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Quantification of human diamine oxidase

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ABSTRACT

Objectives: Diamine oxidase (DAO) is essential for extracellular degradation of histamine. For decades activity assays with inherent limitations were used to quantify the relative amounts of DAO. No reference DAO standard is available. Absolute DAO amounts cannot be determined. Controversy exists, whether DAO is circulating or not in non-pregnant individuals. The role of DAO as biomarker in various diseases is ambiguous. It is not clear, whether precise quantification of human DAO antigen using commercially available enzyme-linked immunosorbent assays (ELISAs) is possible. The objective was to develop a precise and robust ELISA to quantify DAO in various biological fluids.

Design and methods: A research prototype ELISA was established using a mouse monoclonal antibody for capturing and a polyclonal rabbit serum IgG fraction for the detection of human DAO. The limit of blank (LoB), limit of detection (LoD) and estimated limit of quantification (eLoQ) and normal DAO concentrations in serum and plasma were determined.

Results: The LoB, LoD and eLoQ derived from 42 standard curves are 0.27, 0.48 and 0.7 ng/mL respectively. The detection range using the LoD as the lower and the highest DAO standard as the upper boundary is 0.5 to 450 ng/mL. Serum and plasma mean/median concentrations are between 0.5 and 1.5 ng/mL in healthy volunteers ($n = 58$) and mastocytosis patients ($n = 19$) and plateau at approximately 145 ng/mL ($n = 16$) during pregnancy. Accurate quantification was not influenced by heparin (DAO is a heparin-binding protein), lipaemic or hemolytic serum. The measured DAO antigen concentrations are in close agreement with published enzymatic activity data using radioactive putrescine as substrate.

Conclusions: This research prototype ELISA is able to reliably and accurately quantify human DAO in different biological fluids. The potential of DAO as biomarker in various diseases can be evaluated.

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1. Introduction

Human diamine oxidase (hDAO) belongs to the group of copper-containing amine oxidases [1]. They oxidatively deaminate primary amines to the corresponding aldehydes, ammonia and hydrogen-peroxide (H_2O_2). Human DAO preferentially degrades histamine and various polyamines like putrescine or spermidine [2]. Constitutive DAO expression in the intestine and kidney is high. Heparin is able to release DAO into the circulation in animals and humans [3]. It might compete with extracellular binding of DAO mediated by its heparin-binding motif [1,2]. DAO is also stored in the granules of neutrophils and released upon activation ([4]; unpublished data).

The concentration of human DAO compared to non-pregnant individuals increases several hundred-fold during pregnancy [5]. The plasma concentration of hDAO starts to increase at the end of the first trimester and reaches a plateau at gestation week 20. DAO activity was considered as a prognostic tool for pregnancy complications [6].

The enzymatic activity of hDAO seems to be an important protection mechanism against endogenous and exogenous histamine. DAO is considered the main enzyme for extracellular degradation of histamine. In a porcine model inhibition of DAO activity using aminoguanidine, a potent, irreversible and relatively specific DAO inhibitor, followed by oral histamine challenge, induced anaphylactic shock-like symptoms in almost all pigs, whereas control animals suffered no comparable signs [7]. In guinea pigs aminoguanidine increased the shock and death rate after histamine injections mimicking anaphylaxis [8]. After inducing severe anaphylaxis by insect-sting challenge in human subjects the mean

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histamine concentration reached life-threatening 142 ng/mL [9]. Although not published using human samples, plasma DAO seems to increase in animal models of anaphylaxis [10,11]. The enzyme might be a potential marker for the diagnosis of anaphylactic events. Serum concentrations of DAO have been measured also to monitor intestinal mucosal integrity [12].

For the last decades the quantification of DAO was solely based on various activity assays. DAO activity during pregnancy is readily measurable, but controversy exists about the presence of DAO in serum or plasma of non-pregnant healthy subjects. The following references used the “gold standard” DAO activity assay based on the spontaneous conversion of oxidized radioactive putrescine into delta-1-pyrroline, first described by Okuyama and Kobayashi [13]. Delta-1-pyrroline can be separated from the substrate using organic solvent extraction and quantified. In 77 healthy volunteers Tufvesson [14] measured a mean serum DAO activity of 3.9 pmol/mL/min. Schwelberger wrote that DAO is normally not present in blood plasma [15]. The same group failed to detect DAO in human serum by Western Blotting and suggested that enzymatic activity measured in plasma or serum is likely mediated by vascular adhesion protein-1 (VAP-1) but not DAO [16].

Enzyme activity assays have known inherent limitations. Substrate inhibition can be avoided by using lower concentrations. If the substrate is consumed too fast, the amount of enzyme will be underestimated or the assay must be repeated with different incubation times or dilutions. The dynamic range of the assay will be compromised. Substrate specificity problems might be avoided by using low concentrations of natural substrates with preferentially high affinity for the enzyme, but the assay sensitivity might be reduced. The presence of other enzymes capable of oxidizing the same substrate is not easily avoidable. Some of the measured “DAO activity” was assigned to VAP-1 [16], which is present in serum of healthy volunteers at 88 ng/mL [17]. Evidence of a circulating DAO inhibitor was published [18]. Sattler found that several medications substantially block porcine, canine and human intestinal DAO activity measurements [19,20]. It was also published that DAO activity is inhibited by ethanol and stimulated by acetaldehyde [21,22], although we could not confirm these data [23]. Finally, sensitive luminescence assays using horseradish peroxidase (HRP) H_2O_2 -coupling cannot be readily performed in body fluids with high antioxidant capacity like serum or plasma (unpublished data). The high antioxidant activity will reduce or even eliminate a specific signal at relevant substrate and enzyme concentrations. Such an assay will also not circumvent some of the other issues described above.

The availability of a precise and accurate enzyme-linked immunosorbent assay (ELISA) using hDAO as standard could circumvent many described limitations. Nevertheless, we are not aware of a commercially available hDAO ELISA, which can accurately quantify the amount of hDAO in biological samples. We tested two commercially available ELISAs, but their performance was not satisfying. One ELISA was not able to measure DAO levels in third trimester pregnancy samples and the ELISA DAO standards were not recognized by the second ELISA. The first ELISA was not further evaluated. In the manual of the second ELISA the difference in the median between DAO levels in second or third trimester and healthy controls is only 2-fold. Nevertheless, during pregnancy DAO activities increase several hundredfold [5].

To evaluate whether DAO might be a diagnostic marker for anaphylaxis, gastrointestinal ischemia, pregnancy complications and potentially other pathologic conditions like excessive neutrophil activation, a method for robust and accurate quantification of the DAO antigen must be available. Consequently, we developed an in-house research prototype DAO ELISA and the main characteristics are presented.

2. Material and methods

If not otherwise stated, reagents were purchased from Sigma-Aldrich, Vienna, Austria. High molecular weight heparin (Heparin

Gilvasan 1000 units/mL) was from Gilvasan Pharma GmbH, Vienna, Austria. Low molecular weight heparin (enoxaparin) was obtained from Sanofi Austria. All serum and plasma blood withdrawals tubes were from Becton Dickinson and Company. Octaplas (Octapharma, Vienna, Austria) is pooled citrate plasma from healthy subjects.

2.1. ELISA for endogenous and recombinant human DAO (rhDAO)

A cell culture-purified monoclonal antibody raised against human DAO isolated from Caco-2 cell supernatant [24] was coated onto white high protein binding microtiter plates (Greiner bio-one #655074, Kremsmuenster, Austria) at 5 µg/mL (100 µL) in 50 mM carbonate-bicarbonate buffer overnight at 4 °C and blocked for 50 minutes (min) using 1% BSA in PBS pH 7.4 containing 0.01% Thimerosal. The hybridoma cell line (clone anti-DAO8/119) was provided by Prof. Andrea Quaroni (Cornell University, Ithaca, NY). Twenty microliters of the standards or samples were mixed with 80 µL LowCross-Buffer (LCB, CANDOR bioscience, Wangen, Germany), added to the coated plates and incubated for 50 min. All determinations were performed in duplicates and at room temperature (RT). The washing solution was 0.1% Tween-20 in PBS. Human DAO was detected with a polyclonal rabbit IgG serum fraction (Rabbit-407) diluted 1 to 1000 in 0.2% Tween-20, 2% BSA HEPES Buffered Saline (HBS, pH 7.4). Rabbits were immunized with purified rhDAO ([33]; Eurogentec, Seraing, Belgium). The bound antibodies were detected with a 1 to 32,000 dilution of donkey anti-rabbit IgG HRP-labelled antibodies (Sigma-Aldrich #SAB3700928) diluted in HBS with 0.2% Tween-20 and 2% BSA. Donkey IgG was added at 10 µg/mL (ChromPure #017-000-003; Jackson ImmunoResearch, PA).

