

## Markers of Oxidative Stress and Antioxidant Status in the Plasma, Urine and Saliva of Healthy Mice

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### Summary

Oxidative stress markers are usually measured in plasma, a stable environment for biomarkers. Blood collection is invasive, but the use of alternative biofluids is limited, due to high variability. In this study, we aimed to establish reference values for oxidative stress markers in plasma, urine and saliva of adult, healthy mice and to identify some sources of variability. Samples were obtained from 41 female and 37 male adult, healthy mice of the CD-1 strain, aged 95-480 days, weighing 21-55 grams. Reference ranges of TBARS (thiobarbituric acid reactive substances), AOPP (advanced oxidation protein products), fructosamine, GSH/GSSG (reduced and oxidized glutathione) ratio, TAC (total antioxidant capacity), and FRAP (ferric reducing antioxidant power) were measured in plasma and urine, and TBARS, GSH/GSSG ratio, TAC and FRAP in saliva, using standard spectrophotometric and fluorometric methods. Salivary GSH/GSSG and urinary AOPP were higher in females. Urinary fructosamine, GSH/GSSG and FRAP were higher in males. Urinary TAC and FRAP negatively correlated with age, and urinary GSH/GSSG positively correlated with weight. We determined that urine and saliva can be obtained non-invasively from mice, in sufficient amounts for reliable oxidative status assessment. Further studies are needed to uncover whether these biofluids reflect systemic oxidative status in diseases.

### Key words

Antioxidant status • Sex difference • Biomarker • Saliva • Urine

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### Introduction

Reactive oxygen species (ROS) play an important role in the defense mechanisms and in the destruction of damaged cells, they can act as intra- and extracellular messengers and contribute to homeostasis. However, excessive production of ROS may have detrimental effects on the organism, so endogenous and exogenous antioxidant systems help to maintain balance. An imbalance between the production of ROS and their elimination by antioxidants leads to oxidative stress (Sies 1997). Oxidative stress affects all macromolecules, impairs organ functions, and is involved in the pathogenesis of various diseases. Markers of oxidative stress and antioxidant status can be measured in plasma or in tissues in order to evaluate or monitor the disease state and progression (Valko *et al.* 2007).

It is important to note that oxidative stress is a phenomenon that is difficult to characterize. The great diversity in oxidative stress between diseases and conditions has to be taken into account when selecting the appropriate biomarkers (Frijhoff *et al.* 2015). Our study focused on some of the most commonly used. Lipid peroxidation is mainly assessed by measuring

malondialdehyde, and protein oxidation by measuring advanced oxidation protein products (AOPP) (Capeillère-Blandin *et al.* 2004). The ratio of reduced and oxidized glutathione (GSH/GSSG) is used as a general marker of oxidative stress (Zitka *et al.* 2012). To avoid the need for measuring several individual antioxidants, total antioxidant capacity (TAC) or ferric reducing antioxidant power (FRAP) is analyzed (Benzie and Strain 1996, Erel 2004). Carbonyl stress is an irreversible form of non-enzymatic glycation (Bargnoux *et al.* 2009). The determination of fructosamine, an Amadori product, is used to assess non-enzymatic glycation (Kishabongo *et al.* 2015).

Oxidative stress markers are usually measured in plasma or serum, as both represent a relatively stable environment for systemic biomarkers. However, blood collection represents a significant stress to patients. Thus, other biofluids are being tested as alternatives to plasma. These biofluids should be easier to obtain and in sufficient amounts (Čolak 2008). Urine has the potential to be an alternative biofluid for oxidative stress assessment, as its collection is less invasive and requires less effort, equipment and trained staff than blood collection. Besides, urine can be obtained in large volumes repeatedly during the day. Also, urine has been argued to be a better environment for oxidative stress measurement than plasma, as it has a lower organic and inorganic metal content, and lower levels of ROS promoters, therefore, urine is less liable to artificial increase of oxidative stress markers during sampling and storage (Il'yasova *et al.* 2012). Saliva collection is also inexpensive, easy, fast, and non-invasive. During the collection of both of these biofluids, there is no risk of vessel injury or infection, and they could be collected without the help of a nurse or a doctor. Saliva collection is preferred by non-compliant patients, such as children or elderly people. Saliva and urine are also suitable for home use, since they are easy to store and transport (Lee and Wong 2009).

Despite the possibility to analyze a wide range of markers in urine or saliva, their use in clinical practice is still limited. The main problem is the high intra- and inter-individual variability of the measured markers (Behuliak *et al.* 2009, Kamodyová *et al.* 2013, Tóthová *et al.* 2015). The sources of this variability remain largely unknown. Therefore, it is necessary to identify the underlying factors of technical and biological variability. Because it is difficult to eliminate bias in human studies, research in healthy animals and animal models of various

diseases is the only way to study factors that might influence the variability of urinary and salivary biomarkers. Based on studies conducted in humans, an effect of gender on oxidative stress has been proposed, as females might be more protected against oxidative stress, by the antioxidant effects of estrogens (Bloomer and Fisher-Wellman 2008). It has also been postulated that systemic oxidative stress can play a role in the aging process, and that oxidative stress increases with bodyweight and can be induced by obesity (Harman 1956, Chrysohoou *et al.* 2007, Fernández-Sánchez *et al.* 2011, Serra *et al.* 2013, Savini *et al.* 2013). Whether this is true for experimental animals and their body fluids, is unknown.

The wide use of oxidative stress markers calls for standardization and the determination of whether these markers correlate between individual body fluids. Oxidative stress markers measured in various body fluids might reflect the local oxidative status, rather than the systemic, at least in healthy animals. The aim of this study was to establish reference values for oxidative status markers in plasma, urine and saliva of adult healthy mice of both sexes and to identify the underlying factors of high inter-individual variability.

## Materials and Methods

### Animals

Healthy female (n=41) and male (n=37) adult mice of the CD-1 strain (Anlab, Prague, Czech Republic) were used in this experiment. The age of the mice was between 95-480 days ([Supplementary Fig. S4](#)) and their weight between 21-55 g. The animals were housed in standard cages with wood bedding, and had free access to water and standard rodent pellet chow Ssniff R/M-H (Anlab, Prague, Czech Republic). All mice were kept in a room with a 12/12 h light/dark cycle. Experiments were performed in full compliance with the EU Guidelines for Scientific Experimentation on Animals, and were approved by the Ethics committee of the Institute of Molecular Biomedicine, Slovakia (010/2016/SKP1012).

