

# Gene Expression in Patients With Abdominal Aortic Aneurysm – More Than Immunological Mechanisms Involved

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## Summary

Abdominal aortic aneurysm (AAA) is a serious condition of unclear pathogenesis and progression. Two samples were collected from 48 patients during AAA surgery. One sample was collected from the aneurysm, the other from the aneurysm proximal neck where the tissue did not exhibit any aneurysmal changes. Subsequently, gene expression profiles using microarrays (Illumina) were compared in RNA extracted from the samples. Overall, 2,185 genes were found to be upregulated and 2,100 downregulated; from which 158 genes had a different expression with  $FDR < 0.05$  (False Discovery Rate) and  $FC \geq 2$  (Fold Change). Of this number, 115 genes were over-expressed and 43 under-expressed. The analysis of the gene list based on their biological pathways revealed that the regulation of inflammation was mediated by chemokine and cytokine signaling pathways, the integrin signaling pathway, and T and B cell activation. Moreover, a change was identified in the expression of genes involved in both intercellular and intracellular signaling systems.

## Key words

Abdominal aortic aneurysm • Gene expression • Immunological mechanisms • Signaling systems • Pathways

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## Introduction

Abdominal aortic aneurysm (AAA) is a serious condition with high mortality rate (Go *et al.* 2014). The mechanisms of its formation and reasons for its progression have not been clarified yet. Its pathogenesis is multifactorial and still being investigated (Golledge *et al.* 2010, Maegdefessel *et al.* 2014, Davis *et al.* 2014, Kuivaniemi *et al.* 2015). AAA is a chronic inflammatory disease characterized by inflammatory infiltration of arterial wall, degeneration and elastin fragmentation. The inflammatory process is present in the entire arterial wall and leads to extracellular matrix degradation. The only therapeutic treatment is a surgical procedure: aneurysm removal or use of a stent graft. No pharmacotherapy has been found so far for its effective treatment. Smoking is the most important risk factor for AAA. The only way known to minimize the risk of its progression and rupture is its elimination. Immunological mechanisms represent an important pathogenic factor involved in AAA development. The localized inflammatory reaction in AAA is characterized by the involvement of monocytes, macrophages, T and B lymphocytes (T and B cells) and polymorphonuclear cells. The activity of cytokines and leukotrienes, together with the dysregulation of adhesive molecules, result in the damage of smooth arterial muscle, extracellular matrix degeneration, and neovascularization (Shimizu *et al.* 2006, Rizas *et al.* 2009, Peshkova *et al.* 2016, Swedenborg *et al.* 2011, Lindholt and Shi 2006). The

**Table 1.** Demographic and clinical characteristics of patients.

	<b>n</b>	<b>m</b>	<b>f</b>	<b>Age (Mean)</b>	<b>DM</b>	<b>HT</b>	<b>CHD</b>	<b>Smoking</b>
<i>Patients</i>	48	43	5	69	9	40	17	41

DM – Diabetes mellitus, HT – Hypertension, CHD – Coronary heart disease.

involvement of genetic factors in the development of the disease is indisputable (Blanchard *et al.* 2000, Sandford *et al.* 2007, Jones *et al.* 2017). Receptor mutations for TGF-beta (Transforming Growth Factor beta) and fibrillin were found in both thoracic and abdominal aortic aneurysms (Kim and Stansfield 2017). In recent years, several studies on gene expression in altered aneurysmal tissue have been published. In our opinion, no study has been carried out to compare a sample of aneurysmal and non-aneurysmal aortic tissue in the same patient. Our study compared gene expression in the tissue of AAA and in the healthy part of the proximal neck of the aorta in the same patient.

## Methods

The total of 48 patients with AAA who required an open surgical procedure were included into the study. The average diameter of all aneurysms exceeded 5.5 cm. Two samples (5 x 5 mm in size) comprising the entire thickness of the arterial wall were collected from each patient – a total of 96 samples were examined – 48 pairs of samples from 48 patients. One sample was obtained from the aneurysm bulge at the largest dilatation macroscopically, the second sample was taken from the proximal neck of the aneurysm without any macroscopically detected aneurysmal changes. Subsequently, both samples underwent Illumina microarray analysis of DNA. Baseline demographics included age and gender; data were also collected with regard to the presence of diabetes (DM), hypertension (HT), coronary heart disease (CHD) and smoking (Table 1).