A standard curve from 0 to 450 ng/mL rhDAO purified and quantified as described [25] was prepared in 1% human serum albumin (HSA; Alburnorm 20%, Octapharma, Vienna, Austria) PBS. Single-use aliquots of a 10 µg/mL rhDAO solution in 100 mM potassium phosphate buffer pH 7.4 containing 1% HSA were used to prepare the rhDAO dilution row. Each concentration of the rhDAO standard was diluted 1 to 5 with LCB and 100 µL added to the microtiter plate. Single-use aliquots of serum from a subject with 8 ng/mL endogenous DAO concentration were used as control on each plate.

Bound HRP molecules were quantified using SuperSignal™ ELISA Pico Chemiluminescent Substrate (Thermo Fisher Scientific #37070, Vienna, Austria). After adding detection solution the plate was incubated 10 min at 30 °C and measured using a Perkin Elmer Victor2™ 1420 Plate Reader.

The signals of the DAO standards were used to calculate the DAO concentrations via 4PL non-linear regression using OriginPro 8.5 software. The “calculated DAO concentration” of the standard blank with no DAO added was subtracted from all samples. If the blank DAO standard recalculation or the calculation of the DAO sample concentration was negative, the concentration was set to zero. The limit of blank (LoB) is 0.27 ng/mL (1.645 * standard deviation (SD) of the blank). The limit of detection (LoD) is 0.48 ng/mL (LoB + 1.65 [$= c_{\beta}$ calculated using $1.645 / (1 - 1/4 \times f)$; f = degrees of freedom] * weighted SDs of the 0.21 and 0.62 ng/mL DAO standard). The estimated limit of quantification (eLoQ; LoD + 1.65 [$= c_{\beta}$] * weighted SDs of the 0.21 and 0.62 ng/mL DAO standard) is 0.70 ng/mL. LoB, LoD and eLoQ are calculated using 42 standard curve determinations. The dynamic range of the ELISA is approximately 900 ng/mL using the LoD of 0.48 ng/mL as the lower and 450 ng/mL, the highest standard, as the upper limit.

DAO concentrations measured in citrate plasma are multiplied with 1.17 because of dilution. Citrate is not penetrating cells and therefore the dilution factor is not 10. The citrate solution dilutes only plasma. The dilution factor is 14.3% (correction factor 1.17) using a hematocrit of 40 or 16.7% at a hematocrit value of 50 (correction factor 1.20).

2.2. Conversion of DAO activity to ng/mL antigen

Published DAO activity data were converted to ng/mL using units per mg enzyme activity values from two sources. The enzymatic activities with putrescine as substrate per mg DAO were extracted from published data using purified SF9 insect cell-derived [2] and purified Chinese hamster ovary (CHO) cell-expressed rhDAO [25]. Elmore et al. [2] used an HRP H₂O₂-coupled assay and Gludovacz et al. [25] measured the absorbance of the fusion product between oxidized putrescine, which spontaneously cyclizes to delta-1-pyrroline, and ortho-aminobenzaldehyde (DAOABA assay; [26]).

The DAO plateau activity (Fig. 8 in [2]) was manually extracted ($V_{\text{plateau}} = 1.4 \mu\text{mol/L/min}$) and converted to $\mu\text{mol/L/min/mg DAO}$. Peak or plateau activity was used, because the V_{max} was not described and at higher putrescine concentrations substrate inhibition was present. The copper content was extrapolated to 2 atoms per mol dimer using a correction factor of 1.7, derived by linear regression using data from Table 2 in Elmore et al. [2]. The activity of an “ideal” Elmore DAO batch was 2.93 units/mg DAO or 1 mg DAO oxidizes 2.93 $\mu\text{mol/min}$ putrescine.

Gludovacz et al. [25] published a V_{max} of 1.98 $\mu\text{mol/min/mg DAO}$. Nevertheless, the V_{max} value is an extrapolated value. The maximum measured DAO activity was seen at 400 μM putrescine. This was the highest concentration used for V_{max} calculations, because at higher values substrate inhibition was seen. Therefore, the V_{plateau} (1.83 $\mu\text{mol/min/mg DAO}$) was used for calculations. The rhDAO batch contained 1.99 copper per mol DAO dimer and no correction for copper content was necessary. Nevertheless, Gludovacz et al. [25] performed the assay at 30 °C and Elmore at 37 °C. To extrapolate from 30 °C to 37 °C we measured DAO activity using DAO from trophoblast cell culture supernatants, purified and non-purified rhDAO and porcine kidney DAO (Sigma-Aldrich #D7876) in the DAOABA and the DAOLUM assay (both described in [25]) at 30 °C and 37 °C. The DAOLUM assay measures the luminescence generated via HRP H₂O₂-coupling [27]. The mean conversion factor from 30 °C to 37 °C was 1.8 (data not shown), which is in excellent agreement with published data. Mondovi et al. [28] used porcine kidney DAO and measured oxygen consumption using putrescine, cadaverine and histamine at different temperatures. We calculated a mean conversion factor of 1.76 after extracting the data from Mondovi. Applying the temperature conversion factor increases the V_{plateau} from 1.83 to 3.24 $\mu\text{mol/min/mg}$. This is the enzymatic activity of an “ideal” rhDAO batch from CHO cells [25].

Elmore et al. [2] published an inhibitory constant for putrescine substrate inhibition of 430 μM . When we used the published data (Fig. 8A in [2]) and performed linear regression with extrapolation to 500 μM , DAO activity was only 54% compared to V_{plateau} . We saw 40% inhibition in the DAOABA assay using 600 versus 400 μM putrescine. Substrate inhibition using high putrescine concentrations was included in the analysis. Morgan and Illei [29] used high spermine concentrations in the DAO activity assay but we did not find appropriate numbers to include substrate inhibition for spermine.

Published enzyme activity data were converted to ng/mL using these considerations and the data are summarized in Tables 2 and 3. Only pregnancy samples after 120 days of pregnancy were used, when DAO activity was relatively stable and reached a plateau. DAO activity was extracted from the mean activity curves during the plateau concentration. The nine pregnancy samples used from Hanson et al. [31] were withdrawn at parturition.

2.3. Ethics

The local ethics committee of the Medical University of Vienna approved the study of patients with mastocytosis, recurrent abortions and blood withdrawals and urine collection from healthy volunteers. All patients and healthy individuals provided informed consent before collection of blood and urine samples.

2.4. Statistical analyses

Only standard statistical methods were used. They are listed with the data and the corresponding p-values.

3. Results

3.1. Description of the ELISA standard curve and determination of the LoB, LoD and eLoQ

The mean recalculated DAO concentrations of 42 standard curve determinations are shown in Fig. 1A. The coefficients of variation (CVs) are shown in Fig. 1B. The CV of the DAO standard curve is $>20\%$ $<0.07 \text{ ng/mL}$ after subtracting the mean blank value of 49 pg/mL. The CV is $<10\%$ $>0.65 \text{ ng/mL}$. The corresponding values after applying the 5-fold dilution factor are 0.36 and 3.3 ng/mL.

The standard curves are reproducible and accurate. Nevertheless, when we performed the ELISA with single-use rhDAO standard aliquots frozen at -20°C , we realized that the control endogenous DAO serum concentration increased after several weeks of storage. The mean was 8.0 ng/mL ($n = 9$; $\text{CV} = 8.9\%$) within the first 30 days but increased to 10.4 ng/mL DAO ($n = 12$; $\text{CV} = 13.5\%$; $p\text{-value} < 0.0001$) measured over 21 days two months after freezing. The most likely explanation for the increased values of the endogenous DAO control is that the rhDAO in the single-use aliquots is not stable after freezing at -20°C . After longer storage the values continued to increase (data not shown). We have data from many samples (unpublished data; see also below) that endogenous DAO is stable over years in serum or plasma. The pattern of increased DAO concentrations after storage of rhDAO at 10 $\mu\text{g/mL}$ for a few months was seen several times (data not shown). If samples are measured within a few weeks, this issue is not relevant. In the supplement we present data that, if necessary, we can also relatively precisely measure DAO samples over several months by normalization based on endogenous serum or plasma control samples (Supplement Fig. S1, Table S1). We are currently testing different formulations for long term stability of rhDAO after freezing.

3.2. Recovery rates after spiking rhDAO into plasma or serum

We decided to use 1% HSA in PBS as matrix because of interference with endogenous DAO. In Fig. 2A the mean recovery between 1% HSA PBS and serum or plasma with 99% confidence intervals (CIs) of spiked rhDAO added to EDTA, citrate plasma and serum is presented. At 450 ng/mL recovery is approximately 10% higher in plasma or serum compared to the 1% HSA PBS. The R^2 of a linear regression line is 0.998. Recovery at DAO levels $< 10 \text{ ng/mL}$ is affected by endogenous DAO concentrations (data not shown).

