There are potential benefits of tissue ACEI and β1-selective BB. Angiotensin receptor blockers are an alternative to ACEI and their slightly different mechanism of action may confer certain advantages or disadvantages, the significance of which is not entirely clear. Diuretics are employed to achieve a euvolemic state. Digoxin is used more frequently in children than in adults. Elevated heart rate should be reduced effectively according to the patient's tolerance. The exact role of newer drugs such as ARNI (sacubitril-valsartan), ivabradine and SGLT2 inhibitors has yet to be established.

Abbreviations

ACE, angiotensin converting enzyme; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; ARNI, angiotensin receptor – neprilysin inhibitor; AT1R, angiotensin II receptor type 1; α1-AR, α1-adrenoreceptor; BB, beta blocker; β1-AR, β1-adrenoreceptor; β2-AR, β2-adrenoreceptor; BB, beta blocker; MRA, mineralocorticoid receptor antagonist; SGL2i, sodium-glucose contransporter 2 inhibitor; V-V interaction, ventriculo-ventricular interaction.

Conflict of Interest

The author received fees from Novartis for participation in clinical trials and lecture activities.

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References

1. Shaddy RE, George AT, Jaecklin T, Lochlainn EN, Thakur L, Agrawal R, Solar-Yohay S, Chen F, Rossano JW, Severin T, Burch M. Systematic Literature Review on the Incidence and Prevalence of Heart Failure in Children and Adolescents. Pediatr Cardiol. 2017;39(3):1-22. https://doi.org/10.1007/s00246-017-1787-2
2. Kantor PF, Lougheed J, Dancea A, McGillion M, Barbosa N, Chan C, Dillenburg R, Atallah J, Buchholz H, Chant-Gambacort C, Conway J, Gardin L, George K, Greenway S, Human DG, Jeewa A, Price JF, Ross RD, Roche SL, Ryerson L, Soni R, Wilson J, Wong K. Presentation, Diagnosis, and Medical Management of Heart Failure in Children: Canadian Cardiovascular Society Guidelines. Canadian Journal of Cardiology. 2013;29(12):1535-1552. https://doi.org/10.1016/j.cjca.2013.08.008
3. Kirk R, Dipchand AI, Rosenthal DN, Addonizio L, Burch M, Chrisant M, Dubin A, Everitt M, Gajarski R, Mertens L, Miyamoto S, Morales D, Pahl E, Shaddy R, Towbin J, Weintraub R. The International Society for Heart and Lung Transplantation Guidelines for the management of pediatric heart failure: Executive summary. Journal of Heart and Lung Transplantation. 2014;33:888-909. https://doi.org/10.1016/j.healun.2014.06.002
4. Rossano JW, Shaddy RE. Update on pharmacological heart failure therapies in children: Do adult medications work in children and if not, why not? Circulation. 2014;129(5):607-612. https://doi.org/10.1161/CIRCULATIONAHA.113.003615
5. Castaldi B, Cuppini E, Fumanelli J, Di Candia A, Sabatino J, Sirico D, Vida V, Padalino M, Di Salvo G. Chronic Heart Failure in Children: State of the Art and New Perspectives. J Clin Med. 2023;12(7). https://doi.org/10.3390/jcm12072611
6. Ahmed H, VanderPluym C. Medical management of pediatric heart failure. Cardiovasc Diagn Ther. 2021;11(1):323. https://doi.org/10.21037/cdt-20-358
7. Loss KL, Shaddy RE, Kantor PF. Recent and Upcoming Drug Therapies for Pediatric Heart Failure. Front Pediatr. 2021;9:11. https://doi.org/10.3389/fped.2021.681224
8. Weisert M, Su JA, Menteer J, Shaddy RE, Kantor PF. Drug Treatment of Heart Failure in Children: Gaps and Opportunities. Pediatric Drugs. 2022;24(2):121-136. https://doi.org/10.1007/s40272-021-00485-9
9. Yamamoto F. Metabolic characteristics of immature myocardium. Gen Thorac Cardiovasc Surg. 2010;58(4):171-173. https://doi.org/10.1007/s11748-009-0541-y
10. Siedner S, Krüger M, Schroeter M, Metzler D, Roell W, Fleischmann BK, Hescheler J, Pfitzer G, Stehle R. Developmental changes in contractility and sarcomeric proteins from the early embryonic to the adult stage in the mouse heart. J Physiol 2003;548(2):493-505. https://doi.org/10.1113/jphysiol.2002.036509
11. Racca AW, Klaiman JM, Pioner JM, Cheng Y, Beck AE, Moussavi-Harami F, Bamshad MJ, Regnier M. Contractile properties of developing human fetal cardiac muscle. J Physiol 2016;594(2):437-452. https://doi.org/10.1113/JP271290
12. Lipsett DB, Frisk M, Aronsen JM, Nordén ES, Buonarati OR, Cataliotti A, Hell JW, Sjaastad I, Christensen G, Louch WE. Cardiomyocyte substructure reverts to an immature phenotype during heart failure. J Physiol 2019;597(7):1833-1853. https://doi.org/10.1113/JP277273
13. Givens Raymond C. and Schulze PC. Molecular Changes in Heart Failure. In: Eisen H, ed. Heart Failure: A Comprehensive Guide to Pathophysiology and Clinical Care. Springer London; 2017:1-26. https://doi.org/10.1007/978-1-4471-4219-5_1
14. Mollova M, Bersell K, Walsh S, Savla J, Das LT, Park SY, Silberstein LE, Dos Remedios CG, Graham D, Colan S, Kühn B. Cardiomyocyte proliferation contributes to heart growth in young humans. Proc Natl Acad Sci U S A. 2013;110(4):1446-1451. https://doi.org/10.1073/pnas.1214608110
15. Huang H, Huang GN, Payumo AY. Two decades of heart regeneration research: Cardiomyocyte proliferation and beyond. WIREs Mechanisms of Disease. 2024;16(1). https://doi.org/10.1002/wsbm.1629
16. Ostadal B, Kolar F, Ostadalova I, Sedmera D, Olejnickova V, Hlavackova M, Alanova P. Developmental Aspects of Cardiac Adaptation to Increased Workload. J Cardiovasc Dev Dis. 2023;10(5). https://doi.org/10.3390/jcdd10050205

17. Canseco DC, Kimura W, Garg S, Mukherjee S, Bhattacharya S, Abdisalaam S, Das S, Asaithamby A, Mammen PPA, Sadek HA. Human Ventricular Unloading Induces Cardiomyocyte Proliferation.; 2015. https://doi.org/10.1016/j.jacc.2014.12.027
18. Michel-Behnke I, Pavo I, Recla S, Khalil M, Jux C, Schranz D. Regenerative therapies in young hearts with structural or congenital heart disease. Transl Pediatr. 2019;8(2):140-150. https://doi.org/10.21037/tp.2019.03.01
19. Patel MD, Mohan J, Schneider C, Bajpai G, Purevjav E, Canter CE, Towbin J, Bredemeyer A, Lavine KJ. Pediatric and adult dilated cardiomyopathy represent distinct pathological entities. JCI Insight. 2017;2(14). https://doi.org/10.1172/jci.insight.94382
20. Woulfe KC, Siomos AK, Nguyen H, SooHoo M, Galambos C, Stauffer BL, Sucharov C, Miyamoto S. Fibrosis and Fibrotic Gene Expression in Pediatric and Adult Patients With Idiopathic Dilated Cardiomyopathy. J Card Fail. 2017;23(4):314-324. https://doi.org/10.1016/j.cardfail.2016.11.006
21. Stauffer BL, Russell G, Nunley K, Miyamoto SD, Sucharov CC. miRNA Expression in Pediatric Failing Human Heart. J Mol Cell Cardiol. 2013;57(1):43. https://doi.org/10.1016/j.yjmcc.2013.01.005
22. Miyamoto SD, Stauffer BL, Nakano S, Sobus R, Nunley K, Nelson P, Sucharov CC. Beta-adrenergic adaptation in paediatric idiopathic dilated cardiomyopathy. Eur Heart J. 2014;35(1):33-41. https://doi.org/10.1093/eurheartj/ehs229
23. Schranz D, Voelkel NF. "Nihilism" of chronic heart failure therapy in children and why effective therapy is withheld. Eur J Pediatr. 2016;175(4):445. https://doi.org/10.1007/s00431-016-2700-3
24. Wachter SB, Gilbert EM. Beta-adrenergic receptors, from their discovery and characterization through their manipulation to beneficial clinical application. Cardiology (Switzerland). 2012;122(2):104-112. https://doi.org/10.1159/000339271
25. Das BB. Current state of pediatric heart failure. Children. 2018;5(7). https://doi.org/10.3390/children5070088
26. Recla S, Schmidt D, Logeswaran T, Esmaeili A, Schranz D. Pediatric heart failure therapy: why β1-receptor blocker, tissue ACE-I and mineralocorticoid-receptor-blocker? Transl Pediatr. 2019;8(2):127. https://doi.org/10.21037/tp.2019.04.08
27. Boudoulas KD, Borer JS, Boudoulas H. Heart rate, life expectancy and the cardiovascular system: therapeutic considerations. Cardiology. 2015;132(4):199-212. https://doi.org/10.1159/000435947
28. Fox K, Borer JS, Camm AJ, Danchin N, Ferrari R, Lopez Sendon JL, Steg PG, Tardif JC, Tavazzi L, Tendera M. Resting Heart rate in cardiovascular disease. J Am Coll Cardiol. 2007;50(9):823-830. https://doi.org/10.1016/j.jacc.2007.04.079
29. Rossano JW, Kantor PF, Shaddy RE, Shi L, Wilkinson JD, Jefferies JL, Czachor JD, Razoky H, Wirtz HS, Depre C, Lipshultz SE. Elevated heart rate and survival in children with dilated cardiomyopathy: A multicenter study from the pediatric cardiomyopathy registry. J Am Heart Assoc. 2020;9(15). https://doi.org/10.1161/JAHA.119.015916
30. Fox K, Ford I, Steg PG, Tendera M, Robertson M, Ferrari R. Heart rate as a prognostic risk factor in patients with coronary artery disease and left-ventricular systolic dysfunction (BEAUTIFUL): a subgroup analysis of a randomised controlled trial. Lancet. 2008;372(9641):817-821. https://doi.org/10.1016/S0140-6736(08)61170-8, https://doi.org/10.1016/S0140-6736(08)61171-X
31. Adorisio R, Pontrelli G, Cantarutti N, Bellettini E, Caiazza M, Mencarelli E, Limongelli G, Poli D, Drago F, Kirk R, Amodeo A. Heart rate reduction as a marker to optimize carvedilol treatment and enhance myocardial recovery in pediatric dilated cardiomyopathy. Front Physiol. 2022;13. https://doi.org/10.3389/fphys.2022.1001752
32. Schranz D, Recla S, Malcic I, Kerst G, Mini N, Akintuerk H. Pulmonary artery banding in dilative cardiomyopathy of young children: review and protocol based on the current knowledge. Transl Pediatr. 2019;8(2):151. https://doi.org/10.21037/tp.2019.04.09
33. Spigel ZA, Razzouk A, Nigro JJ, Karamlou TB, Kavarana MN, Roeser ME, Adachi I. Pulmonary artery banding for children with dilated cardiomyopathy: US Experience. Semin Thorac Cardiovasc Surg Pediatr Card Surg Annu. 2020;23:69-76. https://doi.org/10.1053/j.pcsu.2020.03.002
34. Ponzoni M, Castaldi B, Padalino MA. Pulmonary artery banding for dilated cardiomyopathy in children: returning to the bench from bedside. Children. 2022;9(9). https://doi.org/10.3390/children9091392

35. Heidenreich PA, Bozkurt B, Aguilar D, Allen LA, Byun JJ, Colvin MM, Deswal A, Drazner MH, Dunlay SM, Evers LR, Fang JC, Fedson SE, Fonarow GC, Hayek SS, Hernandez AF, Khazanie P, Kittleson MM, Lee CS, Link MS, Milano CA, Nnacheta LC, Sandhu AT, Stevenson LW, Vardeny O, Vest AR, Yancy CW. 2022 AHA/ACC/HFSA Guideline for the Management of Heart Failure: A Report of the American College of Cardiology/American Heart Association Joint Committee on Clinical Practice Guidelines. Circulation. 2022;145(18):E895-E1032. https://doi.org/10.1161/CIR.0000000000001063
36. McDonagh TA, Metra M, Adamo M, Gardner RS, Baumbach A, Böhm M, Burri H, Butler J, Čelutkienė J, et al. 2023 Focused Update of the 2021 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: Developed by the task force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC) With the special contribution of the Heart Failure Association (HFA) of the ESC. Eur Heart J. August 25, 2023. https://doi.org/10.1093/eurheartj/ehad195
37. McDonagh TA, Metra M, Adamo M, Gardner RS, Baumbach A, Böhm M, Burri H, Butler J, Čelutkienė J, et al. 2021 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failureDeveloped by the Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC) With the special contribution of the Heart Failure Association (HFA) of the ESC. Eur Heart J. 2021;42(36):3599-3726. https://doi.org/10.1093/eurheartj/ehab368
38. Grenier MA, Fioravanti J, Truesdell SC, Mendelsohn AM, Vermilion RP, Lipshultz SE. Angiotensin-converting enzyme inhibitor therapy for ventricular dysfunction in infants, children and adolescents: a review. Prog Pediatr Cardiol. 2000;12:91-111. https://doi.org/10.1016/S1058-9813(00)00061-8
39. Pinto B, Jadhav U, Singhai P, Sadhanandham S, Shah N. ACEI-induced cough: A review of current evidence and its practical implications for optimal CV risk reduction. Indian Heart J. 2020;72(5):345-350. https://doi.org/10.1016/j.ihj.2020.08.007
40. Díez CC, Khalil F, Schwender H, Dalinghaus M, Jovanovic I, Makowski N, Male C, Bajcetic M, Van Der Meulen M, De Wildt SN, Ablonczy L, Szatmári A, Klingmann I, Walsh J, Läer S. Pharmacotherapeutic management of paediatric heart failure and ACE-I use patterns: A European survey. BMJ Paediatr Open. 2019;3(1). https://doi.org/10.1136/bmjpo-2018-000365
41. Shah AD, Arora RR. Tissue angiotensin-converting enzyme inhibitors: Are they more effective than serum angiotensin-converting enzyme inhibitors? Clin Cardiol. 2005;28(12):551. https://doi.org/10.1002/clc.4960281203
42. Raasch W, Dendorfer A, Ball B, Dominiak P. The lipophilic properties of angiotensin i-converting enzyme inhibitors do not influence their diffusion through cultured endothelium. Jpn J Pharmacol. 1999;81(4):346-352. https://doi.org/10.1016/S0021-5198(19)30745-0
43. Ruzicka M, Coletta E, White R, Davies R, Haddad H, Leenen FHH. Effects of ACE inhibitors on cardiac angiotensin II and aldosterone in humans: Relevance of lipophilicity and affinity for ACE. Am J Hypertens. 2010;23(11):1179-1182. https://doi.org/10.1038/ajh.2010.148
44. Piepho RW. Overview of the angiotensin-converting-enzyme inhibitors. American Journal of Health-System Pharmacy. 2000;57(suppl_1):S3-S7. https://doi.org/10.1093/ajhp/57.suppl_1.S3
45. Hirooka K, Koretsune Y, Yoshimoto S, Irino H, Abe H, Yasuoka Y, Yamamoto H, Hashimoto K, Chin W, Kusuoka H. Twice-daily administration of a long-acting angiotensin-converting enzyme inhibitor has greater effects on neurohumoral factors than a once-daily regimen in patients with chronic congestive heart failure. J Cardiovasc Pharmacol. 2004;43(1):56-60. https://doi.org/10.1097/00005344-200401000-00009
46. Belgeri MT. Long-acting angiotensin-converting enzyme inhibitors in congestive heart failure: does multiple-daily dosing provide additional benefit over once-daily dosing? J Pharm Technol 2005;21(4):203-206. https://doi.org/10.1177/875512250502100404
47. Raes A, Malfait F, Aken S Van, France A, Donckerwolcke R, Walle J Vande, Raes A. Lisinopril in paediatric medicine: a retrospective chart review of long-term treatment in children. J Renin-Angiotensin-Aldosterone System. 2007;8(1):3-12. https://doi.org/10.3317/jraas.2007.004
48. Snauwaert E, Vande Walle J, De Bruyne P. Therapeutic efficacy and safety of ACE inhibitors in the hypertensive paediatric population: A review. Arch Dis Child. 2017;102(1):63-71. https://doi.org/10.1136/archdischild-2016-310582