### Sample collection

To obtain plasma samples, mice were anesthetized using the isoflurane drop-method, by placing the mice briefly in a closed jar with a cotton pad wetted with several drops of non-diluted isoflurane. Blood was collected from the retro-orbital plexus into two 500 µl EDTA coated blood collection tubes (up to 250 µl of

blood into each tube) (Sarstedt, Numbrecht, Germany). Plasma was obtained by centrifugation (2,000 g for 7 min), plasma samples were stored at -20 °C. Urine samples were collected during 4 h of fasting in metabolic cages, to the volume of 800 µl on average, and stored at -20 °C prior to analysis. For saliva collection, mice were anesthetized with intraperitoneal injection of ketamine (100 mg/kg, Richter Pharma AG, Wels, Austria) and xylazine (10 mg/kg, Ecuphar N. V., Oostkamp, Belgium). Salivation was induced using pilocarpine (0.5 µg/kg, Unimed Pharma, Bratislava, Slovakia). Saliva samples were collected for 30 min, to the volume of 400 µl, approximately, and stored at -20 °C prior to analysis.

### Biochemical analysis

Markers of oxidative stress and antioxidant status were assessed in plasma, urine and saliva samples. Measurements were performed on a TecanSaphire II Instrument (Grödig, Austria), and the chemicals used in the analyses were purchased from Sigma Aldrich (Steinheim, Germany).

Thiobarbituric acid reactive substances (TBARS) were measured as described previously (Tóthová *et al.* 2013). Briefly, 20 µl of samples or standards (1,1,3,3-tetraethoxypropane) were mixed with 30 µl of distilled water, 20 µl of 0.67 % thiobarbituric acid and 20 µl of glacial acetic acid. The plates were shortly mixed and incubated for 45 min at 95 °C. Afterwards, 100 µl of n-butanol were added into the samples and plates were centrifuged (2,000 g/10 min/4 °C). Seventy µl of the upper phase were transferred into a new microtiter plate and fluorescence was measured at  $\lambda_{\text{ex}}=515$  nm and  $\lambda_{\text{em}}=535$  nm.

AOPP were used as a marker of protein damage. For the analysis, 200 µl of samples and standards (chloramine T mixed with potassium iodide) were mixed with 20 µl of glacial acetic acid. After mixing for 2 min, absorbance was measured at 340 nm (Tóthová *et al.* 2013).

Fructosamine was used as a marker of advanced glycation. Twenty µl of the samples and standards (16 mmol/l 1-deoxy-morpholino-D-fructose) were mixed with 100 µl of 0.25 mmol/l nitro blue tetrazolium containing 1 mmol/l nitro blue tetrazolium and 0.1 mol/l sodium carbonate buffer (pH=10.35). Samples were incubated at 37 °C for 15 min. Absorbance was measured at 530 nm (Tóthová *et al.* 2013).

For the GSH/GSSG ratio, GSH was measured by mixing 10 µl of the samples with 10 µl of the

O-phthalaldehyde solution (1 mg/ml) and 180 µl of the phosphate buffer solution (100 mmol/l with 2.5 mM EDTA- $\text{Na}_2$ ). The mixture was then incubated 15 min at room temperature. Afterwards, specific fluorescence at  $\lambda_{\text{ex}}=350$  nm,  $\lambda_{\text{em}}=460$  nm was measured. GSSG was measured by mixing 25 µl of the samples and 10 µl of N-ethylmaleimide (5 g/ml) and incubation for 40 min at room temperature. Ten µl of the mixture were transferred to new microtiter plate with addition of 10 µl of the O-phthalaldehyde (1 mg/ml) and 180 µl of NaOH (0.1 mol/l). After 15 min incubation at room temperature, specific fluorescence was measured at  $\lambda_{\text{ex}}=350$  nm,  $\lambda_{\text{em}}=460$  nm. GSH values were divided with GSSG values to assess their ratio (Tóthová *et al.* 2013).

For the measurement of TAC, 20 µl of samples were mixed with acetate buffer (pH=5.8). The initial absorbance was measured at 660 nm as blank. When ABTS solution (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid with acetate buffer) was added, the absorbance (660 nm) was measured again. The blank absorbance values were subtracted from the values obtained by the second measurement (Tóthová *et al.* 2013).

FRAP was used as a marker of antioxidant status. Two hundred µl of warmed (37 °C) FRAP reagent (containing acetate buffer, pH=3.6; tripyridyl-s-triazine;  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  and water) were pipetted into a microtiter plate. Absorbance was measured as blank. Afterwards, 20 µl of samples and standards (100 mmol/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ) were added. Absorbance was measured again at 530 nm. The blank absorbance values were subtracted from the values obtained by the second measurement (Tóthová *et al.* 2013).

Creatinine concentrations in the urine samples were measured using the spectrophotometric method by Jaffe (Askenazi *et al.* 2014). Ten µl of samples were mixed with mixture of NaOH and picric acid (ratio 5:1). After 6 min, the absorbance was measured at 492 nm.

For the measurement of proteins in plasma and urine, 10 µl of the samples and standards (bovine serum albumin) were used. Afterwards, according to the manufacturer's instructions, 200 µl of the working solution (bicinchoninic acid and copper sulphate, 49:1 ratio, respectively) were added. Samples were incubated at 37 °C for 30 min. After cooling the samples at room temperature, absorbance was measured at 562 nm.

TBARS, fructosamine, TAC and FRAP were normalized to urinary creatinine in urine samples. AOPP

were normalized to plasma proteins in plasma and urinary proteins in urine. To evaluate intra- and inter-assay variability, coefficient of variation (CV) was calculated. Limit of detection (LOD) was also determined for each assay type.

#### Statistical analysis

GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) was used for the statistical analyses. After testing for normality with the D'Agostino-Pearson omnibus test, data were analyzed with the Mann-Whitney U test. For the correlations, Spearman correlation coefficients were calculated. Outliers were detected using the Grubbs' test and removed from further analyses. A value of  $P < 0.05$  was considered statistically significant. The reference ranges of the markers were calculated as the 5<sup>th</sup> and 95<sup>th</sup> percentiles.