There is no significant difference spiking rhDAO into plasma or serum. Fig. 2B shows that endogenous DAO in serum or EDTA plasma is stable for several months and that there is no relevant difference between serum and plasma samples. The CV including all samples is 5.2%.

3.3. Determination of circulating DAO in healthy individuals and mastocytosis patients

Controversy exists, whether DAO is circulating or not. We measured DAO in 58 healthy volunteers and the results are presented in Fig. 3 and Table 1. Our DAO ELISA measured DAO in serum and citrate samples with 50% of the values below the LoB in citrate compared to 16% in serum samples. This difference is statistically significant (Fisher's exact test $p = 0.04$). No statistically significant differences were found between the percent distribution of serum and citrate above the LoD or above the eLoQ. The p-value using a t-test including all serum and

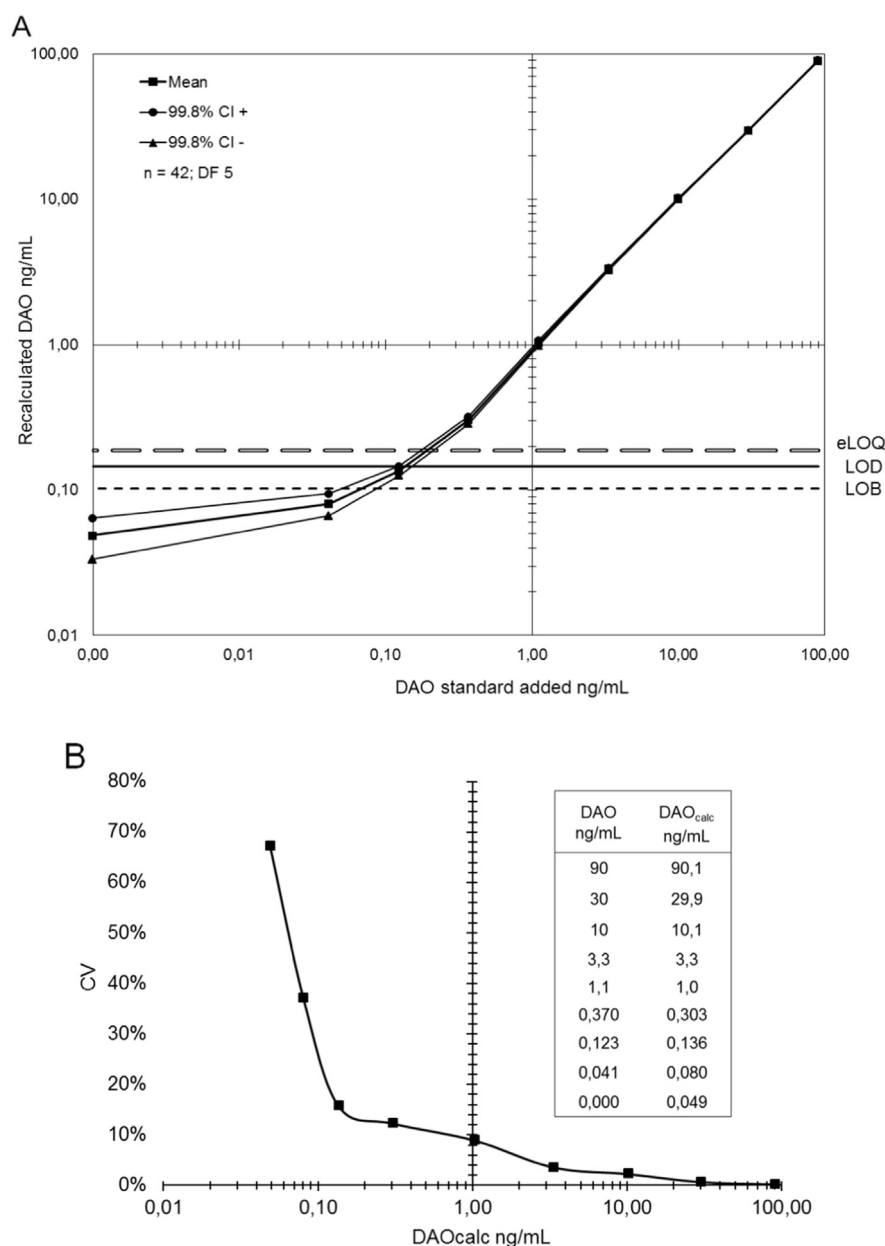


Fig. 1. DAO standard curve, coefficients of variation and detection limit parameters. (A) Single-use aliquots of purified rhDAO diluted with 1% HSA PBS were used at 9 different concentrations (0, 0.21, 0.62, 1.8, 5.6, 16.7, 50, 150 and 450 ng/mL). These standards were diluted 1 to 5 with LowCross-Buffer (LCB). The y-axis shows the mean DAO concentrations with the 99.8 confidence intervals of 42 standard curves calculated by the derived 4PL parameters. The x-axis represents the added concentrations of rhDAO. The 42 plates include 8 single and 17 double (two plates used in one experiment) plate measurements on different days. The presented values must be multiplied by the dilution factor (DF) 5 to obtain the added DAO amounts. The 49 pg/mL mean blank standard value was not subtracted from the graphically presented limit of blank (LoB), limit of detection (LoD) and estimated limit of quantification (eLoQ) values. (B) The coefficients of variation of the calculated DAO concentrations (DAO_{calc}) are presented on the y-axis. The insert shows the nominal (added; left) and calculated (measured; right) DAO values; HSA, human serum albumin; PBS, phosphate buffered saline.

citrate DAO concentrations was statistically significant at 0.011, but the p-value was 0.3 including only the DAO concentrations above the LoB.

We know that the DAO concentrations of endogenous DAO or rhDAO spiked into serum and plasma samples and left for 24 h in the refrigerator, at RT or undergoing one freeze/thaw cycle are not different (data not shown). Consequently, DAO concentrations are unlikely influenced, whether samples are left for a few hours before freezing. Nevertheless, we have data showing that serum tubes left for a few hours at RT contain increased DAO concentrations (data not shown). A potential source of DAO is the neutrophil activated by coagulation and complement activation [4]. EDTA and citrate inhibit coagulation and complement but not completely [30]. The mild outlier and the few DAO

values above 2 ng/mL in Fig. 3 might reflect neutrophil activation in the serum samples.

We performed a study with a total of 43 mastocytosis patients and collected EDTA plasma at baseline, after 24 and 48 weeks. For nineteen patients we have a complete EDTA data set. The corresponding DAO concentrations are presented in Fig. 4.

The mean/median DAO concentration at baseline, 24 and 48 weeks excluding the extreme outlier were 1.0/0.6, 1.1/0.7 and 1.2/0.7 ng/mL respectively. Seventy five, 61 and 46% of samples were above the LoB, LoD and eLoQ. All correlation coefficients (Pearson and Spearman; insert Fig. 4) are above 90%. The DAO concentrations in mastocytosis patients are not different to the serum or citrate samples from healthy