49. Levy BI, Mourad JJ. Renin angiotensin blockers and cardiac protection: from basics to clinical trials. Am J Hypertens. 2022;35(4):293-302. https://doi.org/10.1093/ajh/hpab108
50. Awad K, Zaki MM, Mohammed M, Lewek J, Lavie CJ, Banach M. Effect of the renin-angiotensin system inhibitors on inflammatory markers: a systematic review and meta-analysis of randomized controlled trials. Mayo Clin Proc. 2022;97(10):1808-1823. https://doi.org/10.1016/j.mayocp.2022.06.036
51. Kaschina E, Namsolleck P, Unger T. AT2 receptors in cardiovascular and renal diseases. Pharmacol Res. 2017;125:39-47. https://doi.org/10.1016/j.phrs.2017.07.008
52. Pitt B, Poole-Wilson PA, Segal R, Martinez FA, Dickstein K, Camm AJ, Konstam MA, Riegger G, Klinger GH, Neaton J, Sharma D, Thiyagarajan B. Effect of losartan compared with captopril on mortality in patients with symptomatic heart failure: randomised trial-the Losartan Heart Failure Survival Study ELITE II. The Lancet. 2000;355(9215):1582-1587. https://doi.org/10.1016/S0140-6736(00)02213-3
53. Cohn JN, Tognoni G. A randomized trial of the angiotensin-receptor blocker valsartan in chronic heart failure. New Engl J Med 2001;345(23):1667-1675. https://doi.org/10.1056/NEJMoa010713
54. McMurray JJV, Pfeffer MA, Swedberg K, Dzau VJ. Which inhibitor of the renin-angiotensin system should be used in chronic heart failure and acute myocardial infarction? Circulation. 2004;110(20):3281-3288. https://doi.org/10.1161/01.CIR.0000147274.83071.68
55. Nwaiwu O, Olayemi S, Amao O. Use of angiotensin II receptor blockers in children- a review of evidence. Niger J Paediatr. 2015;42(3):180-187. https://doi.org/10.4314/njp.v42i3.2
56. Das BB. Plasma B-type natriuretic peptides in children with cardiovascular diseases. Pediatr Cardiol. 2010;31(8):1135-1145. https://doi.org/10.1007/s00246-010-9758-x
57. McMurray JJV, Packer M, Desai AS, Gong J, Lefkowitz MP, Rizkala AR, Rouleau JL, Shi VC, Solomon SD, Swedberg K, Zile MR. Angiotensin-Neprilysin Inhibition versus Enalapril in Heart Failure. N Engl J Med. 2014;371(11):993-1004. https://doi.org/10.1056/NEJMoa1409077
58. Shaddy R, Burch M, Kantor PF, Solar-Yohay S, Garito T, Zhang S, Kocun M, Bonnet D. Baseline characteristics of pediatric patients with heart failure due to systemic left ventricular systolic dysfunction in the PANORAMA-HF Trial. Circ Heart Fail. 2023;16(3):E009816. https://doi.org/10.1161/CIRCHEARTFAILURE.122.009816
59. Staessen J, Lijnen P, Fagard R, Verschueren LJ, Amery A. Rise in plasma concentration of aldosterone during long-term angiotensin II suppression. J Endocrinol. 1981;91(3):457-465. https://doi.org/10.1677/joe.0.0910457
60. Pitt B, Zannad F, Remme WJ, Cody R, Castaigne A, Perez A, Palensky J, Wittes J. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. N Engl J Med. 1999;341(10):709-717. https://doi.org/10.1056/NEJM199909023411001
61. Buck ML. Clinical experience with spironolactone in pediatrics. Ann Pharmacother. 2005;39(5):823-828. https://doi.org/10.1345/aph.1E618
62. Li JS, Flynn JT, Portman R, Davis I, Ogawa M, Shi H, Pressler ML. The efficacy and safety of the novel aldosterone antagonist eplerenone in children with hypertension: a randomized, double-blind, dose-response study. J Pediatr. 2010;157(2):282-287. https://doi.org/10.1016/j.jpeds.2010.02.042
63. Willenheimer R, Van Veldhuisen DJ, Silke B, Erdmann E, Follath F, Krum H, Ponikowski P, Skene A, Van De Ven L, Verkenne P, Lechat P. Effect on survival and hospitalization of initiating treatment for chronic heart failure with bisoprolol followed by enalapril, as compared with the opposite sequence: Results of the Randomized Cardiac Insufficiency Bisoprolol Study (CIBIS) III. Circulation. 2005;112(16):2426-2435. https://doi.org/10.1161/CIRCULATIONAHA.105.582320
64. Packer M, Fowler MB, Roecker EB, Coats AJS, Katus HA, Krum H, Mohacsi P, Rouleau JL, Tendera M, Staiger C, Holcslaw TL, Amann-Zalan I, DeMets DL. Effect of carvedilol on the morbidity of patients with severe chronic heart failure: results of the carvedilol prospective randomized cumulative survival (COPERNICUS) study. Circulation. 2002;106(17):2194-2199. https://doi.org/10.1161/01.CIR.0000035653.72855.BF

65. Hjalmarson Å, Goldstein S, Fagerberg B, Wedel H, Waagstein F, Kjekshus J, Wikstrand J, El Allaf D, Vítovec J, Aldershvile J, Halinen M, Dietz R, Neuhaus KL, Jánosi A, Thorgeirsson G, Dunselman PHJM, Gullestad L, Kuch J, Herlitz J, Riekenbacher P, Ball S, Gottlieb S, Deedwania P. Effects of controlled-release metoprolol on total mortality, hospitalizations, and well-being in patients with heart failure: the Metoprolol CR/XL Randomized Intervention Trial in congestive heart failure (MERIT-HF). MERIT-HF Study Group. JAMA. 2000;283(10):1295-1302. https://doi.org/10.1001/jama.283.10.1295
66. Bruns LA, Chrisant MK, Lamour JM, Shaddy RE, Pahl E, Blume ED, Hallowell S, Addonizio LJ, Canter CE. Carvedilol as therapy in pediatric heart failure: An initial multicenter experience. Journal of Pediatrics. 2001;138(4):505-511. https://doi.org/10.1067/mpd.2001.113045
67. Shaddy RE, Boucek MM, Hsu DT, Boucek RJ, Canter CE, Mahony L, Ross RD, Pahl E, Blume ED, Dodd DA, Rosenthal DN, Burr J, LaSalle B, Holubkov R, Lukas MA, Tani LY. Carvedilol for children and adolescents with heart failure: a randomized controlled trial. JAMA. 2007;298(10):1171-1179. https://doi.org/10.1001/jama.298.10.1171
68. Bernstein D, Fajardo G, Zhao M. The role of β-adrenergic receptors in heart failure: Differential regulation of cardiotoxicity and cardioprotection. Prog Pediatr Cardiol. 2011;31(1):35-38. https://doi.org/10.1016/j.ppedcard.2010.11.007
69. Albers S, Meibohm B, Mir TS, Läer S. Population pharmacokinetics and dose simulation of carvedilol in paediatric patients with congestive heart failure. Br J Clin Pharmacol. 2008;65(4):511-522. https://doi.org/10.1111/j.1365-2125.2007.03046.x
70. Sucharov CC, Hijmans JG, Sobus RD, Melhado WFA, Miyamoto SD, Stauffer BL. β-Adrenergic receptor antagonism in mice: a model for pediatric heart disease. J Appl Physiol. 2013;115(7):979. https://doi.org/10.1152/japplphysiol.00627.2013
71. Läer S, Mir TS, Behn F, Eiselt M, Scholz H, Venzke A, Meibohm B, Weil J. Carvedilol therapy in pediatric patients with congestive heart failure: A study investigating clinical and pharmacokinetic parameters. Am Heart J. 2002;143(5):916-922. https://doi.org/10.1067/mhj.2002.121265
72. Gomez AB, Healy M, Donne GD, Bentley S, Makhecha S, Daubeney P, Naqvi N, Till J, Wong L. P10 Initiation of bisoprolol in paediatric patients - experience from a specialist paediatric cardiac centre. Arch Dis Child. 2023;108(5):5. https://doi.org/10.1136/archdischild-2023-NPPG.9
73. Jain S, Vaidyanathan B. Digoxin in management of heart failure in children: Should it be continued or relegated to the history books? Ann Pediatr Cardiol. 2009;2(2):149-152. https://doi.org/10.4103/0974-2069.58317
74. Rathore SS, Curtis JP, Wang Y, Bristow MR, Krumholz HM. Association of serum digoxin concentration and outcomes in patients with heart failure. JAMA. 2003;289(7):871-878. https://doi.org/10.1001/jama.289.7.871
75. Abdel Jalil MH, Abdullah N, Alsous MM, Saleh M, Abu-Hammour K. A systematic review of population pharmacokinetic analyses of digoxin in the paediatric population. Br J Clin Pharmacol. 2020;86(7):1267-1280. https://doi.org/10.1111/bcp.14272
76. Lee AY, Kutyifa V, Ruwald MH, McNitt S, Polonsky B, Zareba W, Moss AJ, Ruwald AC. Digoxin therapy and associated clinical outcomes in the MADIT-CRT trial. Heart Rhythm. 2015;12(9):2010-2017. https://doi.org/10.1016/j.hrthm.2015.05.016
77. Vamos M, Erath JW, Hohnloser SH. Digoxin-associated mortality: A systematic review and meta-analysis of the literature. Eur Heart J. 2015;36(28):1831-1838. https://doi.org/10.1093/eurheartj/ehv143
78. Ziff OJ, Lane DA, Samra M, Griffith M, Kirchhof P, Lip GYH, Steeds RP, Townend J, Kotecha D. Safety and efficacy of digoxin: systematic review and meta-analysis of observational and controlled trial data. BMJ. 2015;351:h4451. https://doi.org/10.1136/bmj.h4451
79. Oster ME, Kelleman M, McCracken C, Ohye RG, Mahle WT. Association of digoxin with interstage mortality: Results from the pediatric heart network single ventricle reconstruction trial public use dataset. J Am Heart Assoc. 2016;5(1):1-7. https://doi.org/10.1161/JAHA.115.002566
80. Brown DW, Mangeot C, Anderson JB, Peterson LE, King EC, Lihn SL, Neish SR, Fleishman C, Phelps C, Hanke S, Beekman RH, Lannon CM. Digoxin use is associated with reduced interstage mortality in patients with no history of arrhythmia after stage I palliation for single ventricle heart disease. J Am Heart Assoc: Cardiovascular and Cerebrovascular Disease. 2016;5(1). https://doi.org/10.1161/JAHA.115.002376

81. Moffett BS, Price JF. National prescribing trends for heart failure medications in children. Congenit Heart Dis. 2015;10(1):78-85. https://doi.org/10.1111/chd.12183
82. DiFrancesco D. Funny channels in the control of cardiac rhythm and mode of action of selective blockers. Pharmacol Res. 2006;53(5):399-406. https://doi.org/10.1016/j.phrs.2006.03.006
83. Bonnet D, Berger F, Jokinen E, Kantor PF, Daubeney PEF. Ivabradine in children with dilated cardiomyopathy and symptomatic chronic heart failure. J Am Coll Cardiol. 2017. https://doi.org/10.1016/j.jacc.2017.07.725
84. Packer M, Butler J, Zannad F, Filippatos G, Ferreira JP, Pocock SJ, Carson P, Anand I, Doehner W, Haass M, Komajda M, Miller A, Pehrson S, Teerlink JR, Schnaidt S, Zeller C, Schnee JM, Anker SD. Effect of empagliflozin on worsening heart failure events in patients with heart failure and preserved ejection fraction: EMPEROR-Preserved Trial. Circulation. 2021;144(16):1284-1294. https://doi.org/10.1161/CIRCULATIONAHA.121.056824
85. McMurray JJV, Solomon SD, Inzucchi SE, Køber L, Kosiborod MN, Martinez FA, Ponikowski P, Sabatine MS, Anand IS, Bělohlávek J, Böhm M, Chiang CE, Chopra VK, de Boer RA, Desai AS, Diez M, Drozdz J, Dukát A, Ge J, Howlett JG, Katova T, Kitakaze M, Ljungman CEA, Merkely B, Nicolau JC, O'Meara E, Petrie MC, Vinh PN, Schou M, Tereshchenko S, Verma S, Held C, DeMets DL, Docherty KF, Jhund PS, Bengtsson O, Sjöstrand M, Langkilde AM. Dapagliflozin in Patients with Heart Failure and Reduced Ejection Fraction. N Engl J Med 2019;381(21):1995-2008. https://doi.org/10.1056/NEJMoa1911303
86. Januzzi JL, Ibrahim NE. Understanding the mechanistic benefit of heart failure drugs matters. J Am Coll Cardiol. 2020;76(23):2752-2754. https://doi.org/10.1016/j.jacc.2020.10.026
87. Lopaschuk GD, Verma S. Mechanisms of cardiovascular benefits of sodium glucose co-transporter 2 (SGLT2) Inhibitors: A State-of-the-Art Review. JACC Basic Transl Sci. 2020;5(6):632-644. https://doi.org/10.1016/j.jacbts.2020.02.004
88. Newland DM, Law YM, Albers EL, Friedland-Little JM, Ahmed H, Kemna MS, Hong BJ. Early clinical experience with Dapagliflozin in children with heart failure. Pediatr Cardiol. 2023;44(1):146-152. https://doi.org/10.1007/s00246-022-02983-0
89. Malik FI, Hartman JJ, Elias KA, Morgan BP, Rodriguez H, Brejc K, Anderson RL, Sueoka SH, Lee KH, Finer JT, Sakowicz R, Baliga R, Cox DR, Garard M, Godinez G, Kawas R, Kraynack E, Lenzi D, Lu PP, Muci A, Niu C, Qian X, Pierce DW, Pokrovskii M, Suehiro I, Sylvester S, Tochimoto T, Valdez C, Wang W, Katori T, Kass DA, Shen YT, Vatner SF, Morgans DJ. Cardiac myosin activation: a potential therapeutic approach for systolic heart failure. Science. 2011;331(6023):1439. https://doi.org/10.1126/science.1200113
90. Teerlink JR, Diaz R, Felker GM, McMurray JJV, Metra M, Solomon SD, Biering-Sørensen T, Böhm M, et al. Effect of ejection fraction on clinical outcomes in patients treated with omecamtiv mecarbil in GALACTIC-HF. J Am Coll Cardiol. 2021;78(2):97-108. https://doi.org/10.1016/j.jacc.2021.04.065
91. Armstrong PW, Pieske B, Anstrom KJ, Ezekowitz J, Hernandez AF, Butler J, Lam CSP, Ponikowski P, Voors AA, Jia G, McNulty SE, Patel MJ, Roessig L, Koglin J, O'Connor CM. Vericiguat in Patients with Heart Failure and Reduced Ejection Fraction. N Engl J Med 2020;382:1883-1893. https://doi.org/10.1056/NEJMoa1915928

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REVIEW

Perinatal Hypoxia and Immune System Activation in Schizophrenia Pathogenesis: Critical Considerations During COVID-19 Pandemic

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Summary

Schizophrenia, a severe psychiatric, neurodevelopmental disorder affecting about 0.29-1 % of the global population, is characterized by hallucinations, delusions, cognitive impairments, disorganized thoughts and speech, leading to significant social withdrawal and emotional blunting. During the 1980s, considerations about diseases that result from complex interactions of genetic background and environmental factors started to appear. One of the critical times of vulnerability is the perinatal period. Concerning schizophrenia, obstetric complications that are associated with hypoxia of the fetus or neonate were identified as a risk. Also, maternal infections during pregnancy were linked to schizophrenia by epidemiological, serologic and genetic studies. Research efforts then led to the development of experimental models testing the impact of perinatal hypoxia or maternal immune activation on neurodevelopmental disorders. These perinatal factors are usually studied separately, but given that the models are now validated, it is feasible to investigate both factors together. Inclusion of additional factors, such as metabolic disturbances or chronic stress, may need to be considered also. Understanding the interplay of perinatal factors in schizophrenia's etiology is crucial for developing targeted prevention and therapeutic strategies.

Keywords

Schizophrenia • Perinatal hypoxia • Perinatal infection • Microbiota, SARS-CoV-2

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Introductory remarks on Professor Jan Herget's contribution to understanding developmental disorders

In this Special issue of Physiological Research, we celebrate the legacy of Professor Jan Herget (1945-2019) of the Second Faculty of Medicine, Charles University in Prague, Czech Republic. Although this review focuses on perinatal factors in schizophrenia, it is pertinent to remember Jan's unique contributions to uncovering the long-term effects of perinatal hypoxemia on responses to decreased oxygen in adulthood [1].