Multivariate regression was performed on the relationship between salivary and plasma markers, or urinary and plasma markers, using gender as a fixed factor and weight and age as covariates, employing the general linear model (GLM), (SPSS v. 20, SPSS Inc., Chicago, IL, USA).

## Results

TBARS, AOPP, fructosamine, GSH/GSSG ratio, TAC and FRAP were detected in the plasma and urine of the mice. TBARS and the GSH/GSSG ratio, along with the markers of antioxidant status TAC and FRAP, were detected in the saliva of adult healthy mice. Salivary fructosamine concentrations were under the detection limit. Salivary AOPP were not measured. LOD was determined for TBARS to be  $0.32 \mu\text{mol/l}$ . For AOPP, LOD was  $13.21 \mu\text{mol/l}$ . LOD calculated for fructosamine was  $0.72 \text{ mmol/l}$ , for GSH, it was  $9.46 \mu\text{mol/l}$ , for GSSG,  $22.8 \mu\text{mol/l}$ . In case of TAC, LOD was calculated as  $8.93 \mu\text{mol/l}$ , and for FRAP, it was  $134.35 \mu\text{mol/l}$ . Reference ranges for the measured plasma, urinary and salivary markers were assessed in mice of both sexes. Plasma TBARS were 10 % higher in males ( $P < 0.05$ ). Plasma fructosamine was higher in females by 15 % ( $P < 0.001$ ) (Table 1). When sex differences were assessed in urinary markers, urinary TBARS and TAC showed no significant differences between the sexes. Urinary AOPP were higher in females by 207 % ( $P < 0.001$ ), while urinary fructosamine was higher in males by 108 % ( $P < 0.001$ ). Urinary GSH/GSSG ratio was higher in males by 201 % ( $P < 0.001$ ) and urinary FRAP was higher in

males by 67 % ( $P < 0.001$ ). Sex differences in salivary TBARS, TAC, and FRAP, were not significant. Significant sex difference in saliva was observed in the GSH/GSSG ratio, which was higher in females by 55 % ( $P < 0.01$ ). Salivary TAC was higher in males by 17 % ( $P < 0.001$ ) (Table 1).

The measured plasma markers did not correlate significantly with the age of the animals (Figs 1A-1F). Urinary TAC negatively correlated with the age of the animals (Fig. 2E;  $r = -0.23$ ,  $P < 0.05$ ) as did urinary FRAP (Fig. 2F;  $r = -0.05$ ,  $P < 0.05$ ). Urinary TBARS, AOPP, fructosamine or GSH/GSSG did not correlate with age significantly (Figs 2A-2D). Salivary markers did not significantly correlate with age (Figs 3A-3D).

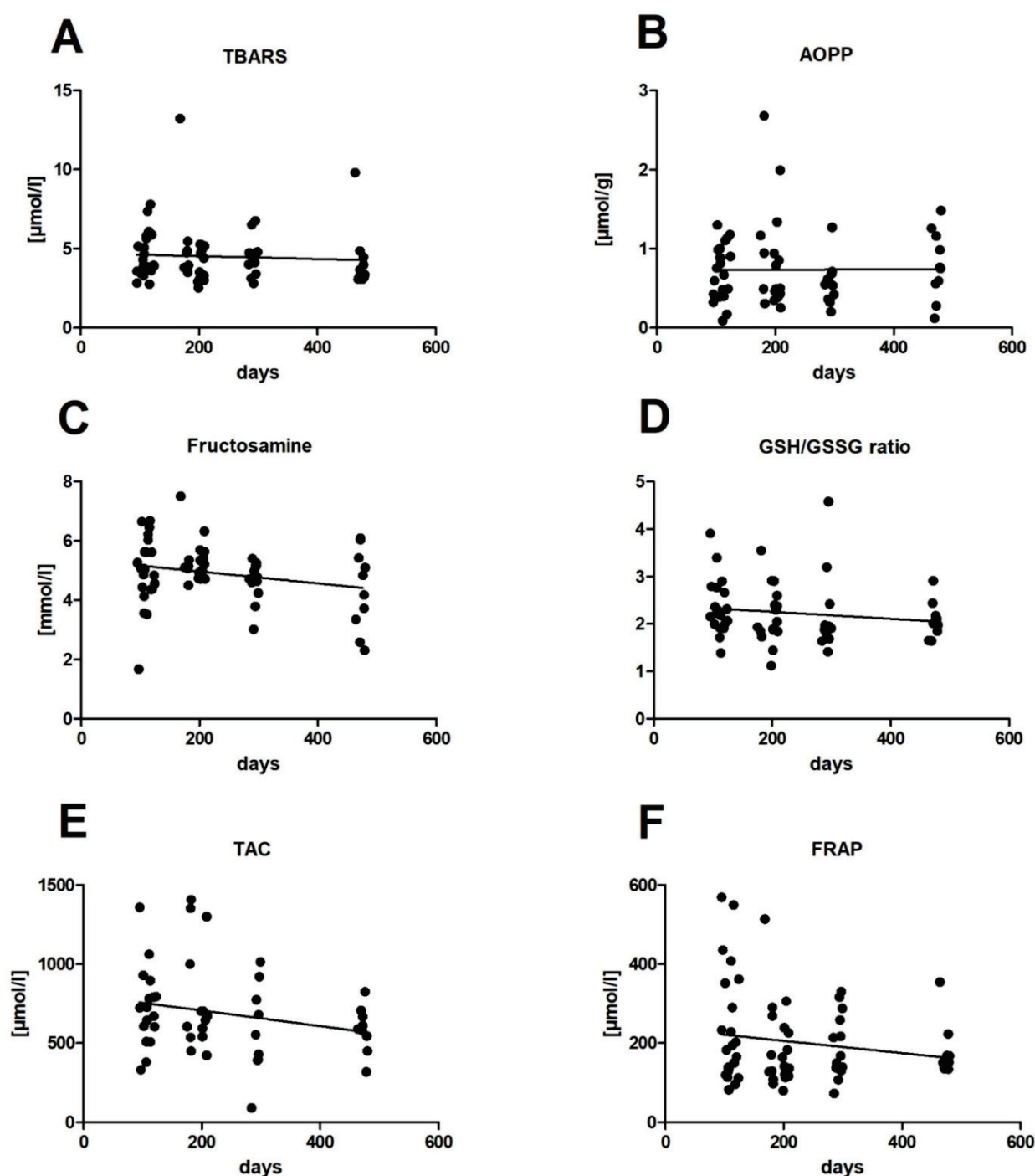
Plasma fructosamine correlated significantly with weight ( $r = -0.28$ ,  $P < 0.05$ ). Plasma TBARS, AOPP, GSH/GSSG, TAC or FRAP did not correlate with weight significantly ([Supplementary Fig. S1](#)). Out of the urinary markers only GSH/GSSG ratio correlated positively with the weight of the animals ( $r = 0.39$ ;  $P < 0.001$ ) ([Supplementary Fig. S2](#)). The measured salivary markers did not correlate with the weight of the animals ([Supplementary Fig. S3](#)). Urinary and plasma concentrations did not correlate significantly in any of the measured markers (Figs 4A-4F). Salivary concentrations did not correlate significantly with plasma concentrations either (Figs 5A-5D). GLM analysis confirmed the results of simple regression analyses on the significant sex differences, on the relationship between age, weight and plasma, urinary and salivary markers, and on the absence of significant relationships between urinary and plasma markers or salivary and plasma markers.