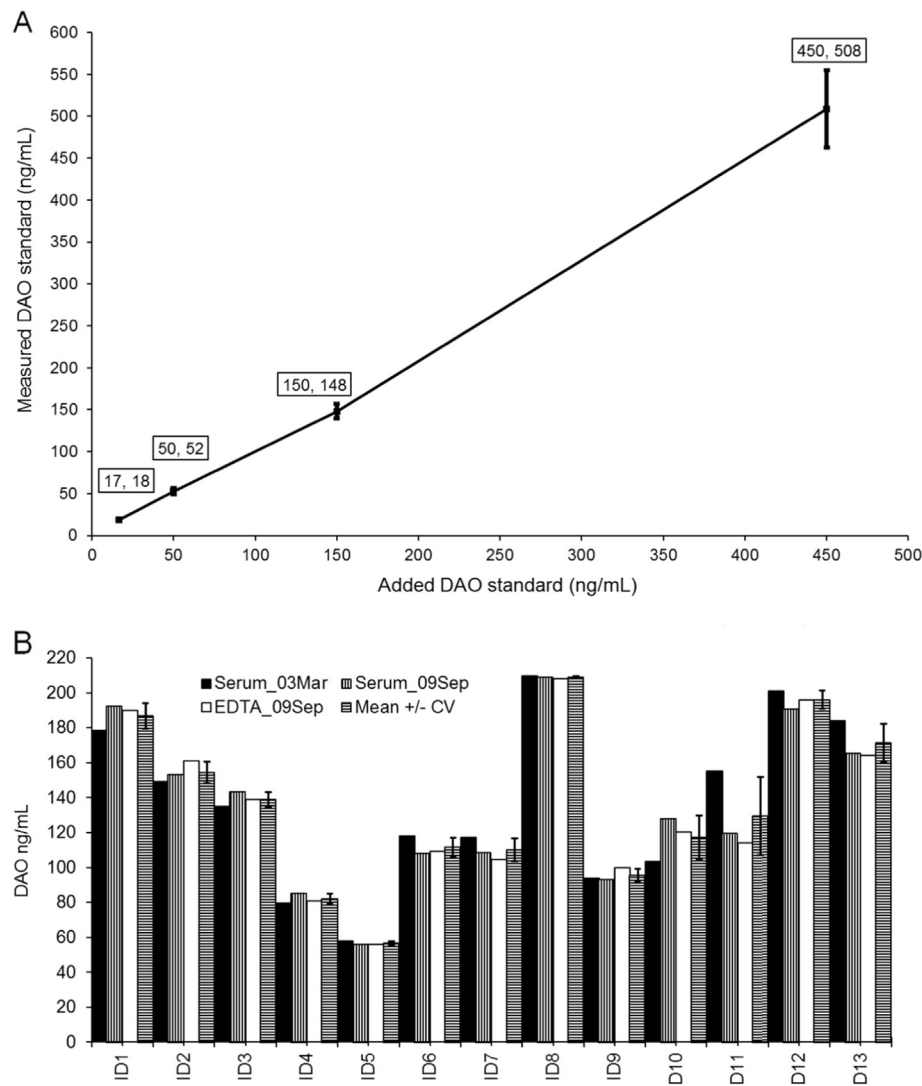


Fig. 2. Recovery of rhDAO spiked into sera and plasma and stability of endogenous DAO measurements. (A) Seven sera, five EDTA plasma and two times Octaplas citrate plasma samples were tested on two days (six different serum and plasma samples on each day; the same Octaplas batch was used twice) after spiking 16.7, 50, 150 and 450 ng/mL rhDAO into the different samples (x-axis). The mean recovered DAO concentrations with the 99% confidence intervals are shown. The first number in the small rectangles represents the measured DAO concentrations in 1% HSA PBS (matrix for the DAO standard). The second number is the recovered DAO concentration after spiking into sera and plasma samples. The R^2 of a linear regression line is 0.998. (B) Thirteen pregnancy sera were measured 6 months apart. EDTA plasma of the same women were also measured after at least 8 months storage at -20°C . The fourth column represents the mean of the three measurements including the coefficients of variation (CVs), which have been converted to ng/mL using the mean values. The overall CV for the means of the three measurements is 5.2%.

volunteers (Fig. 3). Tryptase levels, elevated in mastocytosis patients, did not correlate with DAO values (data not shown). The data in Fig. 4 imply that DAO levels are stable in individual mastocytosis patients and that our ELISA can reliably and accurately measure DAO concentrations in samples frozen for >2 years. The extreme outlier might be caused by activation of mast cells with heparin and consequently heparin-induced DAO release.

We were also able to measure high DAO concentrations far above the eLoQ in Caco-2 cells, placenta explant and trophoblast cell culture supernatants and amnion fluid. Spiking of recombinant DAO indicated no relevant interference in these matrices (data not shown).

A $>30,000$ -fold molar excess of high and low molecular weight heparin over DAO, abnormal concentrations of cholesterol and triglycerides and high concentrations of hemoglobin (tested range from 0.033 to 2.9 g/dL) did not significantly interfere with the DAO ELISA. These data are presented in the Supplement Figs. S2, S3 and S4 and Table S2.

Spiking of DAO into EDTA plasma of patients with combined hepatic and renal insufficiency allowed recovery rates of approximately 90%, but these data must be considered preliminary (see Supplement). We were able to detect DAO in urine samples, but the current protocol does not allow accurate and reliable quantification (see Supplement).

3.4. Comparison of research prototype with commercially available DAO ELISAs

We also compared our ELISA with a commercial (COM) DAO ELISA and some data are presented in the Supplement Fig. S5. This COM ELISA was used to measure DAO antigen levels in several publications (references 10 to 15 in the Supplement).

3.5. Comparison of DAO ELISA with published DAO activity data

The “gold” standard DAO activity assay is based on the oxidation of radioactive putrescine to 4-aminobutyraldehyde, which spontaneously

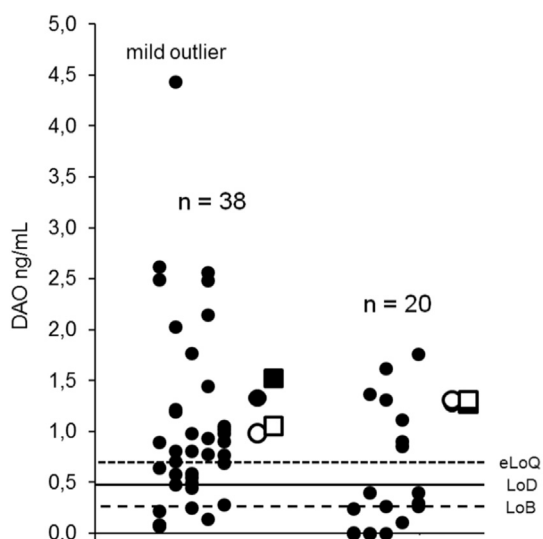


Fig. 3. DAO serum and citrate plasma concentrations in 58 healthy volunteers. DAO concentrations in the sera of 38 healthy individual (mean/median age 33/30; standard deviation (SD) = 9.1) are plotted on the left side. The data point 4.4 ng/mL is a mild but not an extreme outlier (upper inner fence: $Q3 + 1.5 \times IQR = 3.6$ ng/mL; upper outer fence: $Q3 + 3 \times IQR = 5.4$ ng/mL) using only the DAO values above the limit of detection (LoD) for outlier calculations. It is an extreme outlier including all data points. DAO concentrations in citrate plasma of 20 healthy individuals (mean/median age 27/26; SD = 7.7) are plotted on the right side. One value at 9.6 ng/mL was excluded because it is an extreme outlier (upper outer fence: $Q3 + 3 \times IQR = 4.0$ ng/mL) using only the DAO values above the LoD for outlier calculation. The mean (closed symbols) and median (open symbols) of the DAO concentrations above the LoD (circle) and estimated limit of quantification (eLoQ, square) are shown adjacent to the individual values. The horizontal lines at 0.27, 0.48 and 0.7 ng/mL represent the limit of blank (LoB), LoD and eLoQ respectively. Q, quartile; IQR, interquartile range.

cyclizes to delta-1-pyrroline. This ring molecule can be separated from the substrate precursor by organic liquid extraction and precisely quantified. In Tables 2 to 4 we present data that the published activity DAO values are quite congruent to our ELISA concentration measurements. We converted DAO activity data into ng/mL using the published specific activities of two purified rhDAO enzymes. Table 2 lists the published DAO activity values converted to pmol/mL/min and the corresponding ng/mL values. Table 3 provides more detailed information about the utilized assays.

In Table 4 DAO concentrations from non-pregnant and pregnant individuals presented in this study are directly compared to the DAO concentrations extrapolated from the published oxidation rates using the “gold standard” DAO activity assay with appropriate concentrations of isotopic putrescine as substrate.

DAO antigen concentrations in healthy volunteers and mastocytosis patients are about 1 to 2 ng/mL (see Figs. 3 and 4; Table 4). The mean and median of 16 third trimester DAO concentrations in normal pregnancies were 143 and 147 ng/mL respectively (Table 4; data not

shown). The putrescine-based activity assay data are close to our DAO ELISA data. The last three assays listed in Tables 2 and 3 have been performed at substrate concentrations far below or above the K_m . The corresponding calculated DAO values fit neither to the putrescine-based assays nor to our ELISA results. In the Supplement we comment also on a commercially available DAO activity assay based on the conversion of radioactive putrescine at a very low substrate concentration.

4. Discussion

A histamine-degrading enzyme in lung tissue of oxen and horses was first demonstrated by Charles Best in 1929 [35]. Nevertheless, lung tissue contains very little histamine-degrading activity [36] and DAO mRNA compared to kidney, duodenum or jejunum [2]. It is possible that histamine *N*-methyl transferase and/or AOC4, an enzyme homologue of VAP-1 silenced in humans, were responsible for histamine inactivation [15].

Almost ninety years later we are still not able to unambiguously assign measured enzymatic putrescine or histamine oxidation activity in human plasma or serum to a specific enzyme. The presence or absence of DAO in human serum or plasma has not been firmly established [15, 16]. Activity assays have inherent limitations and two commercially available DAO ELISAs did not perform satisfactorily in our hands (see Supplement).

In human serum 61% of samples show an hDAO antigen signal above the eLoQ of our research prototype DAO ELISA. The corresponding percentage in citrate plasma is only 35%. The discrepancy might be explained by neutrophil activation during serum coagulation. Activated neutrophils release DAO (unpublished data; [4]). For accurate quantification of circulating DAO concentrations, we recommend using EDTA or citrate plasma and not serum.