### *Analysis of expression profiles*

The total RNA was isolated using RNeasy Micro Kit (Qiagen, MD, USA) according to the manufacturer's protocol. Quality and concentration of RNA were measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA). The RNA integrity was analyzed by the Bio-analyzer 2100 (Agilent, CA, USA). Only samples with an intact RNA profile were used for expression profiling analyses – RIN>9 (RNA Integrity

Number). Illumina HumanHT-12 v4 Expression BeadChips (Illumina, CA, USA) were used for microarray analysis performed in accordance with the standard protocol. In brief, 200 ng RNA was amplified with Illumina TotalPrep RNA Amplification Kit (Ambion, TX, USA) and 750 ng of labeled RNA was hybridized on the chip according to the manufacturer's protocol. The analysis was performed in two biological replicates per group. The raw data were preprocessed using GenomeStudio software (version 1.9.0.24624; Illumina, CA, USA) and the Limma package (Smyth 2004) of Bioconductor (Gentleman *et al.* 2004), as described elsewhere (Valach *et al.* 2012). The transcription profiles were background-corrected using the normal-exponential model, which was quantile normalized and variance stabilized using base 2 logarithmic transformation.

### *Statistics*

A moderated t-test was used to detect transcripts differentially expressed between the samples and controls (within Limma). False discovery rate values were used to select significantly differentially transcribed genes (false discovery rate (FDR)<0.05). The transcription data represent minimum information about a microarray experiment (MIAME) compliant and were deposited in the ArrayExpress database. Gene set enrichment analysis and determination of gene function were performed using Enrichr web service (Chen *et al.* 2013). The Na Homolce Hospital Ethics Committee approved all protocols and informed consent forms. All patients provided informed consent for participation.

## Results

Different gene expression was established in aneurysmal and non-aneurysmal tissue. Figure 1 shows heat-maps for 1,000 of the most differently expressed genes. The total of 4,285 genes with different gene expression in the aneurysmal and non-aneurysmal tissues of 11,084 transcripts were found. Of this number, 2,185 genes were over-expressed and 2,100 genes under-expressed. Of these genes, 158 were differentially expressed with FDR<0.05 and fold change (FC)≥2. Of

these, 115 were over-expressed and 43 under-expressed. The gene set enrichment analysis (GSEA) method was used to analyze the functions represented by a group of deregulated genes. The analysis performed by means of the protein analysis through evolutionary relationships (PANTHER) and Nature Pathway databases has shown changes in the signal pathways of chemokine and cytokine mediated inflammation reactions, in integrin expression,

and T and B lymphocyte activation in the inflamed aneurysmal tissue. These findings were in agreement with the analysis integrated in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. In addition to immunity mechanisms, signal pathways were involved related to the regulation of inflammation, to the cytoskeleton, and signals of intercellular and intracellular pathways.