Intra-plate variability of TBARS expressed by CV was 3.26 % in the plate where plasma was assessed, 10.12 % in urine, and 12.75 % in saliva. Inter-plate CV was 38.59 %. In case of AOPP, intra-plate CV was 8.17 % in plasma, and 6.85 % in urine. Inter-plate CV was 9.97 %. In fructosamine, the CV of plasma was 7.28 %, and of urine, it was 4 %. Inter-plate CV was 14.2 %. Intra-plate CV of GSH was 7.59 % in plasma, 11.8 % in urine, and 12.06 % in saliva. Inter-plate CV was 12.3 %. In GSSG, CV was 6.12 % in the plasma plate, 2.45 % in urine, and 9.01 % in saliva. Inter-plate CV was 7.84 %. Intra-plate CV of TAC was 5.77 % in the plasma plate, 1.27 % in urine, and 4.95 % in saliva. Inter-plate CV was 14.29 %. Intra-plate CV of FRAP was 11.79 % in plasma, 1.12 % in urine, and 3.45 % in saliva. Inter-plate CV was calculated as 25.09 %.

**Table 1.** Reference values of oxidative stress markers in plasma, urine and saliva of healthy mice.

Markers	Plasma			Urine			Saliva		
	Females	Males	Sex difference	Females	Males	Sex difference	Females	Males	Sex difference
<i>TBARS</i>	2.82-8.09 μmol/l	3.14-7.29 μmol/l	10 % *	21.78-68.85 μmol/mmol	25.18-81.33 μmol/mmol	10 % ns	0.05-1.87 μmol/l	0.04-1.27 μmol/l	2 % ns
<i>AOPP</i>	0.22-2.23 μmol/l	0.22-1.29 μmol/l	6 % ns	325.92-1575.73 μmol/g	99.08-441.37 μmol/g	207 % ***	not measured	not measured	not calculated
<i>Fructosamine</i>	3.90-6.65 mmol/l	2.60-6.03 mmol/l	15 % ***	14.79-49.94 mmol/mmol	17.48-135.11 mmol/mmol	108 % ***	not detectable	not detectable	not calculated
<i>GSH/GSSG</i>	1.65-4.21	1.42-3.36	9 % ns	0.36-1.49	1.30-3.42	201 % ***	1.55-3.98	0.88-2.86	55 % ***
<i>TAC</i>	425.26-1338.67 μmol/l	317.66-1014.92 μmol/l	21 % ns	20.46-48.29 mmol/mmol	19.48-66.20 mmol/mmol	17 % ns	129.29-211.47 μmol/l	159.86-234.27 μmol/l	17 % **
<i>FRAP</i>	108.02-394.61 μmol/l	90.04-487.21 μmol/l	19 % ns	24.96-51.48 mmol/mmol	33.30-100.94 mmol/mmol	67 % ***	12.68-45.22 μmol/l	5.61-73.79 μmol/l	3 % ns

Reference values of plasma, urinary and salivary markers TBARS – thiobarbituric acid reactive substances, AOPP – advanced oxidation protein products, fructosamine, GSH/GSSG – ratio of oxidized/reduced glutathione, TAC – total antioxidant capacity, FRAP – ferric reducing antioxidant power, for female and male mice. Reference ranges of the markers were calculated as the 5<sup>th</sup> and 95<sup>th</sup> percentiles. \* denotes P<0.05, \*\* denotes P<0.01, \*\*\* denotes P<0.001.