About half the plasma samples produce a signal below the LoB meaning that if DAO is present at all, the concentration is <270 pg/mL. In mastocytosis patients 46% of EDTA plasma samples show a signal above the eLoQ. Only 25% are below the LoB. Nevertheless, mastocytosis patients have an increased number of mast cells and consequently elevated circulating tryptase concentrations, which also implies that heparin is continuously released from mast cell granules [37]. Heparin released from abundant mast cells in the gastrointestinal tract might release DAO from its storage sites and might be responsible for the higher (mean of 0.97 versus 0.54 ng/mL) but statistically not significantly (p -value = 0.09) different DAO concentrations in EDTA plasma from mastocytosis patients compared to citrate plasma from healthy volunteers.

DAO quantifications based on radioactive activity assays using substrate concentrations close to the K_m and $V_{plateau}$ and the hDAO ELISA data are comparable in non-pregnant individuals and during pregnancy. We did not expect this high congruency between the published DAO activity data converted to ng/mL and our DAO ELISA data. These calculations were performed with DAO activity data from two different purified recombinant DAO proteins [2,25] and validate both the radioactive activity assay data published over several decades but also the DAO concentrations derived from the new ELISA. Activity data derived using assays with substrate concentrations far below or above the K_m have to be cautiously interpreted.

If a medication inhibits DAO activity, the antigen concentration determined by ELISA should be the same but an activity assay will indicate DAO inhibition or even the absence of DAO. Diminazene aceturate and aminoguanidine, two potent and the later irreversible DAO inhibitors, do not interfere with ELISA measurements (data not shown). A possible natural DAO inhibitor in pregnancy serum was described but not further characterized [18]. The tools to hunt for natural or iatrogenic DAO inhibitors are now available.

Although we can detect hDAO antigen in a certain percentage of citrate plasma samples from healthy volunteers, no obvious physiological role might be assigned to the amount of circulating enzyme

Table 1
DAO concentrations in serum and citrate plasma of healthy subjects.

	Mean	Median	Percent
Serum samples n = 38			
>LoB; n = 32	1,2	0,9	84%
>LoD; n = 29	1,3	1,0	76%
>eLoQ; n = 23	1,5	1,1	61%
Citrate samples n = 20			
>LoB; n = 10	1,0	1,0	50%
>LoD; n = 7	1,3	1,3	35%
>eLoQ; n = 7	1,3	1,3	35%

LoB = 0.27; LoD = 0.48; eLoQ = 0.7. LoB, limit of blank; LoD, limit of detection; eLoQ, estimated limit of quantification.

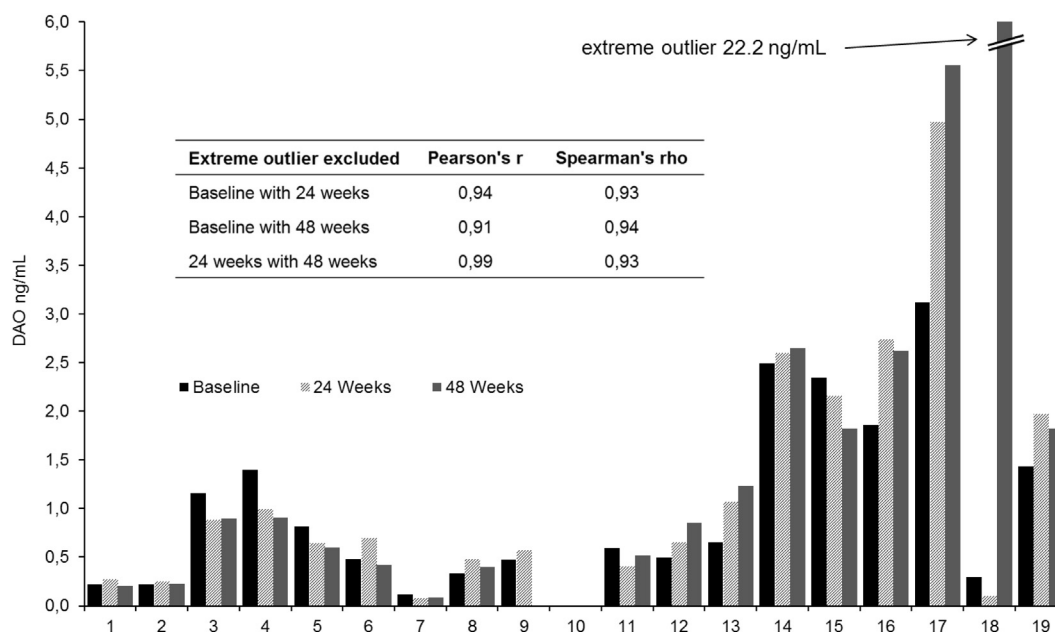


Fig. 4. EDTA plasma DAO levels in 19 mastocytosis patients at three time points. The DAO concentrations of 19 EDTA plasma samples were measured at baseline (black bars), after 24 (light grey bars) and 48 (dark grey bars) weeks. The data point 22.2 ng/mL is an extreme outlier and was excluded for the correlation coefficient calculations. The upper outer fence ($Q3 + 3 \times IQR$) for all data points ($n = 57$) or for only the values greater than the LoD ($n = 35$) was 6.5 and 7.9 ng/mL respectively. The insert shows Pearson's r and Spearman's ρ comparing baseline with 24 and 48 weeks and 24 with 48 weeks; Q , quartile; IQR , interquartile range.

considering that the histamine degradation capacity is rather limited and the physical half-life of histamine in the blood is only a few minutes. One nanogram/milliliters DAO, the level we find on average in non-pregnant individuals, can oxidize histamine only at a rate of 90 pg/mL/min. This number is derived using hDAO activity data from Gludovacz et al. [25] corrected for the lower ($\sim 50\%$) oxidation capacity of DAO towards histamine over putrescine. The true activity rate is certainly lower because the 90 pg/mL/min oxidation rate is calculated using velocity data at the K_m of histamine, which is 2.8 μM [2]. Secondly, this velocity is pathophysiologically not relevant considering that histamine starts to exert symptoms only above a few ng/mL and has a short circulatory half-life of a few minutes. DAO concentrations are approximately 150 ng/mL during the second or third trimester of pregnancy. This concentration of hDAO degrades 13.5 ng histamine per mL/min. Only high levels of histamine might become

hemodynamically relevant during pregnancy, if DAO is not inhibited or expression compromised.

Finally, many pathophysiological states have been associated with altered DAO activity (reviewed in [38,39]). The availability of an accurate and robust ELISA against hDAO should allow to substantiate or to refute the role of DAO as a potential biomarker. The release of DAO during anaphylaxis has only been described in animals and we are not aware of any human data. We are currently attempting to procure samples from anaphylactoid events.

5. Conclusions

Almost ninety years after the discovery of a histamine-degrading enzymatic activity, later renamed diamine oxidase, we are now able to precisely measure the concentrations of hDAO in various biological fluids.

Table 2

Conversion of published DAO activity data into ng/mL – activity conversion.

n (NP; P)	DAO activity			DAO ng/mL				Ref
	Non-pregnant (NP) ^a	Pregnant (P) ^a	Ratio P/NP	Elmore NP	MUV NP	Elmore P	MUV P	
34; 10	1,5	207	139	1,0	0,9	142	128	[18]
25		520				177	160	[6]
45		585				200	181	[5]
77	3,9			2,7	2,4			[14]
9		1470				503	455	[31]
Corr_SI		2450				302	273	
20	2,4			1,6	1,5			[41]
45		1157				395	357	[32]
Corr_SI		1928				237	214	
17; 57	1,7	155	91	2,0	1,8	178	161	[29]
17; 25	0,018	11,7	650	0,04	0,04	27	25	[40]
37	5640			1926	1741			[33]
38	81,7			28	25			[34]

^a Converted into pmol/mL/min from the described units and unit definitions; n, number of subjects (partially multiple measurements in pregnancy samples); Corr_SI, correction for substrate inhibition; NP, non-pregnant; P, pregnant; Elmore refers to [2] and MUV (Medical University of Vienna) to [25]; Ref, reference.

Table 3

Conversion of published DAO activity data into ng/mL – assay characteristics.