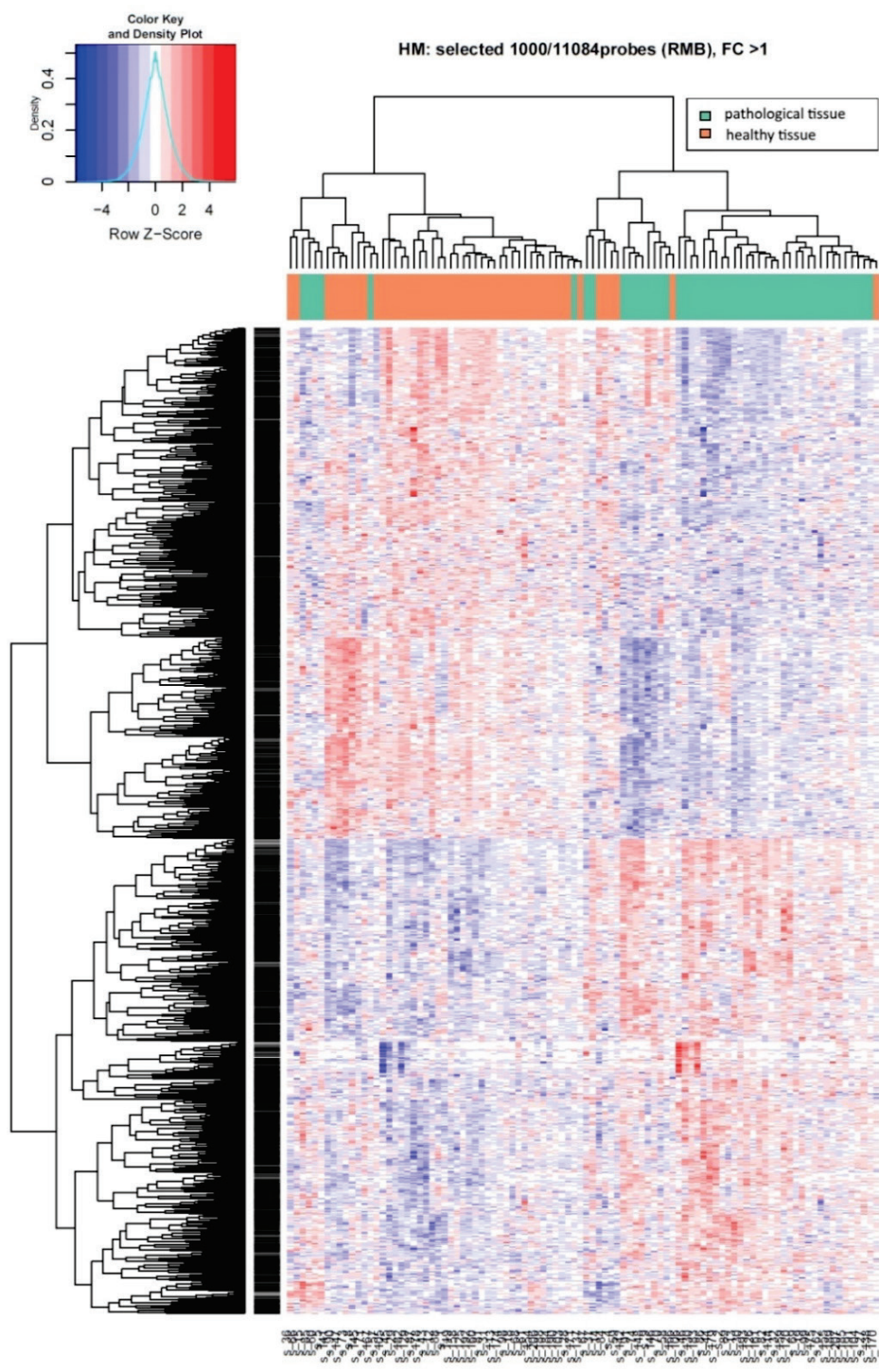


Fig. 1. Heatmaps for 1,000 of the most differently expressed genes.

## Discussion

A number of studies have been published recently that have dealt with gene expression in AAA patients. Their summary is given in the following reviews (Estrelinha *et al.* 2014, Kuivaniemi *et al.* 2014). Gene expression was studied both in patients' plasma and/or biopsy tissue. In our study, we compared aneurysmal tissue with healthy tissue of the affected vessel, i.e. aorta of the same individual. As far as we know, it is the first study of this design. We think that the changes in gene expression manifested in the damaged

tissue are more important than those detected in serum. In serum, they are associated either with a very heterogeneous mixed cell population or with an isolated cell population. With regard to the clear compartmentalization of the immune response with AAA patients, a greater importance can be supposed of the changes found directly in pathologically changed tissue similarly to hemato-oncological or oncological diseases. It is no surprise that a significant part of the changed gene expression is related to genes involved in inflammatory processes, i.e. genes for chemokines, integrins, and those for T and B cell activation, similarly