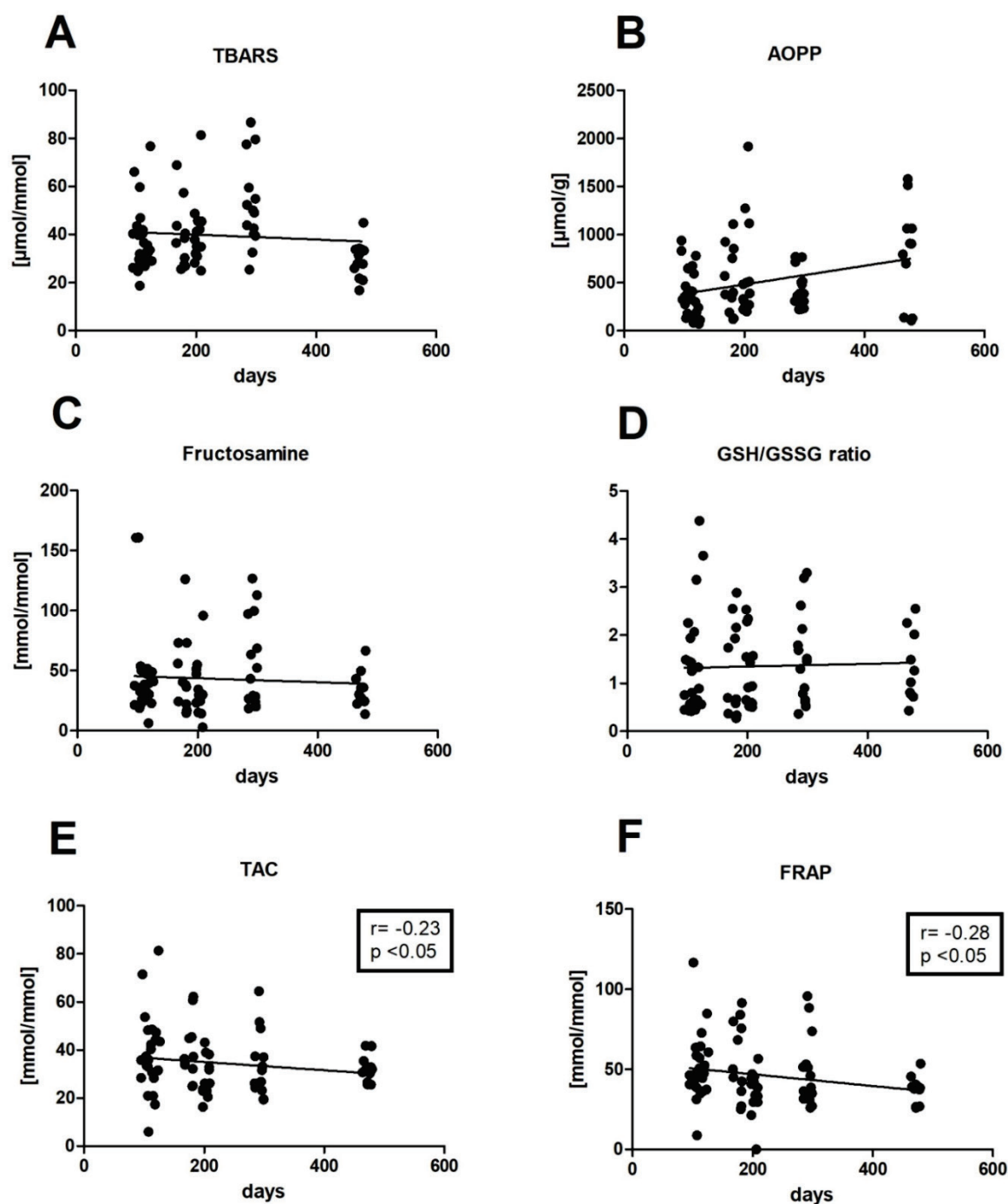


**Fig. 1.** Correlations between age and plasma (A) TBARS – thiobarbituric acid reactive substances, (B) AOPP – advanced oxidation protein products, (C) fructosamine, (D) GSH/GSSG – ratio of oxidized/reduced glutathione, (E) TAC – total antioxidant capacity, (F) FRAP – ferric reducing antioxidant power.

## Discussion

The main goal of this study was the assessment of reference values of plasma, urinary and salivary biomarkers of oxidative stress in healthy mice and the identification of at least some of the sources of their variability. In plasma, several studies established

reference values for some oxidative stress markers including AOPP and isoprostanes in human neonates (Longini *et al.* 2017) or healthy dogs (Tomišić *et al.* 2016). According to our knowledge, studies exploring the reference values of oxidative stress markers in these biofluids of healthy laboratory mice have not been published.