Assay type	[Substrate] μM	Activity at K_m or V_{max}	n (NP; P)	% serum or plasma	Ref
C ¹⁴ -Put	27	K_m	34; 10	95% PI	[18]
C ¹⁴ -Put	270	V_{max}	25	95% PI	[6]
C ¹⁴ -Put	270	V_{max}	45	23% PI	[5]
C ¹⁴ -Put	25	K_m	77	25% S	[14]
C ¹⁴ -Put	500	V_{max} ; SI	9	50% PI	[31]
C ¹⁴ -Put	25	K_m	20	25% S	[41]
oABA Put	1000	V_{max} ; SI	45	40% S	[32]
C ¹⁴ -Spm	4000	V_{max} ; SI?	17; 57	33% S	[29]
H ³ -His	0,075	$\ll K_m$	17; 25	2.5–5% PI	[40]
Cad; HRP	30,000	$\gg K_m$; SI?	37	13% S	[33]
Cad; HRP	30,000	$\gg K_m$; SI?	38	6% S	[34]

C¹⁴-Put, radioactive putrescine; oABA, ortho-aminobenzaldehyde; C¹⁴-Spm, radioactive spermine; H³-His, radioactive histamine; Cad, cadaverine; HRP, horseradish peroxidase (HRP and H₂O₂-coupled assay); SI, substrate inhibition; n, number of subjects (partially multiple measurements with pregnancy samples); NP, non-pregnant; P, pregnant; S, serum; PI, plasma; Ref, reference.

Table 4
Comparison of DAO concentrations measured in serum and plasma with the “gold standard” DAO activity assay using C¹⁴ putrescine and the DAO ELISA.

Assay	Mean (ng/mL)	n	Matrix	Reference
Non-pregnant DAO ELISA	1,2	32	20% serum ^b	This study
	1,0	10	20% plasma ^b	
	1,4	42	20% plasma ^b	
Mean of means	1,2	84		
C ¹⁴ putrescine ^a	0,9	34	95% plasma	[18]
	2,4	77	25% serum	[14]
	1,5	25	25% serum	[41]
Mean of means	1,6	136		
Pregnant DAO ELISA	143	16	20% serum ^b	This study
C ¹⁴ putrescine ^a	128	10	95% plasma	
	160	25	95% plasma	
	181	45	23% plasma	
	273 ^c	9	50% plasma	[31]
Mean of means	156	89		

^a DAO activity was converted to ng/mL using the specific activity of rhDAO in Ref. [25].
^b The means of the DAO concentrations > the LOB are shown.
^c DAO activity was corrected for substrate inhibition as described in [Material and methods](#).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.clinbiochem.2016.12.011>.

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Supplementary data to:

Quantification of human diamine oxidase

Thomas Boehm, Sophie Pils, Elisabeth Gludovacz, Helen Szoelloesi, Karin Petroczi, Otto Majdic, Andrea Quaroni, Nicole Borth, Peter Valent, Bernd Jilma

1. Material and methods:

1.1. Delipidation

The cholesterol and triglyceride concentrations of two individual lipaemic sera, two pools with sera from three donors each and one serum from a healthy volunteer were determined before and after delipidation. Recombinant hDAO was added (0, 2, 20 and 200 ng/mL) before lipid extraction. Delipidation does not influence DAO activity or recovery in the ELISA (data not shown). After adding EDTA (100 μ M) to all samples 2 volumes of a mixture of 40% butanol and 60% di-isopropylether were added and incubated for 45 min using gentle end-over-end rotation [4]. After centrifugation at 2000 rpm for 2 min the aqueous phase was removed. After delipidation sera were clear.

1.2. Hemolysis

Four hundred μ L of EDTA whole blood from two volunteers were mixed with 600 μ L erylysis buffer (155 mM ammonium chloride, 100 μ M EDTA and 10 mM KHCO_3 ; pH=7.4) and incubated for 5 min at RT. After centrifugation at 350 g for 10 min the supernatant (lysate) contained more than 90% of the total hemoglobin. Autologous plasma was used to dilute the lysate. Recombinant hDAO was spiked at 2 and 20 ng/mL. The endogenous plasma DAO concentrations were 1 and 2 ng/mL. The hemoglobin concentrations were measured with the QuantiChrom kit (BioAssay System; QHB-Kit, CA). The samples were frozen overnight before performing the ELISA to mimic frozen hemolyzed samples.

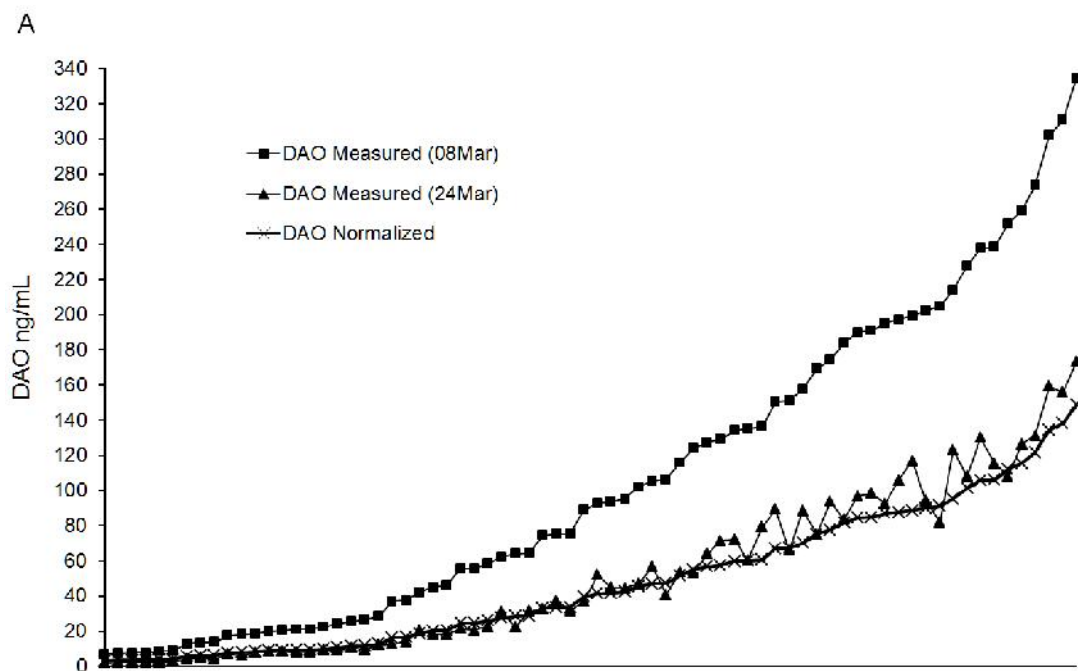
1.3.Commercial DAO ELISA kits tested

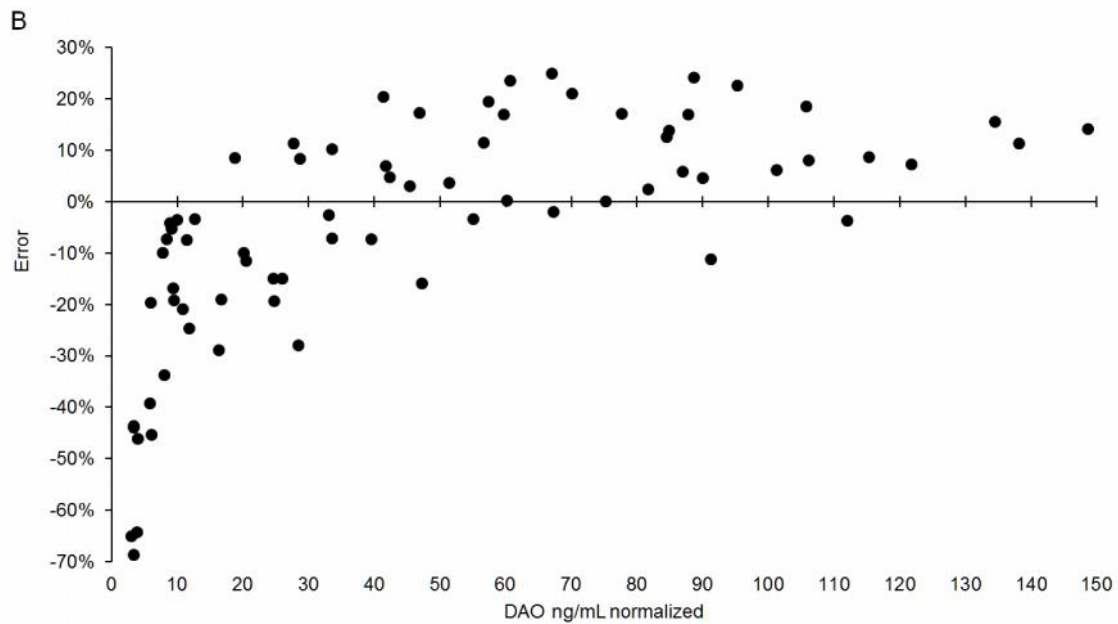
We tested two commercially available ELISAs. The E90656Hu ELISA Kit for Diamine Oxidase (DAO) Homo sapiens from USCN Life Science Inc. (Wuhan, China) and the K8500 human DAO ELISA from Immundiagnostik AG (Bensheim, Germany). The USCN ELISA was not able to measure DAO levels in third trimester pregnancy samples and was not further evaluated. The K8500 DAO ELISA from Immundiagnostik is labelled COM ELISA and was directly compared to our ELISA using the same samples.