**Table 2.** Selected pathways and differentially expressed genes.

KEGG ID	TITLE	No. Genes	No. DEG	SPIA p-value	SPIA FDR	SPIA status
<b>Activated pathways</b>						
hsa05414	Dilated cardiomyopathy	91	32	1.46E-11	9.80E-10	Activated
hsa04062	Chemokine signaling pathway	189	45	5.82E-11	2.22E-09	Activated
hsa04060	Cytokine-cytokine receptor interaction	265	55	4.78E-10	1.28E-08	Activated
hsa04270	Vascular smooth muscle contraction	116	34	1.60E-09	3.06E-08	Activated
hsa04670	Leukocyte transendothelial migration	116	32	1.32E-08	2.21E-07	Activated
hsa05166	HTLV-I infection	263	46	2.92E-07	4.34E-06	Activated
hsa04064	NF-kappa B signaling pathway	92	24	2.57E-06	3.13E-05	Activated
hsa05162	Measles	135	27	3.72E-05	0.000416	Activated
hsa04020	Calcium signaling pathway	184	35	6.79E-05	7.00E-04	Activated
hsa05416	Viral myocarditis	71	18	0.000143	0.00137	Activated
hsa04650	Natural killer cell mediated cytotoxicity	138	21	0.000169	0.00151	Activated
hsa05032	Morphine addiction	92	21	0.000196	0.00165	Activated
hsa04380	Osteoclast differentiation	132	26	0.00036	0.00268	Activated
hsa04660	T cell receptor signaling pathway	108	20	0.00108	0.00725	Activated
hsa04330	Notch signaling pathway	47	12	0.00156	0.0095	Activated
<b>Inhibited pathways</b>						
hsa04810	Regulation of actin cytoskeleton	213	48	6.92E-14	9.28E-12	Inhibited
hsa04510	Focal adhesion	201	49	6.62E-11	2.22E-09	Inhibited
hsa05412	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	74	27	7.20E-10	1.61E-08	Inhibited
hsa04512	ECM-receptor interaction	84	23	2.25E-06	3.01E-05	Inhibited
hsa05132	Salmonella infection	86	19	0.00029	0.00229	Inhibited
hsa04142	Lysosome	121	24	0.000856	0.00603	Inhibited
hsa05100	Bacterial invasion of epithelial cells	70	16	0.0015	0.0095	Inhibited
hsa04540	Gap junction	89	16	0.00238	0.0122	Inhibited
hsa04070	Phosphatidylinositol signaling system	81	16	0.00961	0.0322	Inhibited

KEGG ID – Kyoto encyclopedia of genes and genomes pathway ID, Name – KEGG pathway name, No Genes – Number of genes detected in the experiment and belonging to the pathway, No DEG – Number of differentially expressed genes detected in the experiment and belonging to the pathway, SPIA p-value – SPIA global pathway significance p-value, SPIA FDR – FDR corrected SPIA global pathway significance p-value, SPIA status – SPIA estimate on pathway activation/inhibition.

to the previously published studies (Biros *et al.* 2014, Hinterscher *et al.* 2013, Giusti *et al.* 2009, Butt *et al.* 2016, Tanios *et al.* 2015). Destructive inflammation is the main characteristic of AAA formation and progression; it is therefore understandable that immunological mechanisms are at play. The results show that both B and T lymphocytes (i.e. both cell and humoral immunity) are involved. Regarding the basic pathogenetical mechanism (chronic inflammation), it was not surprising, e.g. activation of B lymphocytes “promotes AAA by producing immune-globulins, cytokines and metalloproteinases resulting in the activation of macrophages, mast cells and complement pathways (Zhang *et al.* 2015). Similarly, our study demonstrated the activation of the pathways regarding the chemokine signaling, cytokine receptor interaction, leukocyte transendothelial migration, and Toll like receptor signaling. The selected pathways and differentially expressed genes discussed in the following text are listed in Table 2. When interpreting our results, we focused on the pathways associated with differently

expressed genes. When analyzing associated signal pathways in 158 genes with the most significant differences in expression, we found 17 inhibited pathways and 27 activated pathways. Many of these pathways are associated with inter and intracellular signaling. Inter and intra-cellular signaling plays an essential role for the correct function of the cells and thus also tissues (Antebi *et al.* 2017). What we consider to be important is the evaluation of the associated pathways and trends of the changes found: whether the activation or inhibition is going on. The detection of activated associated signal pathways is the most common trend. Speaking of individual signal pathways, it is interesting to mention a different expression of genes that are associated with dilated cardiomyopathy (DCM). The pathway of dilated cardiomyopathy with differentially expressed genes are on Figure 2. These findings suggest that the two diseases are related. DCM is a disease that is mostly autosomal dominant and more rarely displaying autosomal recessive heredity or X-linked inheritance (Mestroni *et al.* 2014).