In the pioneering experiments, pregnant rats were placed into the hypoxic chambers and were kept there until their offspring were a week old. After placing the animals into the normoxic air, they recovered from hypoxia and had comparable pressure in pulmonary circulation as control mice unexposed to perinatal hypoxia. However, when these animals were re-exposed to acute hypoxia in adulthood, their responses were more severe than in animals born in normal air [1]. The perinatal exposure to hypoxia also blunted humoral immune responses in adult rats [2]. The mechanisms of these intriguing, lifelong-lasting effects are not yet fully understood. [3]

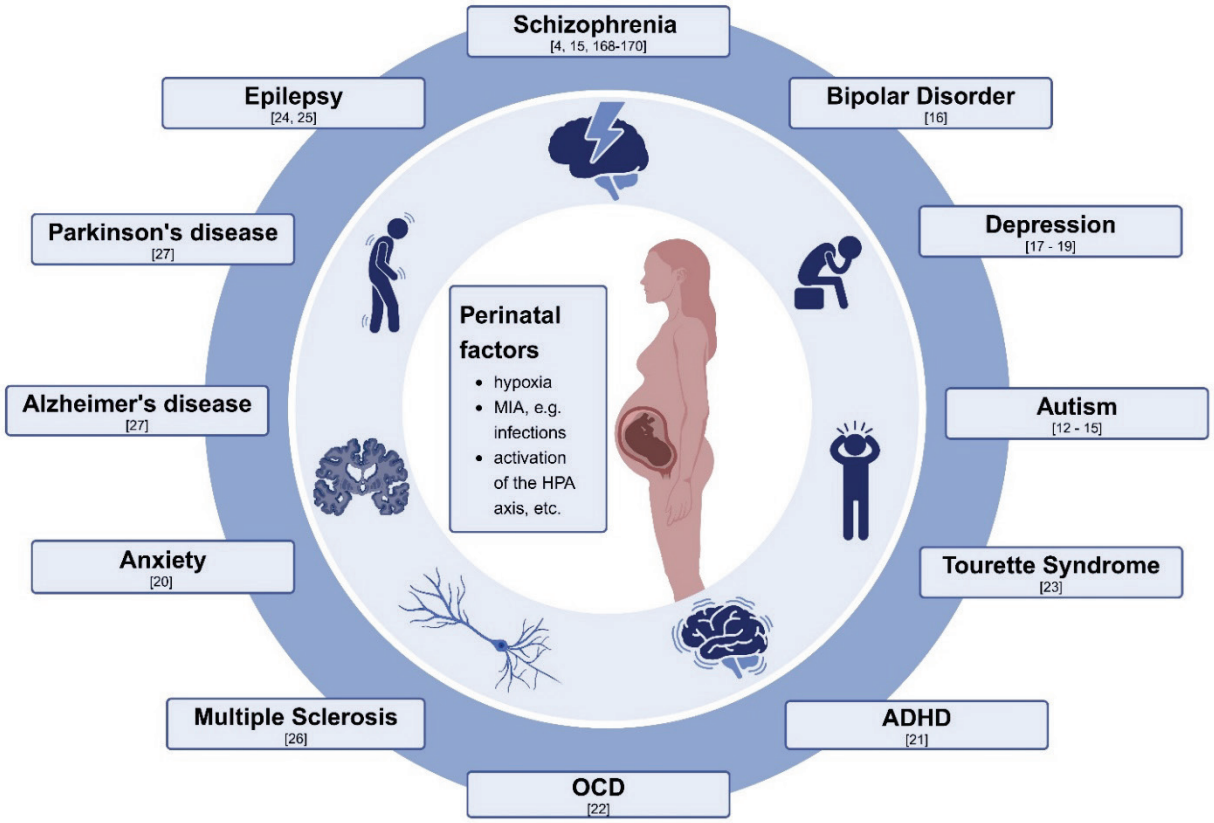

Fig. 1. Multiple factors acting perinatally can increase a risk for neurodevelopmental conditions that may manifest during childhood and adulthood. The picture was made with using a Biorender template.

Jan received the Robert F. Grower Prize from the American Thoracic Society for his research work. He was also an astounding teacher and mentor who profoundly impacted his students and research trainees. His vision that the course of a disease processes needs to be studied from a very early time in life took another dimension with models where perinatal hypoxia and maternal immune activation were identified as critical players in neurodevelopmental conditions.

Perinatal exposures and risk for neurodevelopmental conditions

Many perinatal factors influence the fetus during the perinatal period and affect the inherently complex and highly active brain tissue. For example, perinatal exposures that are related to increased risk of schizophrenia include conditions that cause hypoxemia, infections (especially influenza, rubella and toxoplasmosis) [4,5], maternal and offspring psycho-social stress [6], genetic factors [7], advanced paternal age [8], nutritional deficiencies [9], urbanicity [10] and migration status [11].

Vice versa, if we look at one group factors, e.g., maternal immune activation (MIA) initiated by viruses, bacteria, fungi, autoimmune conditions, they can play a role in a variety of childhood- or adulthood-onset of disorders, as depicted in Figure 1. They include autism [12-15], schizophrenia [4,15,168-170], bipolar disorder [16], depression [17-19], anxiety disorder [20], attention deficit-hyperreactivity disorder [21], obsessive-compulsive disorder [22], Tourette's syndrome [23], epilepsy [24,25], multiple sclerosis [26], Parkinson's disease and Alzheimer disease [27].

The precise mechanisms by which individual perinatal factors increase susceptibility to various neuropsychiatric conditions remain incompletely understood. The interplay of genetic predisposition, the timing of exposure, and the intensity of these factors collectively determine the outcomes. Elucidating these intricate details presents a formidable intellectual challenge. Resolving this complexity is crucial for identifying diagnostic biomarkers that enable clinicians to detect individuals at elevated risk for these conditions and to provide care strategies that may mitigate the likelihood of disease development.

In clinical psychiatry, diagnoses are based on symptoms and co-morbidities are a very common finding. In turn, making accurate diagnoses is arduous. Significant efforts are ongoing to define objective biomarkers that reflect distinct pathological processes, though they present by mixed behavioral symptoms. Understanding the pathophysiology is a prerequisite for diagnostic precision and enhancing effectiveness of treatment of schizophrenic patients [28].

We will focus here on hypoxia and perinatal immune activation as risk factors for schizophrenia. These two conditions have a high prevalence globally and often coincide, and the recent SARS-CoV-2 pandemic, or Severe Acute Respiratory Syndrome Coronavirus 2, very likely enhanced MIA in pregnant women during the pandemic.

Introduction to schizophrenia

In this selective review, we will focus on one of the neurodevelopmental disorders: schizophrenia, a severe psychiatric illness affecting about 0.29 % to 1 % of people worldwide. In the age group of 15-24 year-old individuals, it is the third most frequent mental disease, after anxiety and depression [29,30]. Patients experience symptoms such as hallucinations, delusions, cognitive impairment, disorganized speech and thought processes, which are categorized as “positive symptoms” of the disease. Their altered perception of reality often provokes severe anxiety and leaves them in profound loneliness. Patients have reduced expressions of emotions and typically withdraw from social contact. These symptoms are labeled as “negative symptoms”, and are more difficult to control by pharmacological treatment. Besides the impact of the disease on affected individuals, there is also a collateral toll on patient’s caregivers, families, friends, and colleagues [31-35]. In addition, the annual societal cost is high [36].

The pathogenic disturbances leading to schizophrenia syndrome result from interactions of multiple genetic and environmental factors [37] that start affecting brain circuitries during brain development [38], though the symptoms manifest in late adolescence to early adulthood. Most patients are affected between 15-25 years of age, with males having an earlier onset than females. In about 20 % of patients, the onset of schizophrenia occurs after forty and before 60 years, and rarely also, the disorder begins in childhood or adults above 60 years of age [29]. The differences in age of onset may reflect distinct pathogenic pathways or accumulation of causative factors, as suggested by the multiple-hit hypothesis [39].

Diagnosis of schizophrenia is still based on clinical symptoms because no objective, validated biomarkers exist for clinical use. As a result, diagnosis is not based on mechanistic principles; existing treatments are non-curative, and 30 % of patients remain resistant to existing therapeutics [40]. A better understanding of pathogenesis will help us subtyping patients into groups according to distinct pathological processes in individual patients and treat patients with high precision, as it has already been developed for cancer patients [41]. To this end, we focus here on the early events that increase the chance of schizophrenia development. Extensive research suggests that schizophrenia is a neurodevelopmental disorder, with the pathological processes potentially commencing as early as the in-utero stage [42,43], and progressing to a neurodegenerative condition [44].

Pathophysiology of schizohrenia

Reasons for poor understanding of schizophrenia include the high heterogeneity of patients, the inaccessibility of human brain tissue for biopsy sampling, and the involvement of animal models that do not fully capture the polygenic nature and multitude of environmental factors [45-48]. Our understanding of schizophrenia’s pathophysiology has been to a large degree based on postmortem brain studies, imaging studies, effects of pharmacotherapy and genetic studies.

Morphological considerations

Postmortem brain studies

Studies on brain pathology in schizophrenia initially focused on post-mortem brain specimens of individuals with schizophrenia. One major challenge with postmortem studies is the variability in findings due to factors such as the stage of the disease, medication status, and comorbid conditions at the time of death. Additionally, postmortem changes and tissue preservation issues can complicate interpretations. Despite these challenges, a consistent pattern has been identified in patients with chronic schizophrenia. Macroscopic findings include an enlargement of lateral and third ventricles, reduced brain volume, reduced gray matter involving the cortex, especially the prefrontal cortex, as

well as changes in subcortical gray matter (decreased volumes of hippocampus and thalamus, and increased basal ganglia volume) [49,50].

At the microscopic level, the altered density of neuronal and glial cells was reported in the *prefrontal cortex,* as well as reduced size of pyramidal neurons, reduced number of parvalbumin interneurons, decreased dendritic spine density and reduced neuropil (regions in the gray matter with dense, interwoven network of axons, dendrites, and glial cells), which contribute to disruptions in synaptic connectivity and plasticity [51-56]. In the *hippocampus,* findings in brains of patients with schizophrenia included neuronal disarray, reduced numbers of neurons and interneurons and decreased gene and protein expression of somatostatin-positive and parvalbumin-positive interneurons [57]. *In the thalamus*, numbers of neurons and parvalbumin positive interneurons were reduced [58]. *Basal ganglia* in individuals with schizophrenia are characterized by changes in the density of neurons and cholinergic interneurons [59]. *In the corpus callosum*, reduced myelin, axon density, and gliosis were noted [60].

Brain imaging

The macroscopic structural findings in schizophrenia were later confirmed in *in vivo* brain imaging studies [61]. Structural changes in the gray matter include reduced volume of frontal, prefrontal and temporal cortices with progressive loss over the course of the disease [62]. With regard to the white matter, a smaller total volume at a later stage of the disease [63] and hypoactive connectivity in major networks were reported [64].

Brain imaging also enabled the study of patients at different stages of the disease. Comparing individuals at prodromal state, first-episode of psychosis (FEP) or chronic schizophrenia revealed the progressive nature of brain pathology.

Ultra-high risk (UHR) individuals at prodromal state are people with personal features (e.g., subthreshold psychotic symptoms or a history of self-limiting brief intermittent psychotic symptoms) or a family history (e.g. first degree relative with a psychotic disorder) that puts them into a risk of developing a full-blown psychotic disorder. UHR individuals show early signs of anatomical and neuropathological abnormalities, e.g., reduction in frontal, prefrontal, and temporal cortices, often subtler than in FEP, affecting cortical and subcortical gray matter [65]. In FEP, prefrontal hypoactivity and hippocampal and subcortical hyperreactivity were described [66]. Gray matter changes are most robust within thalamocortical networks and brain activity is most altered in fronto-parietal circuitries [66]. In chronic schizophrenia, reduced gray matter involving the cortex (frontal, prefrontal, temporal), decreased volumes of hippocampus and thalamus, and increased basal ganglia volume were described [62].

Neurochemical considerations

Understanding of biochemical disturbances in schizophrenia has been evolving since 1950s when it was discovered that chlorpromazine has neuroleptic effects during anesthesia (Laborit *et al.*), and later shown to improve symptoms of schizophrenia [67]. Chlorpromazine effects include dopamine receptor inhibition [68]. Studies of postmortem brain showed dysregulation of dopamine synthesis, receptor expression or intracellular signaling in prefrontal and cingulate corte [69,70], hippocampus [69], thalamus [71] and basal ganglia [72,73]. Later it became clear that mono-amine theory of schizophrenia is over-simplified and pathophysiology involves also alterations in glutamatergic and GABAergic systems [74]. In addition, the effectiveness of several medications provided further clinical insight into the roles of neurotransmitters in the pathogenesis of schizophrenia, e.g., blockade of dopamine D2 receptors by a typical antipsychotic (e.g. haloperidol), serotonin 5-hydroxytryptamine (5-HT)-2A receptor inhibition, and 5-HT-21A receptor activation by atypical antipsychotics (e.g. risperidone) and partial glutamatergic agents, such as N-methyl D-aspartate (NMDA) receptor modulators [75,76]. Also, adjunctive treatment of schizophrenia has involved medications affecting GABAergic system, including benzodiazepines, though their use may need to be honed [77].

Genetic factors

Genome-wide association studies revealed over three hundred genes that represent risk factors for schizophrenia [78]. Twin and family studies showed that heritable risk for schizophrenia is about 67 %, which may be inflated due to common environmental conditions of the participants in the study, and thus environmental factors play significant role in development of the disease [79]. Concerning the perinatal factors discussed in this review, risk genes in pathways relevant to hypoxia [80,81] immune [82,83] and gut microbioma [84] responses are significantly enriched.

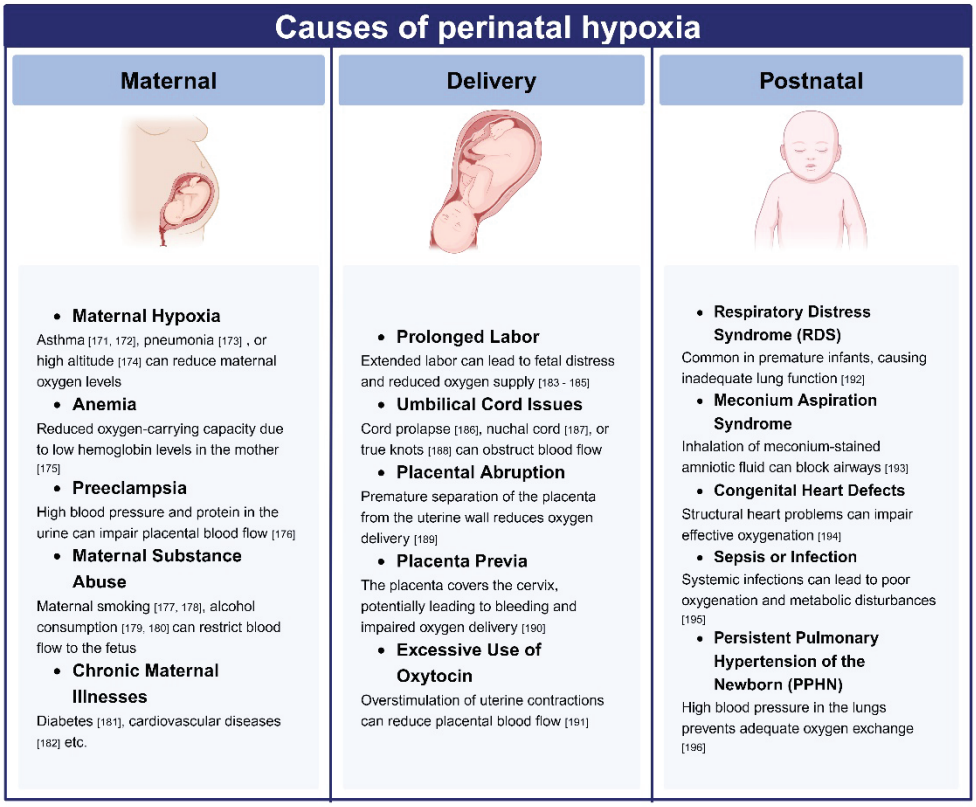

Fig. 2. Maternal, fetal and neonatal causes of decreased oxygen content in the blood supplying offspring's brain. The picture was made with using a Biorender template.

The relation of perinatal hypoxia to schizophrenia

Many conditions are associated with various degrees of maternal and fetal hypoxemia (Fig. 2). Perinatal hypoxia exerts extensive effects on the brain's histopathology, neurophysiology, and long-term health outcomes. The impact of hypoxemia on brain cells is modulated by the degree and duration of hypoxia. Mild hypoxia may induce reversible cellular changes, leading to adaptations and possibly alterations in transcription programs to cope with reduced ATP synthesis. In contrast, severe or prolonged hypoxia can result in irreversible damage and cell death. Additionally, the effects of hypoxia are influenced by the activity at a specific brain site and time, which is complex in healthy infants and in premature babies.

Clinical findings relating perinatal hypoxia to schizophrenia

The hypothesis that the development of psychotic conditions may be associated with brain hypoxia in the perinatal period was formulated in 1975 by a child psychiatrist, H. Allen Handford, based on the clinical history of patients seen in his practice, highlighting at that time that additional factors than genetics are at play [85].

Epidemiological studies then showed that hypoxia-associated obstetric complications significantly increase the risk for schizophrenia with early onset, before the age of 22 years (odds ratio, OR, 2.16) [86], and the degree of hypoxia-associated obstetric complications correlates with risk for developing schizophrenia [87]. After adjusting for other obstetric complications (e.g. maternal history of psychotic illness and social class), the association between signs of asphyxia at birth and schizophrenia reached odds ratio (OR) 4.4 [88].

Concerning the impact of perinatal hypoxia on the brain tissue of newborn, brain imaging studies revealed that the most susceptible areas include injuries within watershed areas, hippocampus, basal ganglia, thalamus, hippocampus and white matter. The impact depends on maturation stages of the brain [89]. Prefrontal cortex that is consistently linked to schizophrenia, is supplied by both anterior and middle cerebral arteries and includes the watershed area between them, making it sensitive to hypoxemia. Prefrontal cortex is also

connected to the subcortical nuclei, e.g. thalamus, hippocampus, striatum, and hypoxia-induced white matter injury may affect the connectivity. Also, post-mortem brain analysis of the posterior hippocampus in patients with schizophrenia revealed a negative correlation between events associated with hypoxia and the numbers of pyramidal cells in CA4, a deep polymorphic layer of dentate gyrus [90]. Correspondingly, brain imaging studies in patients with psychotic disorder uncovered a decreased hippocampal volume in individuals with schizophrenia as compared to healthy controls, and those volumes were further decreased in patients with hypoxia events in their early life [91]. In summary, hypoxic insults, though highly variable in their strength and timing, appear to impact many areas shown to be affected also in schizophrenia.