2.Results:

2.1.Normalization of DAO measurements

Figure S1A shows repeated DAO concentration measurements of 72 samples from 20 different women during early and late pregnancy using an rhDAO standard frozen for several months at -20°C (DAO Measured [08Mar]) versus a freshly frozen rhDAO standard (DAO Measured [24Mar]). The third curve (DAO normalized) represents the normalized values.





Supplement Figure S1A and B

Supplement Figure S1: Normalization of measured DAO concentrations.

(A) DAO concentrations in 72 EDTA plasma samples from twenty women were measured with an “old” [DAO Measured (08Mar)] and “new” [DAO Measured (24Mar)] single-use rhDAO standard aliquot for calculating the standard curve. The endogenous serum DAO control was the same. The DAO Normalized values were calculated by using the ratio of the endogenous DAO concentrations measured with the old and new DAO standard control (2.25). The DAO standard frozen for several months overestimated the DAO concentration by 225%. All DAO concentrations [DAO Measured (08Mar)] were divided by this factor. (B) The DAO normalized concentrations with the corresponding error in relation to the measured DAO concentrations (24Mar) are plotted. A positive error means underestimation of the DAO concentration.

The normalization factor of 2.25 was calculated by dividing the DAO concentrations of the same endogenous DAO serum control sample measured using either an “old”, frozen for several months (08Mar), or a “new”, frozen for a few days (24Mar), rhDAO standard. This ratio is close to the mean/median (2.36 and 2.3) of the 72 ratios of DAO concentrations

calculated by dividing the DAO concentrations measured with the old (08Mar) and new (24Mar) DAO standard. Figure S1B shows the errors of measured minus calculated in percent of measured (DAO Measured [24Mar]) DAO concentrations on the y-axis and the normalized DAO concentrations on the x-axis. Table S1 describes the relative and absolute errors in some detail. DAO concentrations after normalization are on average 25.2% different between 0 and 25 ng/mL and 10.4 to 12.3% between 26 and 150 ng/mL. The relative errors are -24.5% between 0 and 25 ng/mL, 0.5% between 26 and 51 ng/mL and about 10% between 51 and 151 ng/mL (Table S1). This error rates seem acceptable, if it were really necessary to use this normalization procedure. We currently test different formulations for long term stability of single-use rhDAO standard aliquots frozen at -20°C.

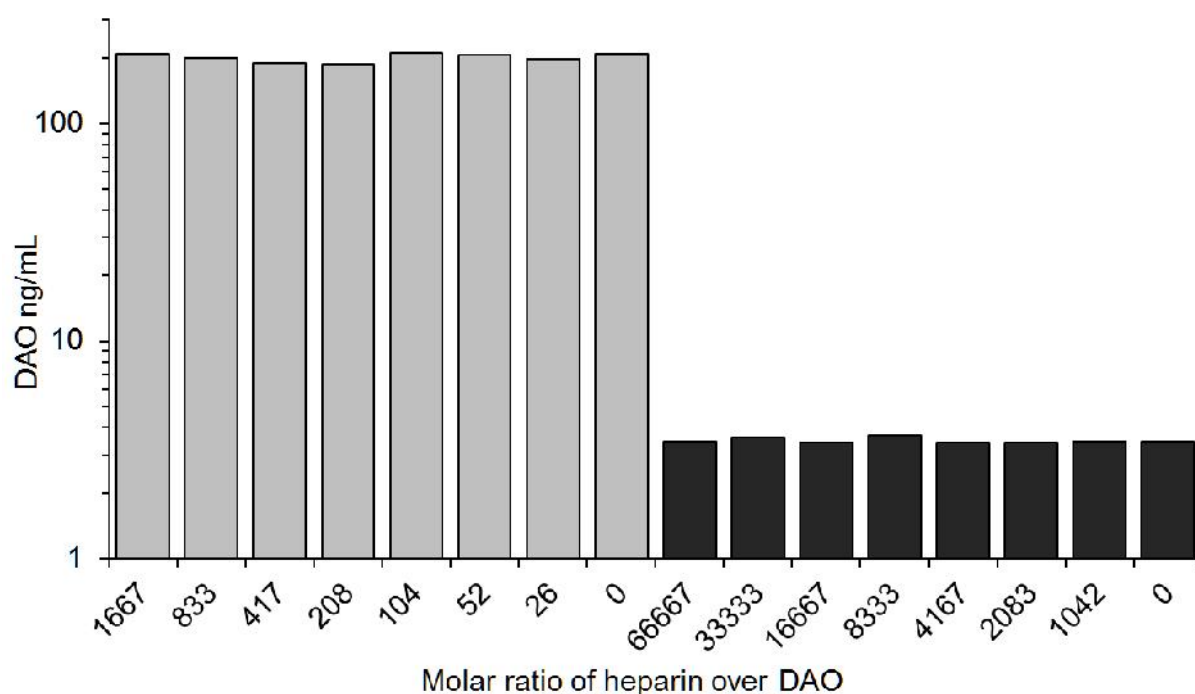
Supplement Table S1: Percent error after normalization

DAO_norm Range	Mean	Median	SD	SEM	n
Absolute error					
0-25 ng/mL	25,2%	19,3%	19,7%	3,7%	28
26-50 ng/mL	11,3%	9,4%	7,2%	1,9%	14
51-100 ng/mL	12,3%	12,7%	8,6%	1,9%	21
100-150 ng/mL	10,4%	8,7%	4,9%	1,6%	9
Relative error					
0-25 ng/mL	-24,5%	-19,3%	20,5%	3,9%	28
26-50 ng/mL	0,5%	3,9%	13,8%	3,7%	14
51-100 ng/mL	10,7%	12,7%	10,6%	2,3%	21
100-150 ng/mL	9,6%	8,7%	6,5%	2,2%	9

SD, standard deviation; SEM, standard error of the mean; DAO, diamine oxidase.

2.2.No influence of heparin, lipaemia and hemolysis on DAO antigen measurements

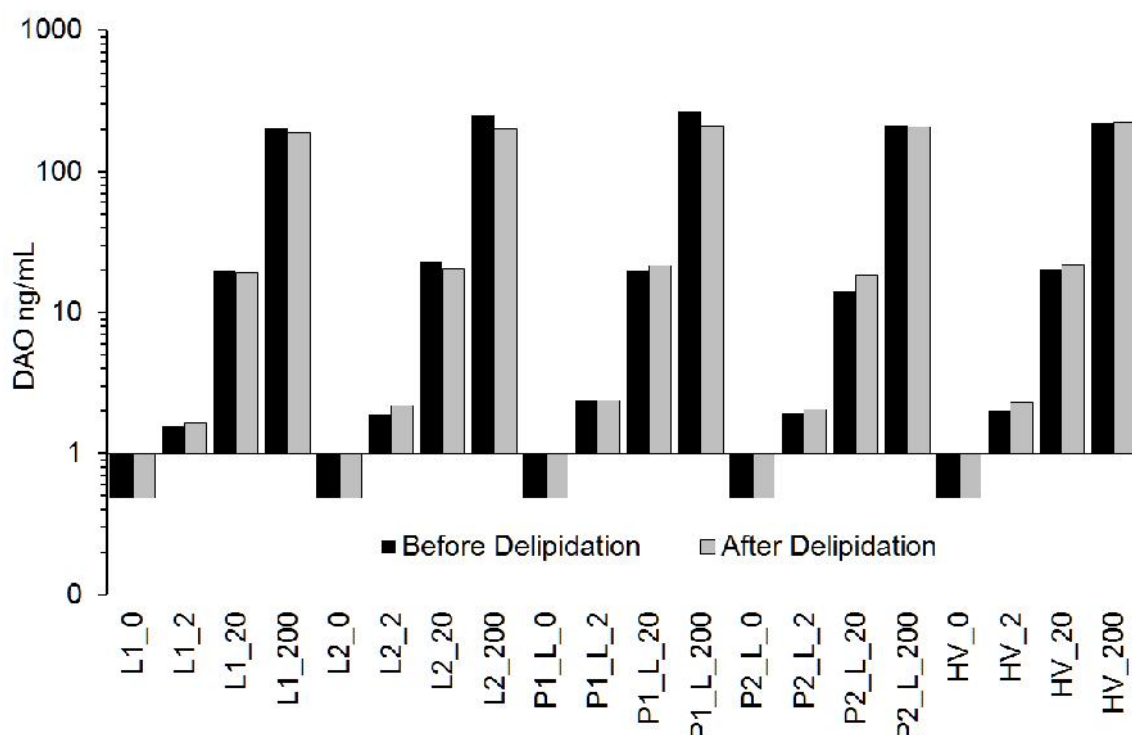
Intravenously administered high molecular weight heparin is releasing DAO into the circulation and it might accomplish this effect by competition with extracellular binding of DAO mediated by its heparin-binding motif [1; 2; 3]. Heparin or heparan sulfate containing proteoglycans could mask antibody binding sites on the molecular DAO surface severely interfering with our ELISA measurements. We therefore tested our assay in the presence of different concentrations of unfractionated high molecular weight heparin (Figure S2). The highest tested molar excess of heparin over DAO was more than sixty thousand-fold. The CV for spiking 5 and 200 ng/mL DAO (n=8) were 3.0% and 4.5% respectively. We performed this experiment also using a standard antibody dilution buffer (2% BSA, 0.02% Tween 20 in PBS) instead of the LowCross-Buffer and obtained similar results (data not shown). A thirty-three thousand-fold excess of low molecular weight heparin (enoxaparin) did not influence the measurements of 30 ng/mL DAO (data not shown).