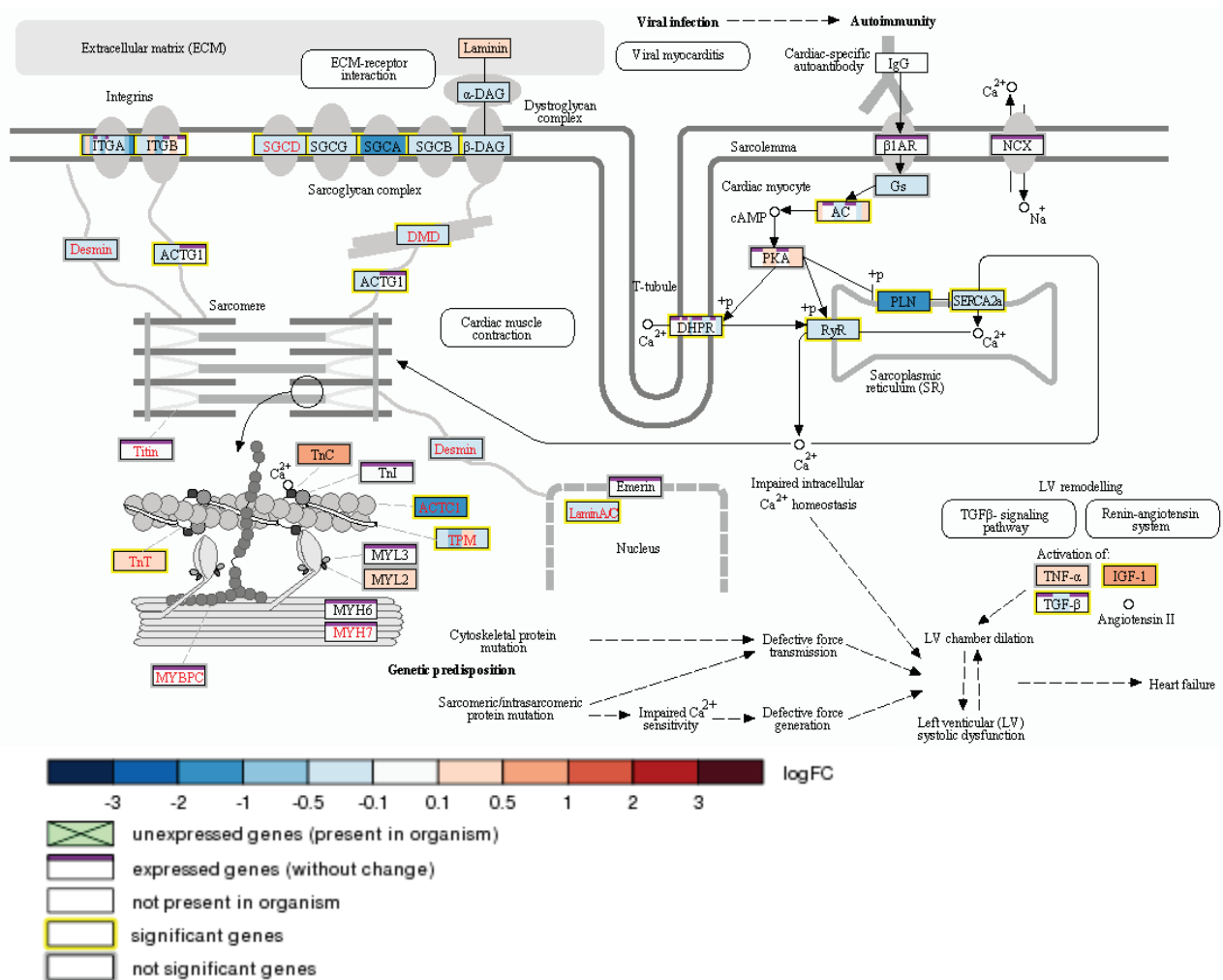


Fig. 2. Pathway of dilated cardiomyopathy.