It was suggested that the level of susceptibility to hypoxia may depend on genetic background. To this end, Nicodemus *et al.* tested thirteen hypoxia-regulated genes related to neurovascular functions. Changes in single nucleotide polymorphisms (SNP) in four genes were identified: AKT1 (AKT serine/threonine kinase 1; three SNPs), BDNF (brain-derived neurotrophic factor; two SNPs), DNTBP1 (dystrobrevin-binding protein 1; one SNP) and GRM3 (S-adenosyl-L-methionine-dependent methyltransferases superfamily protein; one SNP). These findings support the gene-environment interactions in schizophrenia [92]. Concerning functional responses to fetal hypoxia, a protein product of one of the factors – BDNF- was increased in cord blood in control subjects without schizophrenia, while in cases with schizophrenia, BDNF levels were decreased by hypoxia [93]. HMGA1 (High mobility group A protein 1a) was found to be a hypoxia-inducible RNA-binding trans-acting factor for aberrant splicing of presenilin-2 pre-mRNA. Morikawa *et al.* found increased HMGA1a mRNA and protein in patients with schizophrenia [94]. Recently it was also shown that hypoxia-inducible factor induces MIF expression (macrophage migration inhibitory factor, a neuroprotective cytokine at the cross-road of inflammatory and stress responses) by binding to hypoxia-response element at the MIF promoter and that SNP at this site represents a risk factor for schizophrenia by reducing production of MIF in response to hypoxia [95]. Hypoxia also affects the extensively studied pathway of DISC1 (Disrupted in schizophrenia 1, a scaffold protein that interacts with many other proteins and is required for synaptogenesis, neurite outgrowth, and neuronal migration) by reducing the half-life of DISC1 protein [96].

Experimental studies in animal models

Experimental models of schizophrenia induced by perinatal hypoxia typically involve oxygen deprivation during critical developmental periods. For example, a rodent brain at postnatal days 7-8 corresponds to the late gestational period in humans. It represents a critical period for dendritic outgrowth, formation of synapses, and maturation of neuronal tissue [97], neuronal networks with alterations in the glutamatergic receptors (switch subunits of NMDA receptors from GluN2B to GluN2A subtypes) [98] and transformation of GABAergic system from excitatory to inhibitory effects [97]. All the parameters studied during developmental stage can now be linked using 3D eMouse atlas [99] that builds on morphological staging developed by Karl Theiler [100].

The immature brain in this sensitive age is quite vulnerable and depends on the timing and duration of hypoxic insult as it affects dynamic functions, such as neuronal proliferation, migration, and maturation. The models mimic obstetric complications, e.g., C-section, perinatal/postnatal hypoxia, or placental insufficiency [101]. Experimental hypoxia includes several protocols that employ acute or chronic oxygen reduction at sea level barometric pressure or hypobaric conditions where the percentage of oxygen remains the same, but decreased barometric pressure leads to less oxygen delivered to the lungs' alveolo-capillary membrane.

In relation to schizophrenia, neuregulin-1 (a key factor seen elevated in patients with schizophrenia) was 32 % higher in the frontal cortex of adult rats exposed to 7-day neonatal hypoxemia [102]. In a recent study, perinatal hypoxia was shown to dysregulate spontaneous activity patterns critical for forming functional templates for generating cortical architecture and guidance for establishing thalamocortical and intracortical circuits. These circuits are affected in patients with schizophrenia [103].

Hypoxia-induced behavioral changes resembling schizophrenia

C-section results in greater amphetamine-induced locomotion in adult rats, both in animals born in normoxia or hypoxia as compared to vaginal delivery. Amphetamine increases dopamine levels, and increased locomotion in response to amphetamines indicates heightened sensitivity to dopamine, which models the dopamine dysregulation seen in schizophrenia. The rats

also spent more time sniffing than grooming, and hypoxia during C-section was linked to prolonged rearing in adult rats [104]. In animal models, spending more time sniffing the environment and less time grooming can be indicative of increased anxiety or hyperactivity, both of which are observed in individuals with schizophrenia. Prolonged rearing indicates increased exploratory behavior and hyperactivity. In schizophrenia models, this behavior can reflect the hyperdopaminergic state associated with positive symptoms of schizophrenia, such as agitation and hyperactivity.

Adult guinea pigs had also increased amphetamine-induced locomotion and disrupted pre-pulse inhibition (PPI) of acoustic startle, but hypoxia during C-section reduced amphetamine-induced locomotion [105]. PPI is a neurological phenomenon used to measure sensory gating, the brain's ability to filter out unnecessary information. The test consists of the startle reflex, a rapid, involuntary response to a sudden loud noise or other sensory solid stimulus. When a weak pre-stimulus (pre-pulse) is presented shortly before a strong startling stimulus, PPI occurs, and the startle response to the subsequent more substantial stimulus is reduced. Disrupted PPI in animals indicates impaired sensory gating, a key feature of schizophrenia. In another study, postnatal hypoxia induced by bilateral, continuous occlusion of the common carotid artery in 12-day-old male rats resulted in schizophrenia-like behavior, including locomotor activity in pubertal rats (postnatal day 35) and impaired PPI in post-pubertal males (postnatal day 50) [106].

Hypoxia-induced impact on neurotransmitters and/or their receptors related to schizophrenia.

All three central neurotransmitter systems are affected by hypoxia:

1) *Dopamine.* Animal models of C-section hypoxia resulted in altered levels of dopamine or dopamine receptors, the key neurotransmitter associated with schizophrenia. Decreased levels of dopamine were found in the prefrontal cortex [107,108], while dopamine release was increased in the nucleus accumbens [108] and amygdala [109];

2) *Glutamatergic system*. NMDA receptor binding decreased, and transcription of NR1 subunit increased in frontal and temporal regions, nucleus accumbens, and hippocampus. NR2A subunit expression was downregulated in hippocampal sub-regions. On day 120 postnatally, gene expression of NR1 was still increased in hippocampal, frontal, and temporal sub-regions, as well as nucleus accumbens - a pre-pulse inhibition deficit points to schizophrenia-like behavior in 4-month-old rats. Compensatory upregulation of NR1 expression may occur due to NMDA receptor hypofunction. A subset of glutamate receptors, kainite receptors, increased after exposure to hypoxia [104]. Neuregulin -1, a protein that interacts with glutamate receptors, was elevated after hypoxia in 7-day rats [102];

3) *GABA* in the hippocampus increased in 7-day old rats after 1hr ligation of the left carotid artery and exposure to air where the content of oxygen was reduced from 21 to 8 % [110].

In summary, perinatal hypoxia has been linked to many neuropsychiatric conditions. Concerning schizophrenia, the link was established by epidemiological and genetic studies, in vitro experiments on human cells, and *in vivo* experimental studies in several animal species.

Perinatal immune system activation and the brain

Perinatal infections encompass a range of infectious diseases transmitted from mother to fetus in-utero or during birth or occur shortly after delivery. Any infectious microorganisms, including bacteria, viruses, fungi, or parasites, can cause these infections. Immune responses to invading microorganisms are associated with local and systemic activation of immune cells and the production of soluble molecules, including interleukins, cytokines, complement peptides, and antibodies. The presence of these molecules alters the brain development.

Clinical studies in patients with schizophrenia

Epidemiological evidence.

The link between perinatal infection and schizophrenia started to be considered more than three decades ago. In a Finish birth cohort study, Mednick *et al.* showed that mothers in the second trimester of their pregnancy during the 1957 influenza endemic had children who were much more likely to be admitted by 26 years in an inpatient facility with the diagnosis of schizophrenia [5]. Subsequent birth cohort study employed an improved design by involving pregnant women whose respiratory infection was recorded by a physician and whose offspring had continued follow-up with a diagnosis of schizophrenia established by face-to-face interview [111]. Second-trimester infection represented an increased risk for schizophrenia with

a relative rate of 2.13 [111]. Going beyond the in-utero period, a two-fold risk for schizophrenia was found in adults who experienced childhood infections, especially influenza, as a meta-analysis revealed. These findings highlight that the critical developmental period continues in the postnatal period [112]. A recent population-based nationwide cohort study addressed the hazard ratio for neuropsychiatric conditions in children of mothers with autoimmune diseases. The hazard risk with regards to schizophrenia was 1.35, demonstrating increased risk in offspring of women with conditions such as autoimmune diabetes or rheumatoid arthritis [113].

Serologic findings

Other efforts focused on finding infectious microorganisms responsible for these observations. In a nested case-control study, blood samples of mothers of children who turned out to be schizophrenic patients in adulthood were measured. The samples were collected at the end of their pregnancy and were found to have elevated total immunoglobulin (Ig)G and IgM and elevated IgG specific against herpes virus type 2 glycoprotein gG2 [114]. In another nested case-control study, archived blood samples of mothers pregnant between 1959 and 1966 were tested, and offspring were followed for psychiatric disorders for 30-38 years. Influenza infection during the first trimester increased the risk for schizophrenia 7-fold and 3-fold after broadening gestational periods to early to mid-pregnancy [115].

The studies on viral pathogens also expanded to protozoan parasites, toxoplasmosis gondii, and bacterial infections. Xiao *et al.* developed new antibodies for enzyme-linked immunosorbent assay to distinguish three distinct clonal lineages of toxoplasma and then tested the sera of pregnant mothers whose children developed schizophrenia and schizoaffective disorder with sera of mothers of unaffected children. Serological positivity for Toxoplasma type I, Ukrainian infection, increased risk for the development of psychoses with an odds ratio of 1.94. For affective psychoses, the odds ratio was 5.24 [116]. Bacterial infections during pregnancy also represent a significant risk for the development of schizophrenia (adjusted odds ratio 1.8, primarily when the infection affects multiple systems, which raises the adjusted odds ratio to 2.9 [117].

Neuroanatomic considerations

In patients with schizophrenia, postmortem analyses and brain imaging studies done by the early 1990s established that pathology occurs within fronto-striatal-temporal regions [118]. As further details were learned, more details were identified, and the frontal cortex, hippocampus ([119], cerebellar vermis [120], substantia nigra [121] were added to the neuroanatomical areas related to schizophrenia. A recent review of meta-analyses concluded that schizophrenia is characterized by lower grey matter volumes and cortical thickness, accelerated grey matter loss over time, abnormal gyrification patterns, and lower regional SV2A levels (Synaptic Vesicle Glycoprotein 2A is a protein that plays a crucial role in the regulation of neurotransmitter release at synapses) and metabolic markers in comparison to controls (effect sizes from ~ -0.11 to -1.0), and that critical regions affected include frontal, anterior cingulate and temporal cortices and the hippocampi [42].

Concerning the association between immune system activation and schizophrenia, metanalysis revealed a significant increase in the density of microglia, especially in the temporal cortex, while densities of macroglia (astrocytes and oligodendrocytes) did not differ significantly. On the molecular level, increased expression of proinflammatory genes on transcript and protein levels was seen in schizophrenia, while anti-inflammatory gene expression levels did not differ between schizophrenia and controls [122].

Complex developmental trajectories were detected in the brains of patients with autism and schizophrenia spectrum disorders, which are distinct disorders where autism starts in early childhood and schizophrenia in young adulthood. However, patients with autism are three times more likely to develop schizophrenia later in their life [123]. This association may result from interactions between genetically-defined abnormalities and many environmental factors to which each individual is likely exposed at different times. For a better understanding of the mechanisms of this complex phenomenon, animal models of maternal immune activation (MIA) were developed.

Experimental studies in MIA model

Robert Sidwell's group established foundations for the MIA by involving C57/BL6 pregnant mice infected with human influenza virus on gestational day 9 and assessing offspring on day 0 after the birth and at 14 weeks. They were the first to report short- and long-lasting impacts both on adult offspring's behavior and brain neuropathology, including macrocephaly and pyramidal cell atrophy [124]. Paul Patterson and his team

then employed the synthetic double-stranded RNA polyinosinic-polycytidylic acid (Poly (I:C)) instead of the influenza virus and demonstrated a similar impact on brain structures and functions [125]. The deficits in pre-pulse inhibition in the acoustic startle response linked the model to autism and schizophrenia spectrum disorders [126]. Using synthetic mimetics of the influenza virus significantly simplified the methodology of this model and facilitated subsequent studies that involved different Toll-Like Receptor (TLR) ligands administered to pregnant dams at different stages of pregnancy [127].

Paul Patterson's group was pivotal in developing the model's neuropathology and identifying behavioral abnormalities (e.g., deficits in social interaction, increased anxiety, and cognitive impairments), which helped to draw parallels between the animal model findings and symptoms of patients with autism and schizophrenia [15,128]. They also established essential roles of cytokines, particularly interleukin (IL)-6, in mediating the effects of MIA on neurological abnormalities in exposed offspring [126]. Another critical cytokine, IL-17, is required for elicitation of abnormal cortical and altered behavior in offspring, as discovered by Gloria Choi [13]. Her group further refined the MIA model by establishing neuronal circuitry that is affected by IL-17 [129,130].

Urs Meyer's group demonstrated the relevance of the model to schizophrenia and the multi-hit hypothesis. In their experiments, MIA-exposed animals received stressful stimuli in the peripubertal period, which resulted in synergistic effects on brain pathology and behavior in adulthood. The MIA exposure significantly increased the vulnerability of the pubescent offspring [131]. Given that the onset of schizophrenia occurs in young adults, this model involving a combination of perinatal and peripubertal challenges likely reflects real-world scenarios.

In summary both preclinical and clinical studies have linked inflammation and maternal immune activation to pathogenesis of schizophrenia. The critical questions that need to be resolved are when the inflammatory processes within the brain are beneficial and when they are detrimental, and how can the injurious events be therapeutically inhibited without impacting the whole immune system and rendering treated individuals more susceptible to infections.

The complexity of interactions in the MIA model is evident from this outline. However, another layer of complexity was added when Sarkis Maznamian's group reported that MIA alters the gut microbiome, significantly affecting exposed offspring's neurodevelopment [132].

Gut microbiome and the brain

The gut microbiota comprises over 100 trillion bacteria, viruses, and fungi, which form an essential physiological system. The interactions between the large mass of microorganisms and the gastrointestinal wall are highly regulated by the gut-associated lymphoid tissue, which represents the immune tolerance's cardinal site. The gut microbiota interacts with other organs through a multidirectional communication network, via which it also influences brain development and functions [133].

The microbiota communicates with the brain *via* nerves and in an endocrine fashion. Regarding nerves, autonomic parasympathetic, vagal, and splanchnic plexus fibers are directly wired to the central nervous system. The endocrine role of gut microbiota is reflected in the release of many substances that then travel through interstitial fluid or blood to local or distant targets [134].

The most relevant molecules produced or metabolized by the microbiota are short-chain fatty acids (produced by bacteria), bile acids (from the liver and metabolized by the microbiota), and tryptophan (an essential amino acid originating mainly in a diet and then metabolized by the microbiota) [135].

The microbiota can transform tryptophan into indole and other aryl hydrocarbon receptor ligands, critical for maintaining epithelial cell renewal and integrity and controlling intraepithelial leukocyte interactions [135]. Tryptophan is also metabolized by indoleamine-2,3-deoxygenase in epithelial and immune cells to kynurenine and downstream products, which regulate inflammation, adaptive immune responses, and neurotransmission [135]. Another critical role of tryptophan is being a precursor for serotonin. It is produced by two enzymes, tryptophan-hydroxylase 1 and 2, located in the gut and brain. About 95 % of serotonin is found in the gut, produced by enterochromaffin cells [136]. Serotonin acts as a hormone and neurotransmitter in the peripheral and central nervous systems. In the gut, serotonin influences intestinal peristalsis and motility, secretion from gastrointestinal glands, and vasodilation. Serotonin is also a part of the content of platelet and mast cell granules and contributes significantly to inflammatory responses.

Clinical studies on microbiota in schizophrenia

Zheng and his team established the relationship between gut microflora and schizophrenia. They showed that schizophrenia patients exhibited reduced diversity in bacterial species, and revealed a correlation between discriminative microbial markers and the severity of schizophrenia symptoms [137]. A positive correlation was found for Lachnospiraceae OTU (Operational taxonomic unit) 477, Lachnospiraceae OTU629, Ruminococcaceae, Bacteroidaceae OTU 172, and streptococcaceae OTU834, and negative correlation was reported for veillonellaceae OTU191 and Rumino-coccaceae OTU725 [137]. The authors then went beyond establishing the clinically obtainable correlative relationships and addressed the pathogenic role of gut microflora in a translational experiment where fecal microbiota from schizophrenic patients versus healthy controls were transplanted into experimental mice. Animal behavior in group recipients of the stool from schizophrenic patients corresponded to behavior considered characteristic in experimental models of schizophrenia. These changes were accompanied by biochemical alterations in the cortex and hippocampus, which are consistently reported to be affected in schizophrenia [137].

In another study, Li *et al.* show that microbiota in patients with schizophrenia is related to structural changes in their brains. Structural magnetic resonance imaging revealed a reduction in gray matter volume and regional homogeneity in several brain regions in patients. Alpha diversity of the gut microbiota in patients showed a strong linear relationship with the values of both MRI parameters. These results further strengthened the argument that gut microbiome may play a role in the neuropathogenesis of schizophrenia [138].

Evidence for clinical MIA affecting brain development via the impact on microbiota

Whether maternal infection during pregnancy affects the child's brain development at least partially *via* gut microbiota has not been documented. However, fragmented clinical evidence suggests that such a pathway exists. First, even a minor infection affects the composition of gut microbiota, as shown by a longitudinal study on patients with mild, asymptomatic SARS-CoV-2 infection. Their stool was collected during the infection, and then after they turned seronegative for the SARS-CoV-2 virus. The microbiota showed more microbial evenness during infection, and Bacteroidetes species were depleted. When seronegativity for the SARS-CoV-2 virus was reached, the microbiota was comparable with healthy controls [139]. Second, maternal microbiota changes affect an infant's microbiota composition [140]. Third, antibiotics taken during pregnancy alter maternal microbiota and are associated with the development of metabolic and allergic disorders later in childhood, including obesity and asthma [141]. In the context of existing information, maternal microbiota alterations likely affect human offspring's brain development.