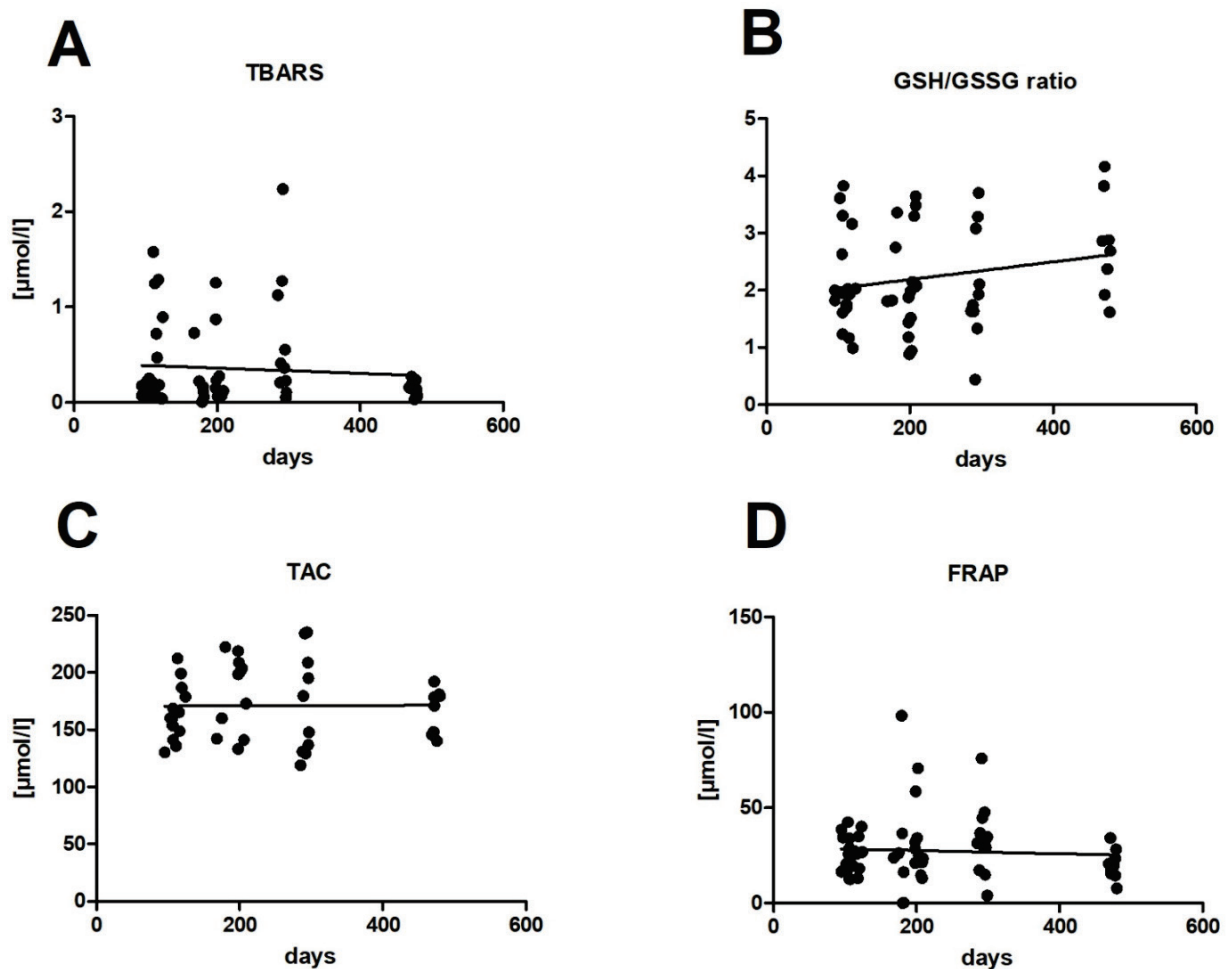


**Fig. 2.** Correlations between age and urinary (A) TBARS – thiobarbituric acid reactive substances, (B) AOPP – advanced oxidation protein products, (C) fructosamine, (D) GSH/GSSG – ratio of oxidized/reduced glutathione, (E) TAC – total antioxidant capacity, (F) FRAP – ferric reducing antioxidant power. Significant correlations are shown between age and TAC, and between age and FRAP.

Most studies in humans show that females are more protected against oxidative stress compared to males, presumably by the effects of estradiol (Sculley and Langley-Evans 2003, Bloomer and Fisher-Wellman 2008, Bloomer and Fisher-Wellman 2010). Studies assessing the sex differences of oxidative stress markers in biofluids are limited. However, a metabolite of 8-isoprostaglandin F<sub>2α</sub> has been shown to be higher in

the urine of females compared to males, and salivary TAC and FRAP to be lower in females (Sculley and Langley-Evans 2003, Il'yasova *et al.* 2005, Lettrichova *et al.* 2016). Although the oxidative stress markers are commonly measured in rodents, sex differences and reference values themselves were not described. In our experiment, significant sex differences were shown in plasma TBARS, which was higher in male mice.





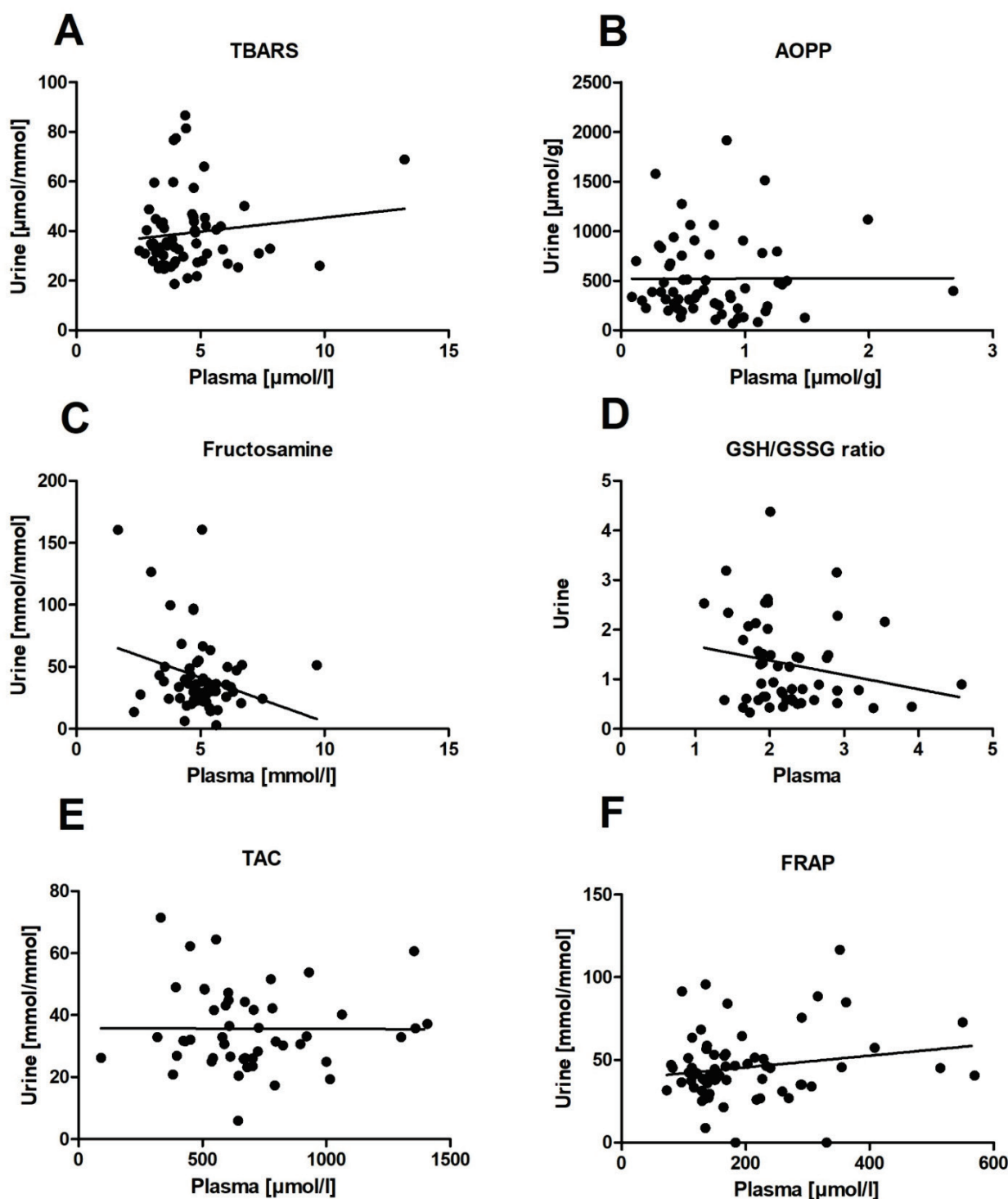
**Fig. 3.** Correlations between age and salivary (A) TBARS – thiobarbituric acid reactive substances, (B) GSH/GSSG – ratio of oxidized/reduced glutathione, (C) TAC – total antioxidant capacity, (D) FRAP – ferric reducing antioxidant power.