More than 40 genes related to DCM (Human Gene Mutation Database) have been detected so far (Tayal *et al.* 2017). When the genes involved in the signal pathway for DCM were activated, a reduced expression of genes associated with intracellular signaling in aneurysmal tissue was found. Inhibition of the pathways associated with the regulation of cytoskeleton shows a potential insufficient function of the mechanisms modulating actin cytoskeleton, which is a very important mechanism in

cardiovascular diseases (Tang and Gerlach 2017). The other example of changes related to intercellular communications is the pathway of Gap junctions (Fig. 3). Ions, hormones and neurotransmitters. These intercellular channels comprise a protein family known as connexins. Gap junctions (GJs) represent an intercellular network of protein channels that facilitate the cell-to-cell passage of GJs are surface membrane structures that allow direct communication between cells. They were discovered in

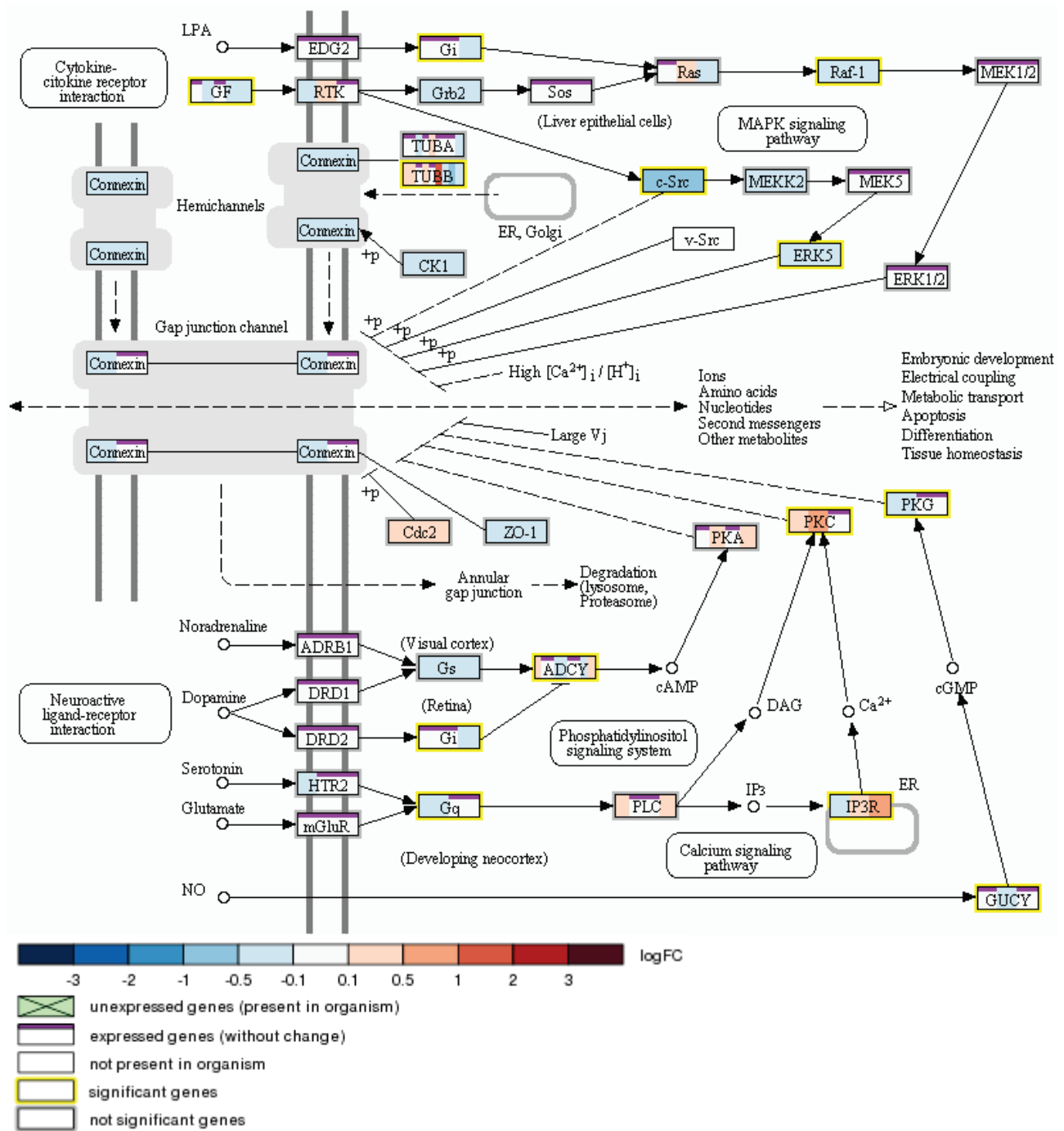
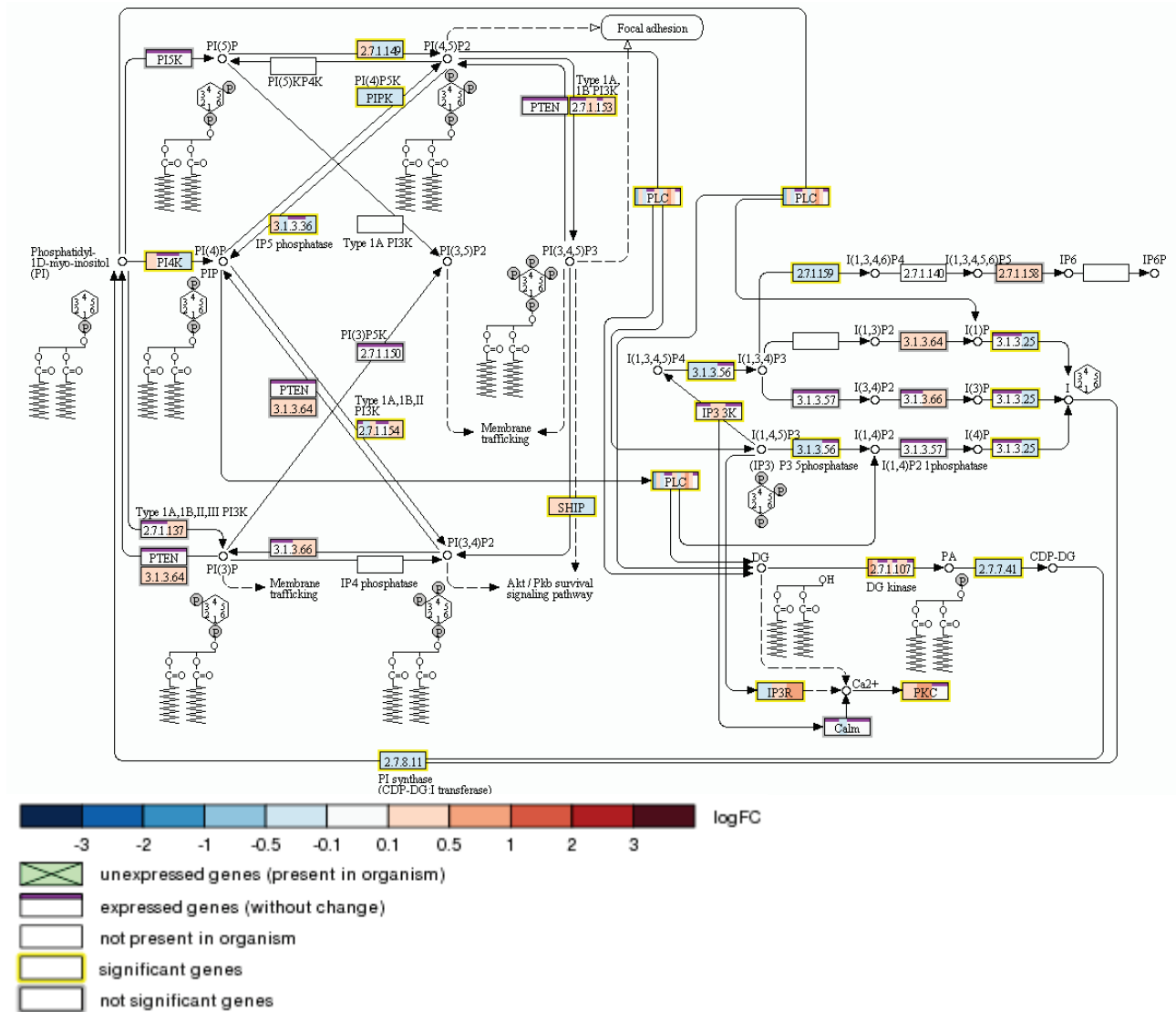