Experimental studies on the impact of MIA on gut microbiome

Mazmanian's team reported first that MIA-impacted gut flora influences the severity of behavioral and neuropathological phenotypes in offspring by breaking the immune tolerance in the gut and activating the microbiota-gut-brain axis [132]. Oral administration of common commensals, Bacteroides fragilis, corrected gut permeability and microbial composition, improving communicative, stereotypic, anxiety-like, and sensorimotor behaviors [132].

A meta-analysis was performed to assess MIA's effect on microbiota and neurodevelopmental conditions in rodents. Combining the results of thirteen studies revealed that maternal microbiome disturbances affect the brain, as reflected by a decrease in offspring's sociability and an increase in stereotypic behaviors [142], which supports the validity of the concept.

Since indigenous spore-forming bacteria from the mouse and human microbiota promote serotonin biosynthesis from colonic enterochromaffin cells [143], MIA's impact on the serotonin pathway was tested by MacDowell *et al.* [144]. MIA reduced serotonin content in brain tissue and promoted changes in the expression of serotonin transporter, 5-HT2A, and 5-HT2C receptors. Long-term paliperidone treatment (a dopamine D2 and serotonin 5HT receptor antagonist) counteracted the MIA-induced changes [144]. These findings provide insight into mechanisms by which MIA's impact on the microbiota can affect the brain.

Microbiota can be altered by antibiotic usage. Except for a few, antibiotics have been considered safe for pregnant women. As we learn more about antibiotics' effects on microbiota, their safety may need to be re-assessed, and the benefit ratio may need to be considered individually. One of the safest antibiotics is penicillin. Administration at a low dose during the third trimester of

mouse pregnancy resulted in behavior changes of adult offspring [145-147]. The behavioral changes were sex-dependent. Female adult mice showed decreased anxiety patterns, while they had abnormal social behavior. The immune system was affected, as evidenced by a decrease in splenic FOXP3+ regulatory T cells, major players in preventing the development of autoimmunity [145].

In similar experimental conditions, Lebovitz *et al.* reported decreased expression of Cx3cr1 (a chemokine receptor for neuron-derived fractalkine) in the microglia of the prefrontal cortex [148]. In another report, dysbiosis-induced microglial expression of CxC3cr1 was restored by treating mice with oral Lactobacillus species [146].

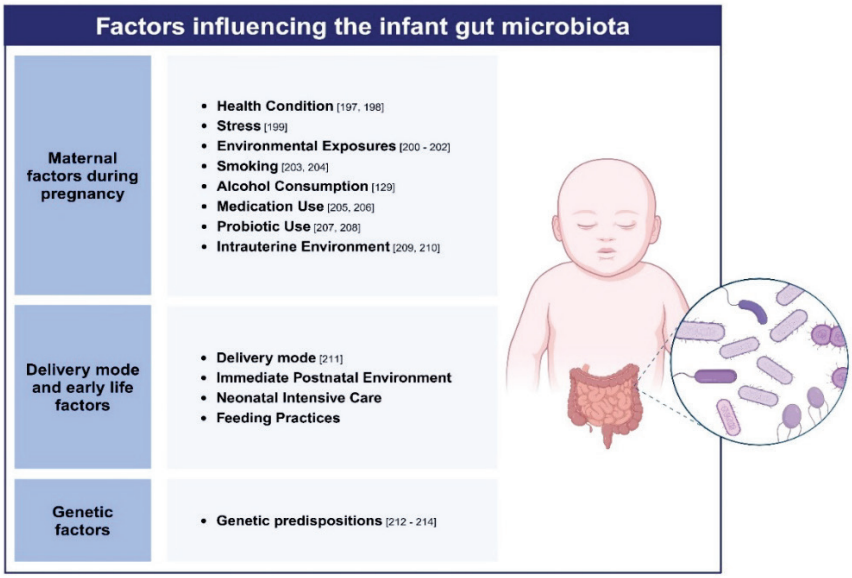

Fig. 3. Various factors that affect maternal and offspring microbiota, which may alter neurodevelopment in the child through gut-microbiota-brain axis. The picture was made with using a Biorender template.

Other causes of perinatal alterations of maternal or infant's microbiota

Both maternal and infant microbiota play crucial roles in shaping an infant's brain development, but their influences are intertwined and impact the infant at different stages. Maternal gut microbiota influences the immune environment and metabolic state during pregnancy, which can impact fetal brain development. During vaginal delivery, infants acquire microbiota from the mother's vaginal and intestinal flora, which is beneficial for early immune system development. After birth, the infant's microbiota continues to develop and is influenced by factors such as breastfeeding and environmental exposures. Breast milk contains beneficial bacteria and prebiotics that help shape the infant's gut microbiota. Figure 3 depicts conditions that can influence maternal or offspring microbiota.

MIA by SARS-CoV-2

World Health Organization declared global SARS-CoV-2 pandemic in March 2020 and announced its end in May 2023. Statistics vary about the number of women who were pregnant during the COVID-19 pandemic. It is, however, clear that SARS-CoV-2 infection worsened pregnancy outcomes. For example, a recent study reported outcomes of INTERCOVID study infections of the omicron variant of SARS-CoV-2 in pregnant women and their babies. Maternal, Neonatal, and Perinatal Morbidity and Mortality indices (MMI) relative risk were 1.16, 1.23, and 1.21, respectively. In unvaccinated women, Maternal MMI was 1.36; in women with severe COVID-19 symptoms, 2.51; and in unvaccinated women with severe COVID-19 symptoms, 2.88 [149].

Vertical transmission of the virus is believed to occur only rarely [150] and the human placenta has been considered a sound barrier that protects the fetus efficiently [151]. However, in a recent experimental study using mice that express human angiotensin-converting enzyme 2 that allows intracellular entry of SARS-CoV-2, the virus is found in the brain within 48 hours after the infection, indicating direct exposure of brain cells to the virus. All cell types within the brain (endothelium, neurons, glia, and astrocytes) can be infected [152]. Even if direct exposure

to human fetuses continues to be refuted, the placenta of infected women (including mild cases of SARS-CoV-2 infection) was reported to have vascular abnormalities (consistent with malperfusion) and villitis [153,154].

SARS-CoV-2 enters cells primarily via binding to Angiotensin Converting Enzyme-2 and Transmembrane Serine Protease 2. The virus also interacts with the host immune system through multiple TLRs, mainly TLR2, TLR4, TLR7, and TLR8, which were all shown before to play a role in MIA [23]. These interactions activate innate immune responses, including producing pro-inflammatory cytokines critical for controlling viral infection and contributing to MIA.

The abnormal fetus oxygenation, local inflammation, and the impact of SARS-CoV-2 on microbiota are all conditions, in which the neural development of the fetus may be affected. These mechanisms may be behind the findings in a recent retrospective study that examined one-year-old children exposed to SARS-CoV-2 in utero (confirmed by polymerase chain reaction test). The study revealed that exposure is associated with a higher rate of neurodevelopmental diagnoses, with an OR of 1.86. When the SARS-CoV-2 occurred in the third trimester, the OR was higher - 2.34 [155]. Another retrospective cohort examined electronic health records and uncovered that males but not females born to SARS-CoV-2 infected mothers were more likely to receive a neurodevelopmental diagnosis in the first 12 months after delivery [156].

While these findings will need additional validations, the existing data already warrant more experimental studies that can help explain histopathological mechanisms involved in the increased vulnerabilities to neurodevelopmental conditions and identify diagnostic markers applicable clinically. Meanwhile, professionals taking care of children exposed to SARS-CoV-2 in utero may closely monitor their development and support formulation of clinical practices where children with vulnerabilities receive more support, as it is the case with individuals at high risk for development of autism or schizophrenia where measures, such as diet, exercise [157,158] showed some positive outcomes.

Interactions between MIA and perinatal hypoxia

The impacts of perinatal hypoxia and MIA on the brain overlap in several key areas. Both conditions can lead to similar neurodevelopmental disruptions and are associated with increased risk for neuropsychiatric disorders, including schizophrenia. They both induce neuroinflammation, which involves microglia activation and release of pro-inflammatory cytokines in the developing brain [45,159]. Both conditions can lead to increased production of oxygen radical species and subsequent oxidative stress, which can cause cellular injury and impair neurodevelopmental processes, including synaptogenesis and myelinization [160,161]. Both perinatal hypoxia and MIA can cause epigenetic changes, such as DNA methylation and histone modification, which impact transcriptional programs involved in various developmental functions and adaptive responses [162-164] and may be dependent on severity of the stimulus [165]. Finally, both perinatal hypoxia and MIA alter the dopaminergic system, which is a crucial feature of schizophrenia [166]. To better understand pathways shared between perinatal hypoxia and MIA, we will need to await experimental evidence where MIA, due to the activation of different TLRs, is tested together with different degrees of perinatal hypoxia (including mild hypoxia). Such efforts can help develop biomarkers for new diagnostic panels, targeted interventions, and preventive strategies for at-risk populations.

Conclusions

This review underscores the roles of perinatal hypoxia, immune system activation, and microbiota in neurodevelopmental conditions, to which also belongs schizophrenia. Clinical and experimental studies demonstrate that these perinatal factors are associated with long-term changes in brain structure and function, as reflected in behavioral and neurotransmitter alterations observed also in schizophrenia. Sophisticated experimental models have been developed during the last two decades to address perinatal hypoxia and MIA but their cumulative effects are rarely studied together.

In light of the SARS-CoV-2 pandemic, these considerations may be needed for children exposed to SARS-CoV-2 in utero, particularly in situations where additional factors contributed to increased risk for asymptomatic brain tissue injury, including obstetric complications causing various degree of hypoxia, additional infection, maternal immune activation or psycho-social stress.

Limitations

One of the significant limitations of translational research on schizophrenia is the heterogeneity of the disease and the existence of subsets of patients that we are not yet able to distinguish objectively. Most existing studies approach schizophrenia as one condition that significantly limits the interpretation of data. The heterogeneity of schizophrenia stems from complex genetic backgrounds that make the susceptibility to environmental factors quite variable. In addition to the heterogeneous nature of schizophrenia, also ethical limitations exist that prevent access to the affected brain tissue.

Future directions

To enhance our understanding of schizophrenia development, using advanced animal models and testing more than one perinatal factor per experiment will be critical. The experimental studies linked to longitudinal clinical studies involving sufficient subjects and assessing patients multimodally will promote the translational value of such work. These efforts should identify objective biomarkers for subsets of patients and hopefully reveal objective biomarkers altered by perinatal factors that are sensitive enough to reveal even pathological processes not immediately evident clinically (e.g., sensory, motor or cognitive deficits) and that are possible to use in longitudinal monitoring during individual's development as they encounter further hits during their lives. That such goals are feasible is demonstrated by recent advances in other psychiatric conditions, namely Alzheimer disease, where a biomarker detectable in the blood was identified [167]. A better understanding of molecular mechanisms can lead to more effective individualized treatment. In addition, the focus of the research should not only be on the treatment of pathological status but on preventive measures that can limit the prevalence of neurodevelopmental conditions, including schizophrenia.

Conflict of Interest

There is no conflict of interest.

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References

1. Hampl V, Herget J. Perinatal hypoxia increases hypoxic pulmonary vasoconstriction in adult rats recovering from chronic exposure to hypoxia. Am Rev Respir Dis 1990;142:619-624. https://doi.org/10.1164/ajrccm/142.3.619
2. Vizek M, Dostal M, Soukupova D. Perinatal hypoxia suppresses immune response of adult rats. Physiol Res 1993;42:201-204.
3. Leckman JF, King RA, Gilbert DL, Coffey BJ, Singer HS, Dure LSt, Grantz H et al. Streptococcal upper respiratory tract infections and exacerbations of tic and obsessive-compulsive symptoms: a prospective longitudinal study. J Am Acad Child Adolesc Psychiatry 2011;50:108-18 e3. https://doi.org/10.1016/j.jaac.2010.10.011
4. Brown AS, Derkits EJ. Prenatal infection and schizophrenia: a review of epidemiologic and translational studies. Am J Psychiatry 2010;167:261-280. https://doi.org/10.1176/appi.ajp.2009.09030361

5. Mednick SA, Machon RA, Huttunen MO, Bonett D. Adult schizophrenia following prenatal exposure to an influenza epidemic. Arch Gen Psychiatry 1988;45:189-192. https://doi.org/10.1001/archpsyc.1988.01800260109013
6. Howes OD, Murray RM. Schizophrenia: an integrated sociodevelopmental-cognitive model. Lancet 2014;383:1677-1687. https://doi.org/10.1016/S0140-6736(13)62036-X
7. Casey C, Fullard JF, Sleator RD. Unravelling the genetic basis of Schizophrenia. Gene 2024;902:148198. https://doi.org/10.1016/j.gene.2024.148198
8. Taylor JL, Debost JPG, Morton SU, Wigdor EM, Heyne HO, Lal D, Howrigan DP et al. Paternal-age-related de novo mutations and risk for five disorders. Nat Commun 2019;10:3043. https://doi.org/10.1038/s41467-019-11039-6
9. Brown AS, Susser ES. Prenatal nutritional deficiency and risk of adult schizophrenia. Schizophr Bull 2008;34:1054-63. https://doi.org/10.1093/schbul/sbn096
10. Grover S, Varadharajan N, Venu S. Urbanization and psychosis: an update of recent evidence. Curr Opin Psychiatry 2024;37:191-201. https://doi.org/10.1097/YCO.0000000000000931
11. Robinson N, Ploner A, Muller-Eberstein R, Lichtenstein P, Kendler KS, Bergen SE. Migration and risk of schizophrenia and bipolar disorder: A Swedish national study. Schizophr Res 2023;260:160-7. https://doi.org/10.1016/j.schres.2023.08.022
12. Bilbo SD, Block CL, Bolton JL, Hanamsagar R, Tran PK. Beyond infection - Maternal immune activation by environmental factors, microglial development, and relevance for autism spectrum disorders. Exp Neurol 2018;299:241-51. https://doi.org/10.1016/j.expneurol.2017.07.002
13. Choi GB, Yim YS, Wong H, Kim S, Kim H, Kim SV, Hoeffer CA et al. The maternal interleukin-17a pathway in mice promotes autism-like phenotypes in offspring. Science 2016;351:933-9. https://doi.org/10.1126/science.aad0314
14. Parker-Athill E, Luo D, Bailey A, Giunta B, Tian J, Shytle RD, Murphy T et al. Flavonoids, a prenatal prophylaxis via targeting JAK2/STAT3 signaling to oppose IL-6/MIA associated autism. J Neuroimmunol 2009;217:20-7.10. https://doi.org/10.1016/j.jneuroim.2009.08.012
15. Patterson PH. Immune involvement in schizophrenia and autism: etiology, pathology and animal models. Behav Brain Res 2009;204:313-321. https://doi.org/10.1016/j.bbr.2008.12.016
16. Parboosing R, Bao Y, Shen L, Schaefer CA, Brown AS. Gestational influenza and bipolar disorder in adult offspring. JAMA Psychiatry 2013;70:677-685. https://doi.org/10.1001/jamapsychiatry.2013.896
17. Khan D, Fernando P, Cicvaric A, Berger A, Pollak A, Monje FJ, Pollak DD. Long-term effects of maternal immune activation on depression-like behavior in the mouse. Transl Psychiatry 2014;4:e363. https://doi.org/10.1038/tp.2013.132
18. Ronovsky M, Berger S, Molz B, Berger A, Pollak DD. Animal Models of Maternal Immune Activation in Depression Research. Curr Neuropharmacol 2016;14:688-704. https://doi.org/10.2174/1570159X14666151215095359
19. Ronovsky M, Berger S, Zambon A, Reisinger SN, Horvath O, Pollak A, Lindtner C et al. Maternal immune activation transgenerationally modulates maternal care and offspring depression-like behavior. Brain Behav Immun 2017;63:127-136. https://doi.org/10.1016/j.bbi.2016.10.016
20. Quagliato LA, de Matos U, Nardi AE. Maternal immune activation generates anxiety in offspring: A translational meta-analysis. Transl Psychiatry 2021;11:245. https://doi.org/10.1038/s41398-021-01361-3
21. Rosenberg JB, Richardt Mollegaard Jepsen J, Mohammadzadeh P, Sevelsted A, Vinding R, Sorensen ME, Horner D et al. Maternal inflammation during pregnancy is associated with risk of ADHD in children at age 10. Brain Behav Immun 2024;115:450-457. https://doi.org/10.1016/j.bbi.2023.10.023
22. Jones HF, Han VX, Patel S, Gloss BS, Soler N, Ho A, Sharma S et al. Maternal autoimmunity and inflammation are associated with childhood tics and obsessive-compulsive disorder: Transcriptomic data show common enriched innate immune pathways. Brain Behav Immun 2021;94:308-317. https://doi.org/10.1016/j.bbi.2020.12.035
23. Han VX, Patel S, Jones HF, Dale RC. Maternal immune activation and neuroinflammation in human neurodevelopmental disorders. Nat Rev Neurol 2021;17:564-579. https://doi.org/10.1038/s41582-021-00530-8