This is in accordance with previous findings in humans (Reckelhoff *et al.* 2004). Plasma fructosamine, on the contrary, was observed to be higher in female mice. Lower AOPP and higher FRAP and GSH/GSSG ratio were found in the urine of male mice. This could point to a higher antioxidant status of male urine. In saliva, a higher GSH/GSSG ratio was found in female mice, but salivary TAC was higher in male mice, as opposed to human studies. The possible explanation could come from the fact that human studies generally use unstimulated saliva, and the salivary flow has been shown to be lower in females (Inoue *et al.* 2006). In our study, pilocarpine had to be used to stimulate saliva production, which increases the salivary flow. Therefore, true sex differences observed in humans might be related to sex differences in saliva production and composition.

Aging is causally related to oxidative stress. The free radical theory of aging has been postulated over half a century ago (Harman 1956, Chrysohoou *et al.* 2007, Fernández-Sánchez *et al.* 2011, Serra *et al.* 2013, Savini

*et al.* 2013). It has been shown that in species in which females have a shorter life span than males, females produce more free radicals (Viña *et al.* 2013). A study has shown that aging mice have higher concentrations of oxidative stress markers and lower antioxidant status in saliva (Yamauchi *et al.* 2017). The majority of animal experiments are performed using young to middle aged animals. Thus the reference values were described in this age category, rather than in older animals. In the present study, no correlations were found between the concentrations of the measured plasma or salivary markers, and the age of healthy mice. The relatively stable physiology of young to middle aged rodents could explain the lack of association between oxidative stress and aging in our experiment. On the other hand, it has also been observed that aging (52 weeks of age) Wistar rats display higher urinary TBARS concentrations than young (13 weeks of age) rats (Gomes *et al.* 2009). While the present study did not find any correlations between age and urinary oxidative stress markers, it has been found



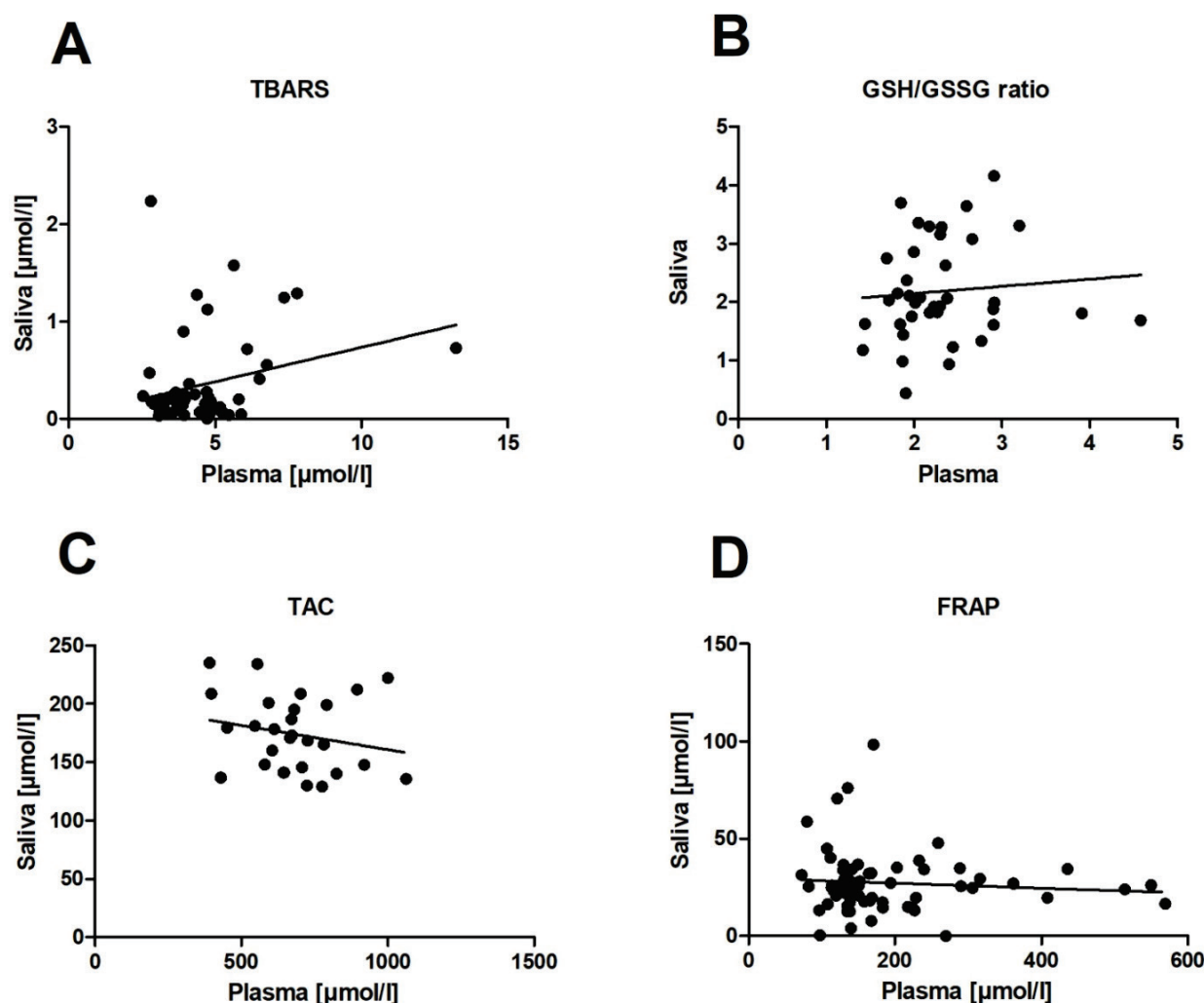


**Fig. 4.** Correlations between plasma and urinary concentrations of (A) TBARS – thiobarbituric acid reactive substances, (B) AOPP – advanced oxidation protein products, (C) fructosamine, (D) GSH/GSSG – ratio of oxidized/reduced glutathione, (E) TAC – total antioxidant capacity, (F) FRAP – ferric reducing antioxidant power.

that urinary TAC and FRAP decrease significantly with age. This could mean that urinary antioxidant status starts to decline in middle age, before changes in plasma antioxidant status manifest.