Fig. 3. Pathway of gap junctions.





**Fig. 4.** Pathway of phosphatidylinositol signaling system.

the 1960s following the convergence of the detection of low-resistance electrical interactions between cells and anatomical studies of intercellular contact points (Evans 2015). GJs are significantly involved in controlling vascular functions (Figuroa and Duling 2009). These findings correspond to the study by Lenk *et al.* (2007) who also proved a significant difference in expression genes associated with GAP and other signaling pathways. The expression of vascular connexins is altered in hypertension. Since our AAA group of patients was characterized by a high incidence of hypertension, it would be interesting to know whether this change is associated with AAA or hypertension. The phosphatidylinositol signaling system is another intracellular signaling system with inhibited expression (Fig. 4). This system regulates various structural and developmental functions

but is also centrally involved in a plethora of signal transduction pathways in all eukaryotic models. They are not only precursors of second messengers but also directly interact with many protein effectors, thus regulating their localization and/or activity (Delage *et al.* 2014). The phosphatidylinositol signaling system is an important lipid signaling system (Wymann and Schreiner 2008). Its altered function in AAA may provide potential evidence of its involvement in AAA pathogenesis. When interpreting the inhibited expression of the above-mentioned signaling systems, we can generalize that what we see is a defect in intracellular communication with all its consequences leading to poor cell functioning. We may only speculate about the causes of these changes, which will no doubt be the subject of further studies. One of the interesting outcomes of our study is the

identification of “local” pathology in relation to a different gene expression. A number of questions arise in this connection. It is known from the clinical practice that there is practically no recurrence with the patients after the operation. Why would the pathology only be limited to a specific section of aorta and significantly depends on the age of the patient? From the existing studies it follows, that aneurysm of chest aorta has significantly lower prevalence and that gene expression TAA differs from gene expression AAA (Matsumoto *et al.* 2014). It might be caused by a different embryonic origin of chest and abdominal aortas, which leads to a different response of the tissue, e.g. to cytokines.

*Limitations of the study.* We cannot be sure that the sample obtained from the neck of aneurysm, represented a healthy vessel tissue. Nevertheless, the heatmaps of the analysis performed provide good evidence for the homogeneity of the samples collected from the neck of aneurysm and that were assessed by a surgeon as “normal” tissue. We did not perform protein analysis of the selected genes that were identified as the most significantly differently expressed. Such a selection, however, would have been problematic owing to their high number. Taking samples from the same patient represents a possible limitation of the study if the genetic factors played a significant role in the onset of the disease. While in the thoracic aortic aneurysms (TTA), we already know a specific list of genes that are undoubtedly associated with the pathogenesis of TAA, no

mutation predicting the occurrence of AAA has yet been found (Carino *et al.* 2018). Therefore, it will certainly not be a monogenic disease. The results of recent meta-analysis have shown only a limited number of AAA-associated nucleotide polymorphisms in the studies performed so far (Bradley *et al.* 2016). The other limitation is the limited number of patients resulted from the financial costs of the study.

In summary, differences in gene expression in the aneurysmal tissue and in the biopsy of healthy tissue of the same patient were found. The changes detected were related to the inflammatory process regulation by means of immune mechanisms – inflammation mediated by chemokine and cytokine signaling pathways, integrin signaling pathway, T and B cell activation. Besides, changes in the expression of genes involved in intercellular and intracellular signaling systems were identified. Knowledge of these mechanisms can help get better understanding of the pathogenesis and treatment of AAAs.

### Conflict of Interest

There is no conflict of interest.

### Acknowledgements

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