24. Corradini I, Focchi E, Rasile M, Morini R, Desiato G, Tomasoni R, Lizier M et al. Maternal Immune Activation Delays Excitatory-to-Inhibitory Gamma-Aminobutyric Acid Switch in Offspring. Biol Psychiatry 2018;83:680-691. https://doi.org/10.1016/j.biopsych.2017.09.030
25. Sun Y, Vestergaard M, Christensen J, Nahmias AJ, Olsen J. Prenatal exposure to maternal infections and epilepsy in childhood: a population-based cohort study. Pediatrics 2008;121:e1100-7. https://doi.org/10.1542/peds.2007-2316
26. Zager A, Peron JP, Mennecier G, Rodrigues SC, Aloia TP, Palermo-Neto J. Maternal immune activation in late gestation increases neuroinflammation and aggravates experimental autoimmune encephalomyelitis in the offspring. Brain Behav Immun 2015;43:159-171. https://doi.org/10.1016/j.bbi.2014.07.021
27. Knuesel I, Chicha L, Britschgi M, Schobel SA, Bodmer M, Hellings JA, Toovey S et al. Maternal immune activation and abnormal brain development across CNS disorders. Nat Rev Neurol 2014;10:643-660. https://doi.org/10.1038/nrneurol.2014.187
28. O'Hara R, Beaudreau SA, Gould CE, Froehlich W, Kraemer HC. Handling clinical comorbidity in randomized clinical trials in psychiatry. J Psychiatr Res 2017;86:26-33. https://doi.org/10.1016/j.jpsychires.2016.11.006
29. Collaborators GBDMD. Global, regional, and national burden of 12 mental disorders in 204 countries and territories, 1990-2019: a systematic analysis for the Global Burden of Disease Study 2019. Lancet Psychiatry 2022;9:137-150. https://doi.org/10.1016/S2215-0366(21)00395-3
30. Solmi M, Seitidis G, Mavridis D, Correll CU, Dragioti E, Guimond S, Tuominen L et al. Incidence, prevalence, and global burden of schizophrenia - data, with critical appraisal, from the Global Burden of Disease (GBD) 2019. Mol Psychiatry 2023;28:5319-27. https://doi.org/10.1038/s41380-023-02138-4
31. Kochhar SS, Mishra AK, Chadda RK, Sood M, Bhargava R. Psychosocial correlates of the experience of caregiving among caregivers of patients with schizophrenia. Cureus 2024;16:e58531. https://doi.org/10.7759/cureus.58531
32. Kretchy IA, Osafo J, Agyemang SA, Appiah B, Nonvignon J. Psychological burden and caregiver-reported non-adherence to psychotropic medications among patients with schizophrenia. Psychiatry Res 2018;259:289-94. https://doi.org/10.1016/j.psychres.2017.10.034
33. Martin-Carrasco M, Fernandez-Catalina P, Dominguez-Panchon AI, Goncalves-Pereira M, Gonzalez-Fraile E, Munoz-Hermoso P, Ballesteros J et al. A randomized trial to assess the efficacy of a psychoeducational intervention on caregiver burden in schizophrenia. Eur Psychiatry 2016;33:9-17. https://doi.org/10.1016/j.eurpsy.2016.01.003
34. Mittendorfer-Rutz E, Rahman S, Tanskanen A, Majak M, Mehtala J, Hoti F, Jedenius E et al. Burden for parents of patients with schizophrenia-a nationwide comparative study of parents of offspring with rheumatoid arthritis, multiple sclerosis, epilepsy, and healthy controls. Schizophr Bull 2019;45:794-803. https://doi.org/10.1093/schbul/sby130
35. Tessier A, Roger K, Gregoire A, Desnavailles P, Misdrahi D. Family psychoeducation to improve outcome in caregivers and patients with schizophrenia: a randomized clinical trial. Front Psychiatry 2023;14:1171661. https://doi.org/10.3389/fpsyt.2023.1171661
36. Lin C, Zhang X, Jin H. The societal cost of schizophrenia: an updated systematic review of cost-of-illness studies. Pharmacoeconomics 2023;41:139-53. https://doi.org/10.1007/s40273-022-01217-8
37. Jauhar S, Johnstone M, McKenna PJ. Schizophrenia. Lancet 2022;399:473-86. https://doi.org/10.1016/S0140-6736(21)01730-X
38. Riglin L, Collishaw S, Richards A, Thapar AK, Maughan B, O'Donovan MC, Thapar A. Schizophrenia risk alleles and neurodevelopmental outcomes in childhood: a population-based cohort study. Lancet Psychiatry 2017;4:57-62. https://doi.org/10.1016/S2215-0366(16)30406-0
39. Bouet V, Percelay S, Leroux E, Diarra B, Leger M, Delcroix N, Andrieux A et al. A new 3-hit mouse model of schizophrenia built on genetic, early and late factors. Schizophr Res 2021;228:519-528. https://doi.org/10.1016/j.schres.2020.11.043
40. Correll CU, Howes OD. Treatment-resistant schizophrenia: definition, predictors, and therapy options. J Clin Psychiatry 2021;82. https://doi.org/10.4088/JCP.MY20096AH1C

41. Messmer MF, Wilhelm EE, Shoulson I. I-SPY 2 breast cancer trial as a model for innovation in Alzheimer disease therapies. JAMA Neurol 2017;74:1027-8. https://doi.org/10.1001/jamaneurol.2017.1528
42. Howes OD, Cummings C, Chapman GE, Shatalina E. Neuroimaging in schizophrenia: an overview of findings and their implications for synaptic changes. Neuropsychopharmacology 2023;48:151-67. https://doi.org/10.1038/s41386-022-01426-x
43. Rund BR. The research evidence for schizophrenia as a neurodevelopmental disorder. Scand J Psychol 2018;59:49-58. https://doi.org/10.1111/sjop.12414
44. Wen J, Antoniades M, Yang Z, Hwang G, Skampardoni I, Wang R, Davatzikos C. Dimensional neuroimaging endophenotypes: neurobiological representations of disease heterogeneity through machine learning. Biol Psychiatry 2024;96:p564-584. https://doi.org/10.1016/j.biopsych.2024.04.017
45. Meyer U. Developmental neuroinflammation and schizophrenia. Prog Neuropsychopharmacol Biol Psychiatry 2013;42:20-34. https://doi.org/10.1016/j.pnpbp.2011.11.003
46. Meyer U, Feldon J. Epidemiology-driven neurodevelopmental animal models of schizophrenia. Prog Neurobiol 2010;90:285-326. https://doi.org/10.1016/j.pneurobio.2009.10.018
47. Powell SB, Swerdlow NR. The relevance of animal models of social isolation and social motivation for understanding schizophrenia: review and future directions. Schizophr Bull 2023;49:1112-1126. https://doi.org/10.1093/schbul/sbad098
48. Uliana DL, Diniz C, da Silva LA, Borges-Assis AB, Lisboa SF, Resstel LBM. Contextual fear expression engages a complex set of interactions between ventromedial prefrontal cortex cholinergic, glutamatergic, nitrergic and cannabinergic signaling. Neuropharmacology 2023;232:109538. https://doi.org/10.1016/ j.neuropharm.2023.109538
49. Harrison PJ. The neuropathology of schizophrenia. A critical review of the data and their interpretation. Brain 1999;122 (Pt 4):593-624. https://doi.org/10.1093/brain/122.4.593
50. Harrison PJ. Postmortem studies in schizophrenia. Dialogues Clin Neurosci 2000;2:349-357. https://doi.org/10.31887/DCNS.2000.2.4/pharrison
51. Glantz LA, Austin MC, Lewis DA. Normal cellular levels of synaptophysin mRNA expression in the prefrontal cortex of subjects with schizophrenia. Biol Psychiatry 2000;48:389-97. https://doi.org/10.1016/S0006-3223(00)00923-9
52. Glantz LA, Lewis DA. Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia. Arch Gen Psychiatry 2000;57:65-73. https://doi.org/10.1001/archpsyc.57.1.65
53. Goldman-Rakic PS, Selemon LD. Functional and anatomical aspects of prefrontal pathology in schizophrenia. Schizophr Bull 1997;23:437-458. https://doi.org/10.1093/schbul/23.3.437
54. Lewis DA, Glantz LA, Pierri JN, Sweet RA. Altered cortical glutamate neurotransmission in schizophrenia: evidence from morphological studies of pyramidal neurons. Ann N Y Acad Sci 2003;1003:102-12. https://doi.org/10.1196/annals.1300.007
55. Rajkowska G, Selemon LD, Goldman-Rakic PS. Neuronal and glial somal size in the prefrontal cortex: a postmortem morphometric study of schizophrenia and Huntington disease. Arch Gen Psychiatry 1998;55:215-24. https://doi.org/10.1001/archpsyc.55.3.215
56. Selemon LD, Goldman-Rakic PS. The reduced neuropil hypothesis: a circuit based model of schizophrenia. Biol Psychiatry 1999;45:17-25. https://doi.org/10.1016/S0006-3223(98)00281-9
57. Heckers S, Konradi C. GABAergic mechanisms of hippocampal hyperactivity in schizophrenia. Schizophr Res 2015;167:4-11. https://doi.org/10.1016/j.schres.2014.09.041
58. Danos P, Baumann B, Bernstein HG, Franz M, Stauch R, Northoff G, Krell D et al. Schizophrenia and anteroventral thalamic nucleus: selective decrease of parvalbumin-immunoreactive thalamocortical projection neurons. Psychiatry Res 1998;82:1-10. https://doi.org/10.1016/S0925-4927(97)00071-1
59. Holt DJ, Bachus SE, Hyde TM, Wittie M, Herman MM, Vangel M, Saper CB et al. Reduced density of cholinergic interneurons in the ventral striatum in schizophrenia: an in situ hybridization study. Biol Psychiatry 2005;58:408-16. https://doi.org/10.1016/j.biopsych.2005.04.007
60. Nasrallah HA, McCalley-Whitters M, Bigelow LB, Rauscher FP. A histological study of the corpus callosum in chronic schizophrenia. Psychiatry Res 1983;8:251-60. https://doi.org/10.1016/0165-1781(83)90013-6

61. Honea R, Crow TJ, Passingham D, Mackay CE. Regional deficits in brain volume in schizophrenia: a meta-analysis of voxel-based morphometry studies. Am J Psychiatry 2005;162:2233-45. https://doi.org/10.1176/appi.ajp.162.12.2233
62. Kuo SS, Pogue-Geile MF. Variation in fourteen brain structure volumes in schizophrenia: A comprehensive meta-analysis of 246 studies. Neurosci Biobehav Rev 2019;98:85-94. https://doi.org/10.1016/j.neubiorev.2018.12.030
63. Cropley VL, Klauser P, Lenroot RK, Bruggemann J, Sundram S, Bousman C, Pereira A et al. Accelerated Gray and White Matter Deterioration With Age in Schizophrenia. Am J Psychiatry 2017;174:286-295. https://doi.org/10.1176/appi.ajp.2016.16050610
64. Dong D, Wang Y, Chang X, Luo C, Yao D. Dysfunction of Large-Scale Brain Networks in Schizophrenia: A Meta-analysis of Resting-State Functional Connectivity. Schizophr Bull 2018;44:168-181. https://doi.org/10.1093/schbul/sbx034
65. Sun D, Phillips L, Velakoulis D, Yung A, McGorry PD, Wood SJ, van Erp TG et al. Progressive brain structural changes mapped as psychosis develops in 'at risk' individuals. Schizophr Res 2009;108:85-92. https://doi.org/10.1016/j.schres.2008.11.026
66. Gong Q, Lui S, Sweeney JA. A Selective Review of Cerebral Abnormalities in Patients With First-Episode Schizophrenia Before and After Treatment. Am J Psychiatry 2016;173:232-43. https://doi.org/10.1176/appi.ajp.2015.15050641
67. Delay J, Deniker P. Neuroleptic effects of chlorpromazine in therapeutics of neuropsychiatry. Int Rec Med Gen Pract Clin 1955;168:318-326.
68. Takesada M, Kakimoto Y, Sano I, Kaneko Z. 3,4-Dimethoxyphenylethylamine and Other Amines in the Urine of Schizophrenic Patients. Nature 1963;199:203-204. https://doi.org/10.1038/199203a0
69. Meador-Woodruff JH, Grandy DK, Van Tol HH, Damask SP, Little KY, Civelli O, Watson SJ, Jr. Dopamine receptor gene expression in the human medial temporal lobe. Neuropsychopharmacology 1994;10:239-248. https://doi.org/10.1038/npp.1994.27
70. Meador-Woodruff JH, Haroutunian V, Powchik P, Davidson M, Davis KL, Watson SJ. Dopamine receptor transcript expression in striatum and prefrontal and occipital cortex. Focal abnormalities in orbitofrontal cortex in schizophrenia. Arch Gen Psychiatry 1997;54:1089-1095. https://doi.org/10.1001/archpsyc.1997.01830240045007
71. Clinton SM, Meador-Woodruff JH. Thalamic dysfunction in schizophrenia: neurochemical, neuropathological, and *in vivo* imaging abnormalities. Schizophr Res 2004;69:237-253. https://doi.org/10.1016/j.schres.2003.09.017
72. Benjamin KJM, Chen Q, Jaffe AE, Stolz JM, Collado-Torres L, Huuki-Myers LA, Burke EE et al. Analysis of the caudate nucleus transcriptome in individuals with schizophrenia highlights effects of antipsychotics and new risk genes. Nat Neurosci 2022;25:1559-1568. https://doi.org/10.1038/s41593-022-01182-7
73. Seeman P, Niznik HB. Dopamine receptors and transporters in Parkinson's disease and schizophrenia. FASEB J 1990;4:2737-2744. https://doi.org/10.1096/fasebj.4.10.2197154
74. Dean B, Boer S, Gibbons A, Money T, Scarr E. Recent advances in postmortem pathology and neurochemistry in schizophrenia. Curr Opin Psychiatry 2009;22:154-160. https://doi.org/10.1097/YCO.0b013e328323d52e
75. Lehman AF, Lieberman JA, Dixon LB, McGlashan TH, Miller AL, Perkins DO, Kreyenbuhl J et al. Practice guideline for the treatment of patients with schizophrenia, second edition. Am J Psychiatry 2004;161:1-56.
76. Lieberman JA, Stroup TS, McEvoy JP, Swartz MS, Rosenheck RA, Perkins DO, Keefe RS et al. Effectiveness of antipsychotic drugs in patients with chronic schizophrenia. N Engl J Med 2005;353:1209-1223. https://doi.org/10.1056/NEJMoa051688
77. Stroup TS, Gerhard T, Crystal S, Huang C, Tan Z, Wall MM, Mathai C et al. Comparative Effectiveness of Adjunctive Psychotropic Medications in Patients With Schizophrenia. JAMA Psychiatry 2019;76:508-515. https://doi.org/10.1001/jamapsychiatry.2018.4489
78. Sullivan PF, Yao S, Hjerling-Leffler J. Schizophrenia genomics: genetic complexity and functional insights. Nat Rev Neurosci 2024;25,611-624. https://doi.org/10.1038/s41583-024-00837-7
79. Wray NR, Gottesman, II. Using summary data from the danish national registers to estimate heritabilities for schizophrenia, bipolar disorder, and major depressive disorder. Front Genet 2012;3:118. https://doi.org/10.3389/fgene.2012.00118