Systemic oxidative stress can be induced by various states and diseases, of which increased weight

and obesity belong to the most elementary. Oxidative stress in overweight and obesity can be triggered *via* various pathways. For instance, superoxide generation from NADPH oxidases, oxidative phosphorylation, chronic inflammation and low antioxidant status account for some of these pathways (Chrysohoou *et al.* 2007,



**Fig. 5.** Correlations between plasma and salivary concentrations of (A) TBARS – thiobarbituric acid reactive substances, (B) GSH/GSSG – ratio of oxidized/reduced glutathione, (C) TAC – total antioxidant capacity, (D) FRAP – ferric reducing antioxidant power.

Fernández-Sánchez *et al.* 2011, Serra *et al.* 2013, Savini *et al.* 2013). In this study, a negative correlation has been found between plasma fructosamine and bodyweight, and no correlations were found between salivary oxidative stress markers and weight. The mice were healthy in our study, despite the variability in bodyweight. Interestingly, it has been postulated, that urinary oxidative stress markers reflect the intensity of oxidative fat metabolism, which can protect against the development of obesity (Zurlo *et al.* 1990, Seidell *et al.* 1992, Valtueña *et al.* 1997, Marra *et al.* 2004, Il'yasova *et al.* 2005). This is reflected in the results of the present study, since a positive correlation between weight and urinary GSH/GSSG ratio has been found.

Urinary oxidative stress markers can reflect local and systemic changes of oxidative status. Nevertheless, these differ within various systemic disorders (Kirschbaum 2001). In the present study, where healthy

mice were examined, no correlations between urinary and plasma TBARS, AOPP, fructosamine, GSH/GSSG, TAC or FRAP concentrations were found. The absence of correlations between plasma and urinary oxidative stress markers suggests that urinary oxidative stress markers mirrors local oxidative status of healthy kidneys in the mice. Similarly, no correlations between salivary and plasma TBARS, GSH/GSSG, TAC or FRAP concentrations were found. In human studies, a variety of biomarkers have been shown to correlate between individual biofluids, such as plasma, urine and saliva suggesting that the analysis of these in alternative biofluids could be helpful in the evaluation of systemic status. For example, a positive correlation between salivary and plasma concentrations of FRAP was described in young athletes. Moreover, a positive correlation of salivary and plasma GSH concentrations has been shown in periodontitis patients as well as

healthy controls (Lyszczarz *et al.* 2002, Öngöz Dede *et al.* 2016). On the other hand, in patients with inflammatory periodontal disease, salivary TBARS has not been shown to correlate with plasma TBARS, which could mean that even a major local process does not necessarily reflect in systemic oxidative stress parameters (Celec *et al.* 2005, Iannitti *et al.* 2012). The discrepancies between our animal experiment and other human studies cannot be further explained as of now, since animal experiments related to this topic are simply missing.

During our experiment, several limitations emerged. It should be mentioned that saliva collection from mice without stimulation would produce unusable amounts. Stimulation by pilocarpine greatly increased salivary flow, but it still did not reach the optimal volume for analysis. Additionally, the stimulated flow probably influenced the composition of saliva, diminishing the sex differences or plasma:saliva correlations. It should also be mentioned that pilocarpine has been shown to induce oxidative stress in the rodent brain, although such an effect has not been studied in body fluids yet (Freitas *et al.* 2005). Salivary AOPP could not be measured, since 200 µl of undiluted saliva are needed for this assay. Even by stimulation, only around 400 µl of saliva were collected. Thus, most of the samples were used up for the measurement of other markers. Samples were not pooled, which could have solved the issue of low sample volume. Fructosamine was under the limit of detection when measured in saliva samples. However, fructosamine concentrations are under the limit of detection in human saliva as well, by using this spectrophotometric method. On the other hand, in urine, both of these markers were measurable.

In human studies, the normalization of salivary markers is still an unsolved question. Saliva flow rate, protein and creatinine concentrations have all been proposed for normalization. In this study, only urinary oxidative markers were normalized, to creatinine, except for AOPP, which was normalized to protein concentrations. Urine was only collected for 4 h during daytime, and the daily diuresis of the mice was calculated based on the volume produced during this period. Thus, the possible circadian rhythm of urine production was omitted.

The volatile anesthesia used for blood collection could also have influenced the measured oxidative stress markers, however, the body of literature on anesthetics

and oxidative stress is quite ambivalent. It has been found that isoflurane increases DNA damage by oxidative stress, but volatile anesthetics like isoflurane or sevoflurane have been found to reduce oxidative stress and inflammation in rodents (Lee *et al.* 2015, Ni *et al.* 2017). One study showed that inhalational anesthetics can induce oxidative stress to some extent, by acting against antioxidant defense systems (Ruxanda *et al.* 2015). Injectable anesthetics, such as ketamine, have been proposed to increase oxidative stress in rodent brains, but other studies describe their antioxidant properties (De Oliveira *et al.* 2009, Gokcinar *et al.* 2013). Moreover, during the measurement of TBARS, no antioxidant was added to the samples before heating. Thus, new, heat induced lipid peroxidation products could have formed.

In conclusion, the present study assessed the physiological values of oxidative stress and antioxidant status markers in the plasma, urine and saliva of healthy young to middle-aged mice. High inter-individual variability is currently a problem leading to minimal clinical use of urinary and salivary biomarkers. The results of saliva and urine analysis are unreliable, since many known (food, tooth-brushing, smoking, etc.), and unknown factors influence individual biomarker concentrations in alternative biofluids. Animal experiments under controlled conditions eliminate this bias and are therefore necessary. The current study proved that urine and saliva could be obtained in sufficient amounts even from small laboratory animals. The markers of oxidative status can be reliably measured in all examined biofluids. Although some of the between subject variability has been explained, other major sources of biological inconsistency are to be uncovered. Further studies should analyze the correlations in animal models of oxidative stress related diseases, to find out if urinary and salivary oxidative stress markers mirror systemic changes of oxidative stress.

## Conflict of Interest

There is no conflict of interest.

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