80. Chen L, Du Y, Hu Y, Li XS, Chen Y, Cheng Y. Whole-exome sequencing of individuals from an isolated population under extreme conditions implicates rare risk variants of schizophrenia. Transl Psychiatry 2024;14:267. https://doi.org/10.1038/s41398-024-02984-y
81. Schmidt-Kastner R, Guloksuz S, Kietzmann T, van Os J, Rutten BPF. Analysis of GWAS-Derived Schizophrenia Genes for Links to Ischemia-Hypoxia Response of the Brain. Front Psychiatry 2020;11:393. https://doi.org/10.3389/fpsyt.2020.00393
82. Hervoso JL, Amoah K, Dodson J, Choudhury M, Bhattacharya A, Quinones-Valdez G, Pasaniuc B et al. Splicing-specific transcriptome-wide association uncovers genetic mechanisms for schizophrenia. Am J Hum Genet 2024;111, 8,1573-1587. https://doi.org/10.1016/j.ajhg.2024.06.001
83. Parker N, Cheng W, Hindley GFL, O'Connell KS, Karthikeyan S, Holen B, Shadrin AA et al. Genetic overlap between global cortical brain structure, c-reactive protein, and white blood cell counts. Biol Psychiatry 2024;95:62-71. https://doi.org/10.1016/j.biopsych.2023.06.008
84. Gong W, Guo P, Li Y, Liu L, Yan R, Liu S, Wang S et al. Role of the gut-brain axis in the shared genetic etiology between gastrointestinal tract diseases and psychiatric disorders: a genome-wide pleiotropic analysis. JAMA Psychiatry 2023;80:360-370. https://doi.org/10.1001/jamapsychiatry.2022.4974
85. Handford HA. Brain hypoxia, minimal brain dysfunction, and schizophrenia. Am J Psychiatry 1975;132:192-194. https://doi.org/10.1176/ajp.132.2.192
86. Rosso IM, Cannon TD, Huttunen T, Huttunen MO, Lonnqvist J, Gasperoni TL. Obstetric risk factors for early-onset schizophrenia in a Finnish birth cohort. Am J Psychiatry 2000;157:801-807. https://doi.org/10.1176/appi.ajp.157.5.801
87. Cannon TD, Rosso IM, Bearden CE, Sanchez LE, Hadley T. A prospective cohort study of neurodevelopmental processes in the genesis and epigenesis of schizophrenia. Dev Psychopathol 1999;11:467-485. https://doi.org/10.1017/S0954579499002163
88. Dalman C, Thomas HV, David AS, Gentz J, Lewis G, Allebeck P. Signs of asphyxia at birth and risk of schizophrenia. Population-based case-control study. Br J Psychiatry 2001;179:403-408. https://doi.org/10.1192/bjp.179.5.403
89. Miller SP, Ferriero DM. From selective vulnerability to connectivity: insights from newborn brain imaging. Trends Neurosci 2009;32:496-505. https://doi.org/10.1016/j.tins.2009.05.010
90. Benes FM, Sorensen I, Bird ED. Reduced neuronal size in posterior hippocampus of schizophrenic patients. Schizophr Bull 1991;17:597-608. https://doi.org/10.1093/schbul/17.4.597
91. Van Erp TG, Saleh PA, Rosso IM, Huttunen M, Lonnqvist J, Pirkola T, Salonen O et al. Contributions of genetic risk and fetal hypoxia to hippocampal volume in patients with schizophrenia or schizoaffective disorder, their unaffected siblings, and healthy unrelated volunteers. Am J Psychiatry 2002;159:1514-1520. https://doi.org/10.1176/appi.ajp.159.9.1514
92. Nicodemus KK, Marenco S, Batten AJ, Vakkalanka R, Egan MF, Straub RE, Weinberger DR. Serious obstetric complications interact with hypoxia-regulated/vascular-expression genes to influence schizophrenia risk. Mol Psychiatry 2008;13:873-7. https://doi.org/10.1038/sj.mp.4002153
93. Cannon TD, Yolken R, Buka S, Torrey EF, Collaborative Study Group on the Perinatal Origins of Severe Psychiatric D. Decreased neurotrophic response to birth hypoxia in the etiology of schizophrenia. Biol Psychiatry 2008;64:797-802. https://doi.org/10.1016/j.biopsych.2008.04.012
94. Morikawa T, Manabe T, Ito Y, Yamada S, Yoshimi A, Nagai T, Ozaki N et al. The expression of HMGA1a is increased in lymphoblastoid cell lines from schizophrenia patients. Neurochem Int 2010;56:736-739. https://doi.org/10.1016/j.neuint.2010.03.011
95. Okazaki S, Boku S, Watanabe Y, Otsuka I, Horai T, Morikawa R, Kimura A et al. Polymorphisms in the hypoxia inducible factor binding site of the macrophage migration inhibitory factor gene promoter in schizophrenia. PLoS One 2022;17:e0265738. https://doi.org/10.1371/journal.pone.0265738
96. Barodia SK, Park SK, Ishizuka K, Sawa A, Kamiya A. Half-life of DISC1 protein and its pathological significance under hypoxia stress. Neurosci Res 2015;97:1-6. https://doi.org/10.1016/j.neures.2015.02.008
97. Ben-Ari Y. Excitatory actions of gaba during development: the nature of the nurture. Nat Rev Neurosci 2002;3:728-39. https://doi.org/10.1038/nrn920

98. Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron 1994;12:529-40. https://doi.org/10.1016/0896-6273(94)90210-0
99. Armit C, Richardson L, Hill B, Yang Y, Baldock RA. eMouseAtlas informatics: embryo atlas and gene expression database. Mamm Genome 2015;26:431-40. https://doi.org/10.1007/s00335-015-9596-5
100. Theiler K. *The House Mouse: Atlas of Embryonic Development*. Springer Berlin, Heidelberg 1989. https://doi.org/10.1007/978-3-642-88418-4
101. Vannucci SJ, Hagberg H. Hypoxia-ischemia in the immature brain. J Exp Biol 2004;207:3149-54. https://doi.org/10.1242/jeb.01064
102. Nadri C, Belmaker RH, Agam G. Oxygen restriction of neonate rats elevates neuregulin-1alpha isoform levels: possible relationship to schizophrenia. Neurochem Int 2007;51:447-450. https://doi.org/10.1016/j.neuint.2007.03.013
103. Molnar Z, Luhmann HJ, Kanold PO. Transient cortical circuits match spontaneous and sensory-driven activity during development. Science 2020;370. https://doi.org/10.1126/science.abb2153
104. El-Khodor BF, Boksa P. Birth insult increases amphetamine-induced behavioral responses in the adult rat. Neuroscience 1998;87:893-904. https://doi.org/10.1016/S0306-4522(98)00194-8
105. Vaillancourt C, Boksa P. Birth insult alters dopamine-mediated behavior in a precocial species, the guinea pig. Implications for schizophrenia. Neuropsychopharmacology 2000;23:654-66. https://doi.org/10.1016/S0893-133X(00)00164-0
106. Tejkalova H, Kaiser M, Klaschka J, Stastny F. Does neonatal brain ischemia induce schizophrenia-like behavior in young adult rats? Physiol Res 2007;56:815-23. https://doi.org/10.33549/physiolres.931056
107. Brake WG, Sullivan RM, Gratton A. Perinatal distress leads to lateralized medial prefrontal cortical dopamine hypofunction in adult rats. J Neurosci 2000;20:5538-43. https://doi.org/10.1523/JNEUROSCI.20-14-05538.2000
108. El-Khodor BF, Boksa P. Long-term reciprocal changes in dopamine levels in prefrontal cortex versus nucleus accumbens in rats born by Caesarean section compared to vaginal birth. Exp Neurol 1997;145:118-29. https://doi.org/10.1006/exnr.1997.6437
109. Laplante F, Brake WG, Chehab SL, Sullivan RM. Sex differences in the effects of perinatal anoxia on dopamine function in rats. Neurosci Lett 2012;506:89-93. https://doi.org/10.1016/j.neulet.2011.10.055
110. Papazisis G, Kallaras K, Kaiki-Astara A, Pourzitaki C, Tzachanis D, Dagklis T, Kouvelas D. Neuroprotection by lamotrigine in a rat model of neonatal hypoxic-ischaemic encephalopathy. Int J Neuropsychopharmacol 2008;11:321-329. https://doi.org/10.1017/S1461145707008012
111. Brown AS, Schaefer CA, Wyatt RJ, Goetz R, Begg MD, Gorman JM, Susser ES. Maternal exposure to respiratory infections and adult schizophrenia spectrum disorders: a prospective birth cohort study. Schizophr Bull 2000;26:287-295. https://doi.org/10.1093/oxfordjournals.schbul.a033453
112. Khandaker GM, Zimbron J, Dalman C, Lewis G, Jones PB. Childhood infection and adult schizophrenia: a meta-analysis of population-based studies. Schizophr Res 2012;139:161-168. https://doi.org/10.1016/j.schres.2012.05.023
113. He H, Yu Y, Liew Z, Gissler M, Laszlo KD, Valdimarsdottir UA, Zhang J, et al. Association of maternal autoimmune diseases with risk of mental disorders in offspring in Denmark. JAMA Netw Open 2022;5:e227503. https://doi.org/10.1001/jamanetworkopen.2022.7503
114. Buka SL, Tsuang MT, Torrey EF, Klebanoff MA, Bernstein D, Yolken RH. Maternal infections and subsequent psychosis among offspring. Arch Gen Psychiatry 2001;58:1032-7. https://doi.org/10.1001/archpsyc.58.11.1032
115. Brown AS, Begg MD, Gravenstein S, Schaefer CA, Wyatt RJ, Bresnahan M, Babulas VP et al. Serologic evidence of prenatal influenza in the etiology of schizophrenia. Arch Gen Psychiatry 2004;61:774-780. https://doi.org/10.1001/archpsyc.61.8.774
116. Xiao J, Buka SL, Cannon TD, Suzuki Y, Viscidi RP, Torrey EF, Yolken RH. Serological pattern consistent with infection with type I Toxoplasma gondii in mothers and risk of psychosis among adult offspring. Microbes Infect 2009;11:1011-1018. https://doi.org/10.1016/j.micinf.2009.07.007

117. Lee YH, Cherkerzian S, Seidman LJ, Papandonatos GD, Savitz DA, Tsuang MT, Goldstein JM et al. Maternal bacterial infection during pregnancy and offspring risk of psychotic disorders: Variation by severity of infection and offspring sex. Am J Psychiatry 2020;177:66-75. https://doi.org/10.1176/appi.ajp.2019.18101206
118. Buchsbaum MS. The frontal lobes, basal ganglia, and temporal lobes as sites for schizophrenia. Schizophr Bull 1990;16:379-389. https://doi.org/10.1093/schbul/16.3.379
119. Soares JC, Innis RB. Neurochemical brain imaging investigations of schizophrenia. Biol Psychiatry 1999;46:600-615. https://doi.org/10.1016/S0006-3223(99)00015-3
120. Supprian T, Ulmar G, Bauer M, Schuler M, Puschel K, Retz-Junginger P, Schmitt HP et al. Cerebellar vermis area in schizophrenic patients - a post-mortem study. Schizophr Res 2000;42:19-28. https://doi.org/10.1016/S0920-9964(99)00103-6
121. van Hooijdonk CFM, van der Pluijm M, Bosch I, van Amelsvoort T, Booij J, de Haan L, Selten JP et al. The substantia nigra in the pathology of schizophrenia: A review on post-mortem and molecular imaging findings. Eur Neuropsychopharmacol 2023;68:57-77. https://doi.org/10.1016/j.euroneuro.2022.12.008
122. van Kesteren CF, Gremmels H, de Witte LD, Hol EM, Van Gool AR, Falkai PG, Kahn RS et al. Immune involvement in the pathogenesis of schizophrenia: a meta-analysis on postmortem brain studies. Transl Psychiatry 2017;7:e1075. https://doi.org/10.1038/tp.2017.4
123. Jutla A, Foss-Feig J, Veenstra-VanderWeele J. Autism spectrum disorder and schizophrenia: An updated conceptual review. Autism Res 2022;15:384-412. https://doi.org/10.1002/aur.2659
124. Fatemi SH, Earle J, Kanodia R, Kist D, Emamian ES, Patterson PH, Shi L et al. Prenatal viral infection leads to pyramidal cell atrophy and macrocephaly in adulthood: implications for genesis of autism and schizophrenia. Cell Mol Neurobiol 2002;22:25-33. https://doi.org/10.1023/A:1015337611258
125. Shi L, Fatemi SH, Sidwell RW, Patterson PH. Maternal influenza infection causes marked behavioral and pharmacological changes in the offspring. J Neurosci 2003;23:297-302. https://doi.org/10.1523/JNEUROSCI.23-01-00297.2003
126. Smith SE, Li J, Garbett K, Mirnics K, Patterson PH. Maternal immune activation alters fetal brain development through interleukin-6. J Neurosci 2007;27:10695-702. https://doi.org/10.1523/JNEUROSCI.2178-07.2007
127. Han VX, Jones HF, Patel S, Mohammad SS, Hofer MJ, Alshammery S, Maple-Brown E et al. Emerging evidence of Toll-like receptors as a putative pathway linking maternal inflammation and neurodevelopmental disorders in human offspring: A systematic review. Brain Behav Immun 2022;99:91-105. https://doi.org/10.1016/j.bbi.2021.09.009
128. Hsiao EY, Patterson PH. Placental regulation of maternal-fetal interactions and brain development. Dev Neurobiol 2012;72:1317-1326. https://doi.org/10.1002/dneu.22045
129. Kim S, Kim H, Yim YS, Ha S, Atarashi K, Tan TG, Longman RS et al. Maternal gut bacteria promote neurodevelopmental abnormalities in mouse offspring. Nature 2017;549:528-32. https://doi.org/10.1038/nature23910
130. Reed MD, Yim YS, Wimmer RD, Kim H, Ryu C, Welch GM, Andina M et al. IL-17a promotes sociability in mouse models of neurodevelopmental disorders. Nature 2020;577:249-53. https://doi.org/10.1038/s41586-019-1843-6
131. Giovanoli S, Engler H, Engler A, Richetto J, Voget M, Willi R, Winter C et al. Stress in puberty unmasks latent neuropathological consequences of prenatal immune activation in mice. Science 2013;339:1095-1099. https://doi.org/10.1126/science.1228261
132. Hsiao EY, McBride SW, Hsien S, Sharon G, Hyde ER, McCue T, Codelli JA et al. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. Cell 2013;155:1451-63. https://doi.org/10.1016/j.cell.2013.11.024
133. Perez-Morales M, Bello-Medina PC, Gonzalez-Franco DA, Diaz-Cintra S, Garcia-Mena J, Pacheco-Lopez G, Neuro-Psycho-Biota C. Steering the Microbiota-Gut-Brain Axis by Antibiotics to Model Neuro-Immune-Endocrine Disorders. Neuroimmunomodulation 2024;31:89-101. https://doi.org/10.1159/000538927
134. Dinan TG, Cryan JF. The microbiome-gut-brain axis in health and disease. Gastroenterol Clin North Am 2017;46:77-89. https://doi.org/10.1016/j.gtc.2016.09.007

135. Agus A, Planchais J, Sokol H. Gut microbiota regulation of tryptophan metabolism in health and disease. Cell Host Microbe 2018;23:716-724. https://doi.org/10.1016/j.chom.2018.05.003
136. Gershon MD, Tack J. The serotonin signaling system: from basic understanding to drug development for functional GI disorders. Gastroenterology 2007;132:397-414. https://doi.org/10.1053/j.gastro.2006.11.002
137. Zheng P, Zeng B, Liu M, Chen J, Pan J, Han Y, Liu Y et al. The gut microbiome from patients with schizophrenia modulates the glutamate-glutamine-GABA cycle and schizophrenia-relevant behaviors in mice. Sci Adv 2019;5:eaau8317. https://doi.org/10.1126/sciadv.aau8317
138. Li S, Song J, Ke P, Kong L, Lei B, Zhou J, Huang Y et al. The gut microbiome is associated with brain structure and function in schizophrenia. Sci Rep 2021;11:9743. https://doi.org/10.1038/s41598-021-89166-8
139. Kim HN, Joo EJ, Lee CW, Ahn KS, Kim HL, Park DI, Park SK. Reversion of gut microbiota during the recovery phase in patients with asymptomatic or mild COVID-19: Longitudinal Study. Microorganisms 2021;9:1237. https://doi.org/10.3390/microorganisms9061237
140. Lundgren SN, Madan JC, Emond JA, Morrison HG, Christensen BC, Karagas MR, Hoen AG. Maternal diet during pregnancy is related with the infant stool microbiome in a delivery mode-dependent manner. Microbiome 2018;6:109. https://doi.org/10.1186/s40168-018-0490-8
141. Isaevska E, Popovic M, Pizzi C, Fiano V, Rusconi F, Merletti F, Richiardi L et al. Maternal antibiotic use and vaginal infections in the third trimester of pregnancy and the risk of obesity in preschool children. Pediatr Obes 2020;15:e12632. https://doi.org/10.1111/ijpo.12632
142. Hassib L, de Oliveira CL, Rouvier GA, Kanashiro A, Guimaraes FS, Ferreira FR. Maternal microbiome disturbance induces deficits in the offspring's behaviors: a systematic review and meta-analysis. Gut Microbes 2023;15:2226282. https://doi.org/10.1080/19490976.2023.2226282
143. Yano JM, Yu K, Donaldson GP, Shastri GG, Ann P, Ma L, Nagler CR et al. Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. Cell 2015;161:264-276. https://doi.org/10.1016/j.cell.2015.02.047
144. MacDowell KS, Munarriz-Cuezva E, Meana JJ, Leza JC, Ortega JE. Paliperidone reversion of maternal immune activation-induced changes on brain serotonin and kynurenine pathways. Front Pharmacol 2021;12:682602. https://doi.org/10.3389/fphar.2021.682602
145. Champagne-Jorgensen K, Mian MF, Kay S, Hanani H, Ziv O, McVey Neufeld KA, Koren O et al. Prenatal low-dose penicillin results in long-term sex-specific changes to murine behaviour, immune regulation, and gut microbiota. Brain Behav Immun 2020;84:154-63. https://doi.org/10.1016/j.bbi.2019.11.020
146. Leclercq S, Mian FM, Stanisz AM, Bindels LB, Cambier E, Ben-Amram H, Koren O et al. Low-dose penicillin in early life induces long-term changes in murine gut microbiota, brain cytokines and behavior. Nat Commun 2017;8:15062. https://doi.org/10.1038/ncomms15062
147. Tejkalova H, Jakob L, Kvasnova S, Klaschka J, Sechovcova H, Mrazek J, Palenicek T et al. The influence of antibiotic treatment on the behavior and gut microbiome of adult rats neonatally insulted with lipopolysaccharide. Heliyon 2023;9:e15417. https://doi.org/10.1016/j.heliyon.2023.e15417
148. Lebovitz Y, Kowalski EA, Wang X, Kelly C, Lee M, McDonald V, Ward R et al. Lactobacillus rescues postnatal neurobehavioral and microglial dysfunction in a model of maternal microbiome dysbiosis. Brain Behav Immun 2019;81:617-629. https://doi.org/10.1016/j.bbi.2019.07.025
149. Villar J, Soto Conti CP, Gunier RB, Ariff S, Craik R, Cavoretto PI, Rauch S et al. Pregnancy outcomes and vaccine effectiveness during the period of omicron as the variant of concern, INTERCOVID-2022: a multinational, observational study. Lancet 2023;401:447-57. https://doi.org/10.1016/S0140-6736(22)02467-9
150. Kotlyar AM, Grechukhina O, Chen A, Popkhadze S, Grimshaw A, Tal O, Taylor HS et al. Vertical transmission of coronavirus disease 2019: a systematic review and meta-analysis. Am J Obstet Gynecol 2021;224:35-53e3. https://doi.org/10.1016/j.ajog.2020.07.049
151. Kim EH, Kim YI, Jang SG, Im M, Jeong K, Choi YK, Han HJ. Antiviral effects of human placenta hydrolysate (Laennec((R))) against SARS-CoV-2 in vitro and in the ferret model. J Microbiol 2021;59:1056-62. https://doi.org/10.1007/s12275-021-1367-2
152. McMahon CL, Castro J, Silvas J, Muniz Perez A, Estrada M, Carrion R, Jr., Hsieh J. Fetal brain vulnerability to SARS-CoV-2 infection. Brain Behav Immun 2023;112:188-205. https://doi.org/10.1016/j.bbi.2023.06.015

153. Patberg ET, Adams T, Rekawek P, Vahanian SA, Akerman M, Hernandez A, Rapkiewicz AV et al. Coronavirus disease 2019 infection and placental histopathology in women delivering at term. Am J Obstet Gynecol 2021;224:382 e1- e18. https://doi.org/10.1016/j.ajog.2020.10.020
154. Shanes ED, Mithal LB, Otero S, Azad HA, Miller ES, Goldstein JA. Placental pathology in COVID-19. Am J Clin Pathol. 2020;154:23-32. https://doi.org/10.1093/ajcp/aqaa089
155. Edlow AG, Castro VM, Shook LL, Kaimal AJ, Perlis RH. Neurodevelopmental Outcomes at 1 Year in Infants of Mothers Who Tested Positive for SARS-CoV-2 During Pregnancy. JAMA Netw Open 2022;5:e2215787. https://doi.org/10.1001/jamanetworkopen.2022.15787
156. Edlow AG, Castro VM, Shook LL, Haneuse S, Kaimal AJ, Perlis RH. Sex-specific neurodevelopmental outcomes in offspring of mothers with SARS-CoV-2 in pregnancy: an electronic health records cohort. medRxiv 2022. https://doi.org/10.1101/2022.11.18.22282448
157. Fernandez-Abascal B, Suarez-Pinilla P, Cobo-Corrales C, Crespo-Facorro B, Suarez-Pinilla M. In- and outpatient lifestyle interventions on diet and exercise and their effect on physical and psychological health: a systematic review and meta-analysis of randomised controlled trials in patients with schizophrenia spectrum disorders and first episode of psychosis. Neurosci Biobehav Rev 2021;125:535-68. https://doi.org/10.1016/j.neubiorev.2021.01.005
158. Firth J, Cotter J, Elliott R, French P, Yung AR. A systematic review and meta-analysis of exercise interventions in schizophrenia patients. Psychol Med 2015;45:1343-61. https://doi.org/10.1017/S0033291714003110
159. Allswede DM, Buka SL, Yolken RH, Torrey EF, Cannon TD. Elevated maternal cytokine levels at birth and risk for psychosis in adult offspring. Schizophr Res 2016;172:41-5. https://doi.org/10.1016/j.schres.2016.02.022
160. Swanepoel T, Moller M, Harvey BH. N-acetyl cysteine reverses bio-behavioural changes induced by prenatal inflammation, adolescent methamphetamine exposure and combined challenges. Psychopharmacology (Berl) 2018;235:351-368. https://doi.org/10.1007/s00213-017-4776-5
161. Thordstein M, Bagenholm R, Thiringer K, Kjellmer I. Scavengers of free oxygen radicals in combination with magnesium ameliorate perinatal hypoxic-ischemic brain damage in the rat. Pediatr Res 1993;34:23-26. https://doi.org/10.1203/00006450-199307000-00006
162. Palma-Gudiel H, Eixarch E, Crispi F, Moran S, Zannas AS, Fananas L. Prenatal adverse environment is associated with epigenetic age deceleration at birth and hypomethylation at the hypoxia-responsive EP300 gene. Clin Epigenetics 2019;11:73. https://doi.org/10.1186/s13148-019-0674-5
163. Su Y, Lian J, Chen S, Zhang W, Deng C. Epigenetic histone acetylation modulating prenatal Poly I:C induced neuroinflammation in the prefrontal cortex of rats: a study in a maternal immune activation model. Front Cell Neurosci 2022;16:1037105. https://doi.org/10.3389/fncel.2022.1037105
164. Labouesse MA, Dong E, Grayson DR, Guidotti A, Meyer U. Maternal immune activation induces GAD1 and GAD2 promoter remodeling in the offspring prefrontal cortex. Epigenetics 2015;10:1143-1155. https://doi.org/10.1080/15592294.2015.1114202
165. Xu Y, Tian Y, Wang Y, Xu L, Song G, Wu Q, Wang W et al. Exosomes derived from astrocytes after oxygen-glucose deprivation promote differentiation and migration of oligodendrocyte precursor cells in vitro. Mol Biol Rep 2021;48:5473-84. https://doi.org/10.1007/s11033-021-06557-w
166. Howes OD, Kapur S. The dopamine hypothesis of schizophrenia: version III--the final common pathway. Schizophr Bull 2009;35:549-562. https://doi.org/10.1093/schbul/sbp006
167. Palmqvist S, Tideman P, Mattsson-Carlgren N, Schindler SE, Smith R, Ossenkoppele R, Calling S et al. Blood biomarkers to detect alzheimer disease in primary care and secondary care. JAMA 2024. https://doi.org/10.1001/jama.2024.13855
168. Wolff AR, Cheyne KR, Bilkey DK. Behavioural deficits associated with maternal immune activation in the rat model of schizophrenia. Behav Brain Res 2011;225:382-387. https://doi.org/10.1016/j.bbr.2011.07.033
169. Hemmerle AM, Ahlbrand R, Bronson SL, Lundgren KH, Richtand NM, Seroogy KB. Modulation of schizophrenia-related genes in the forebrain of adolescent and adult rats exposed to maternal immune activation. Schizophr Res 2015;168:411-420. https://doi.org/10.1016/j.schres.2015.07.006

170. Li Q, Cheung C, Wei R, Hui ES, Feldon J, Meyer U, Chung S et al. Prenatal immune challenge is an environmental risk factor for brain and behavior change relevant to schizophrenia: evidence from MRI in a mouse model. PLoS One 2009;4:e6354. https://doi.org/10.1371/journal.pone.0006354
171. Coleman MT, Rund DA. Nonobstetric conditions causing hypoxia during pregnancy: asthma and epilepsy. Am J Obstet Gynecol 1997;177:1-7. https://doi.org/10.1016/S0002-9378(97)70429-0
172. Cousins L. Fetal oxygenation, assessment of fetal well-being, and obstetric management of the pregnant patient with asthma. J Allergy Clin Immunol 1999;103:S343-9. https://doi.org/10.1016/S0091-6749(99)70260-5
173. Chen YH, Keller J, Wang IT, Lin CC, Lin HC. Pneumonia and pregnancy outcomes: a nationwide population-based study. Am J Obstet Gynecol 2012;207:288 e1-7. https://doi.org/10.1016/j.ajog.2012.08.023
174. Krampl E. Pregnancy at high altitude. Ultrasound Obstet Gynecol 2002;19:535-539. https://doi.org/10.1046/j.1469-0705.2002.00738.x
175. Woodman AG, Care AS, Mansour Y, Cherak SJ, Panahi S, Gragasin FS, Bourque SL. Modest and Severe Maternal Iron Deficiency in Pregnancy are Associated with Fetal Anaemia and Organ-Specific Hypoxia in Rats. Sci Rep 2017;7:46573. https://doi.org/10.1038/srep46573
176. Tong W, Giussani DA. Preeclampsia link to gestational hypoxia. J Dev Orig Health Dis 2019;10:322-333. https://doi.org/10.1017/S204017441900014X
177. Habek D, Habek JC, Ivanisevic M, Djelmis J. Fetal tobacco syndrome and perinatal outcome. Fetal Diagn Ther 2002;17:367-71. https://doi.org/10.1159/000065387
178. Socol ML, Manning FA, Murata Y, Druzin ML. Maternal smoking causes fetal hypoxia: experimental evidence. Am J Obstet Gynecol 1982;142:214-8. https://doi.org/10.1016/S0002-9378(16)32339-0
179. Saha PS, Mayhan WG. Prenatal exposure to alcohol: mechanisms of cerebral vascular damage and lifelong consequences. Adv Drug Alcohol Res 2022;2:10818. https://doi.org/10.3389/adar.2022.10818
180. Bosco C, Diaz E. Placental hypoxia and foetal development versus alcohol exposure in pregnancy. Alcohol Alcohol 2012;47:109-117. https://doi.org/10.1093/alcalc/agr166
181. Teramo K, Klemetti M, Tikkanen M, Nuutila M. [Maternal diabetes and fetal hypoxia]. Duodecim 2013;129:228-234.
182. Hutter D, Kingdom J, Jaeggi E. Causes and mechanisms of intrauterine hypoxia and its impact on the fetal cardiovascular system: a review. Int J Pediatr 2010;2010:401323. https://doi.org/10.1155/2010/401323
183. Wray S, Alruwaili M, Prendergast C. Hypoxia and reproductive health: Hypoxia and labour. Reproduction 2021;161:F67-F80. https://doi.org/10.1530/REP-20-0327
184. Acharya A, Swain B, Pradhan S, Jena PK, Mohakud NK, Swain A, Mohanty N. Clinico-Biochemical Correlation in Birth Asphyxia and Its Effects on Outcome. Cureus 2020;12:e11407. https://doi.org/10.7759/cureus.11407
185. Peebles DM, Spencer JA, Edwards AD, Wyatt JS, Reynolds EO, Cope M, Delpy DT. Relation between frequency of uterine contractions and human fetal cerebral oxygen saturation studied during labour by near infrared spectroscopy. Br J Obstet Gynaecol 1994;101:44-48. https://doi.org/10.1111/j.1471-0528.1994.tb13008.x
186. Boushra M, Stone A, Rathbun KM. *Umbilical Cord Prolapse*. 2023. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024.
187. Peesay M. Nuchal cord and its implications. Matern Health Neonatol Perinatol 2017;3:28. https://doi.org/10.1186/s40748-017-0068-7
188. Gaikwad V, Yalla S, Salvi P. True Knot of the Umbilical Cord and Associated Adverse Perinatal Outcomes: A Case Series. Cureus 2023;15:e35377. https://doi.org/10.7759/cureus.35377
189. Matsuda Y, Ogawa M, Konno J, Mitani M, Matsui H. Prediction of fetal acidemia in placental abruption. BMC Pregnancy Childbirth 2013;13:156. https://doi.org/10.1186/1471-2393-13-156
190. Jenabi E, Bashirian S, Khoshravesh S. The association between of placenta previa and congenital abnormalities: a systematic review and network meta-analysis. BMC Pediatr 2023;23:606. https://doi.org/10.1186/s12887-023-04433-z
191. Johnson N, van Oudgaarden E, Montague I, McNamara H. The effect of oxytocin-induced hyperstimulation on fetal oxygen. Br J Obstet Gynaecol 1994;101:805-7. https://doi.org/10.1111/j.1471-0528.1994.tb11951.x

192. Sweet DG, Carnielli VP, Greisen G, Hallman M, Klebermass-Schrehof K, Ozek E, Te Pas A et al. European Consensus Guidelines on the Management of Respiratory Distress Syndrome: 2022 Update. Neonatology 2023;120:3-23. https://doi.org/10.1159/000528914
193. Monfredini C, Cavallin F, Villani PE, Paterlini G, Allais B, Trevisanuto D. Meconium Aspiration Syndrome: A Narrative Review. Children (Basel) 2021;8:230. https://doi.org/10.3390/children8030230
194. Ottolenghi S, Milano G, Cas MD, Findley TO, Paroni R, Corno AF. Can Erythropoietin Reduce Hypoxemic Neurological Damages in Neonates With Congenital Heart Defects? Front Pharmacol 2021;12:770590. https://doi.org/10.3389/fphar.2021.770590
195. Yadav P, Yadav SK. Progress in Diagnosis and Treatment of Neonatal Sepsis: A Review Article. JNMA J Nepal Med Assoc 2022;60:318-24.10.31729/jnma.7324. https://doi.org/10.31729/jnma.7324
196. Nandula PS, Shah SD: Persistent Pulmonary Hypertension of the Newborn. In: StatPearls. (eds), Treasure Island (FL), 2024.
197. Soderborg TK, Carpenter CM, Janssen RC, Weir TL, Robertson CE, Ir D, Young BE et al. Gestational Diabetes Is Uniquely Associated With Altered Early Seeding of the Infant Gut Microbiota. Front Endocrinol (Lausanne) 2020;11:603021. https://doi.org/10.3389/fendo.2020.603021
198. Lemas DJ, Klimentidis YC, Aslibekyan S, Wiener HW, O'Brien DM, Hopkins SE, Stanhope KL et al. Polymorphisms in stearoyl coa desaturase and sterol regulatory element binding protein interact with N-3 polyunsaturated fatty acid intake to modify associations with anthropometric variables and metabolic phenotypes in Yup'ik people. Mol Nutr Food Res 2016;60:2642-53. https://doi.org/10.1002/mnfr.201600170
199. Mepham J, Nelles-McGee T, Andrews K, Gonzalez A. Exploring the effect of prenatal maternal stress on the microbiomes of mothers and infants: A systematic review. Dev Psychobiol 2023;65:e22424. https://doi.org/10.1002/dev.22424
200. Naspolini NF, Meyer A, Moreira JC, Sun H, Froes-Asmus CIR, Dominguez-Bello MG. Environmental pollutant exposure associated with altered early-life gut microbiome: Results from a birth cohort study. Environ Res 2022;205:112545. https://doi.org/10.1016/j.envres.2021.112545
201. Banerjee S, Suter MA, Aagaard KM. Interactions between Environmental Exposures and the Microbiome: Implications for Fetal Programming. Curr Opin Endocr Metab Res 2020;13:39-48. https://doi.org/10.1016/j.coemr.2020.09.003
202. Zordao OP, Campolim CM, Yariwake VY, Castro G, Ferreira CKO, Santos A, Norberto S et al. Maternal exposure to air pollution alters energy balance transiently according to gender and changes gut microbiota. Front Endocrinol (Lausanne) 2023;14:1069243. https://doi.org/10.3389/fendo.2023.1069243
203. McLean C, Jun S, Kozyrskyj A. Impact of maternal smoking on the infant gut microbiota and its association with child overweight: a scoping review. World J Pediatr 2019;15:341-9. https://doi.org/10.1007/s12519-019-00278-8
204. Peng Y, Tun HM, Ng SC, Wai HK, Zhang X, Parks J, Field CJ et al. Maternal smoking during pregnancy increases the risk of gut microbiome-associated childhood overweight and obesity. Gut Microbes 2024;16:2323234. https://doi.org/10.1080/19490976.2024.2323234
205. Huang H, Jiang J, Wang X, Jiang K, Cao H. Exposure to prescribed medication in early life and impacts on gut microbiota and disease development. EClinicalMedicine 2024;68:102428. https://doi.org/10.1016/j.eclinm.2024.102428
206. Morreale C, Giaroni C, Baj A, Folgori L, Barcellini L, Dhami A, Agosti M et al. Effects of Perinatal Antibiotic Exposure and Neonatal Gut Microbiota. Antibiotics (Basel) 2023;12:258. https://doi.org/10.3390/antibiotics12020258
207. Cuinat C, Stinson SE, Ward WE, Comelli EM. Maternal Intake of Probiotics to Program Offspring Health. Curr Nutr Rep 2022;11:537-562. https://doi.org/10.1007/s13668-022-00429-w
208. Sanz Y. Gut microbiota and probiotics in maternal and infant health. Am J Clin Nutr 2011;94:2000S-5S. https://doi.org/10.3945/ajcn.110.001172
209. Yang J, Hou L, Wang J, Xiao L, ZhangJ, Yin N, Yao S et al. Unfavourable intrauterine environment contributes to abnormal gut microbiome and metabolome in twins. Gut 2022;71:2451-2462. https://doi.org/10.1136/gutjnl-2021-326482

210. Jeong S. Factors influencing development of the infant microbiota: from prenatal period to early infancy. Clin Exp Pediatr 2022;65:439-447. https://doi.org/10.3345/cep.2021.00955
211. Reyman M, van Houten MA, van Baarle D, Bosch A, Man WH, Chu M, Arp K et al. Impact of delivery mode-associated gut microbiota dynamics on health in the first year of life. Nat Commun 2019;10:4997. https://doi.org/10.1038/s41467-019-13014-7
212. Palmeira O, Matos LRB, Naslavsky MS, Bueno HMS, Soler JP, Setubal JC, Zatz M. Longitudinal 16S rRNA gut microbiota data of infant triplets show partial susceptibility to host genetics. iScience 2022;25:103861. https://doi.org/10.1016/j.isci.2022.103861
213. Matsuki T, Yahagi K, Mori H, Matsumoto H, Hara T, Tajima S, Ogawa E et al. A key genetic factor for fucosyllactose utilization affects infant gut microbiota development. Nat Commun 2016;7:11939. https://doi.org/10.1038/ncomms11939
214. Qin Y, Havulinna AS, Liu Y, Jousilahti P, Ritchie SC, Tokolyi A, Sanders JG et al. Combined effects of host genetics and diet on human gut microbiota and incident disease in a single population cohort. Nat Genet 2022;54:134-42. https://doi.org/10.1038/s41588-021-00991-z