Supplement Figure S2: Heparin does not interfere in the DAO antigen measurements.

The incubation buffer 1% HSA PBS was spiked with 5 and 200 ng/mL (60 pM and 2.4 nM) rhDAO and different concentrations of unfractionated high molecular weight heparin were added. One unit was considered to correspond to 5 μ g heparin and an average molecular weight of 15000 Da was used for calculations. One molecule heparin was assumed to bind to one DAO dimer. The highest heparin concentration was 6 units/mL, 2 μ M or 30 μ g/mL. The x-axis shows the molar fold-excess of heparin over DAO. The different samples were incubated for 15 min at room temperature before dilution with LCB and addition to anti-DAO antibody coated microtiter plates. The means of duplicates are shown; HSA, human serum albumin; PBS, phosphate buffered saline; LCB, LowCross-Buffer.

High triglyceride concentrations resulting in milky, turbid plasma or serum are not uncommon in clinical samples and might interfere with accurate DAO quantifications. Therefore we tested the effect of lipaemia on the performance of the DAO ELISA. We spiked rhDAO at different concentrations into two single lipaemic sera, two pools of three individuals and one serum from a healthy subject with no lipaemia. After DAO spiking the samples were split and one half delipidated using the method by Cham [4]. The lipid concentrations before and after delipidation are summarized in Table S2. The triglyceride and cholesterol levels are reduced between 8- and 33-fold. We did not detect any relevant influence on DAO antigen measurements at high or low triglyceride and cholesterol concentrations. The results are shown in Figure S3.



Supplement Figure S3: Lipaemia does not interfere with DAO antigen measurements.

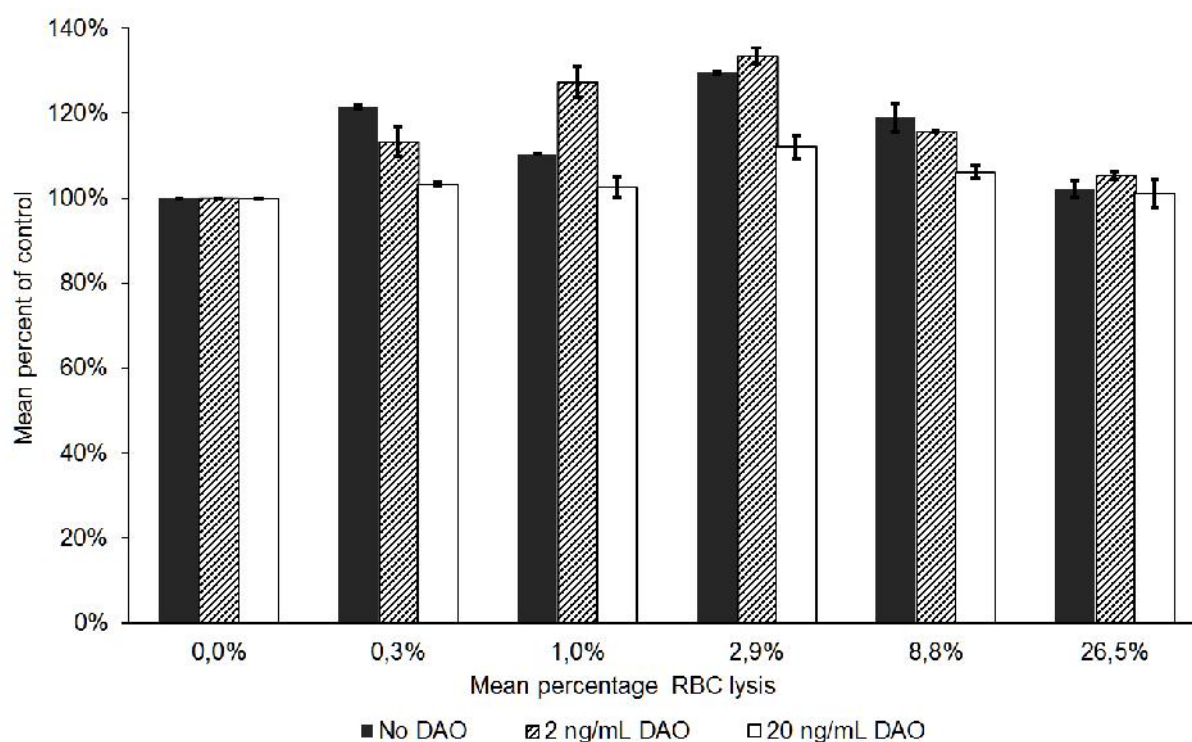
Two single lipaemic sera (L1, L2), two pools of three individuals (P1, P2) and one serum from a healthy volunteer (HV) were spiked with different rhDAO concentrations (0, 2, 20 and 200 ng/mL). After spiking the sera were delipidated according to Cham [4] and the DAO concentrations were measured with and without delipidation. All DAO concentrations without DAO spiking were below the LoD but the LoD value of 0.48 is shown for better graphical presentation. The y-axis shows the mean of duplicates. L, lipaemic serum; P, pool of lipaemic sera; HV, healthy volunteer.

Supplement Table S2: Triglyceride and cholesterol concentrations before and after delipidation.

	Before	After	Ratio
	Delipidation		Before/After
	Delipidation		Delipidation
L1_TG	982	29	33
L1_Chol	133	17	8
L2_TG	436	24	18
L2_Chol	256	20	13
P1_L_TG	1518	46	33
P1_L_Chol	351	23	15
P2_L_TG	2982	89	33
P2_L_Chol	450	25	18
HV_TG	148	18	8
HV_Chol	185	19	10

TG, triglycerides (mg/dL); Chol, cholesterol (mg/dL); L, lipaemic serum; P, pool of lipaemic sera; HV, healthy volunteer.

We also tested the influence of hemolyzed erythrocytes on the DAO ELISA performance. Whole blood from two healthy volunteers was artificially hemolyzed and mixed with autologous EDTA plasma at several ratios. After spiking different concentrations of DAO into the samples they were frozen for at least 24 hours before DAO ELISA measurements to mimic freezing of hemolyzed serum or plasma. No significant effect on DAO measurements was evident starting the dilution row with 2.9 g/dL free hemoglobin (Figure S4). This concentration corresponds to approximately 25% hemolyzed erythrocytes.



Supplement Figure S4: Hemolysis does not influence DAO antigen measurements.

The RBCs of whole blood of two healthy volunteers were lysed using an ammonium chloride-based lysis buffer and hemolyzed plasma was mixed at different ratios with autologous EDTA plasma. Recombinant hDAO was spiked into hemolyzed plasma at 0, 2 and 20 ng/mL. The mean endogenous DAO concentration of the two samples was 1.5 ng/mL. The mean DAO concentrations of the two plasma samples as percent of control EDTA plasma without hemolysis (100% bars on the left) are shown. The highest hemoglobin concentration (26.5% mean percentage RBC lysis) in the plasma samples corresponds to 2.9 g/dL. The error bars represent the CVs. All CVs are below 4%; RBCs, red blood cells; CVs, coefficients of variation.

2.3.Measurement of DAO concentrations in patients with liver and kidney damage

In a preliminary experiment we measured DAO concentrations in EDTA plasma from a few patients (n=6) with combined hepatic and renal failure. DAO spiking recovery rates using 2 and 20 ng/mL were 92 and 84% (CVs 18 and 23%) for all six samples. Nevertheless, the

recovery rates of one patient was only about 50% and excluding this patient the DAO recovery rates increase to 97 and 91% (CVs 12 and 9%) for 2 and 20 ng/mL rhDAO spiking. Four out of 6 patients showed pathological creatinine or bilirubin levels. Two patients including the “outlier” had both creatinine and bilirubin elevated. Dialyses of serum or plasma overnight might further improve recovery and might allow measuring DAO concentrations reliably and accurately in patients with liver and/or kidney disease.

2.4.Measurement of DAO in urine

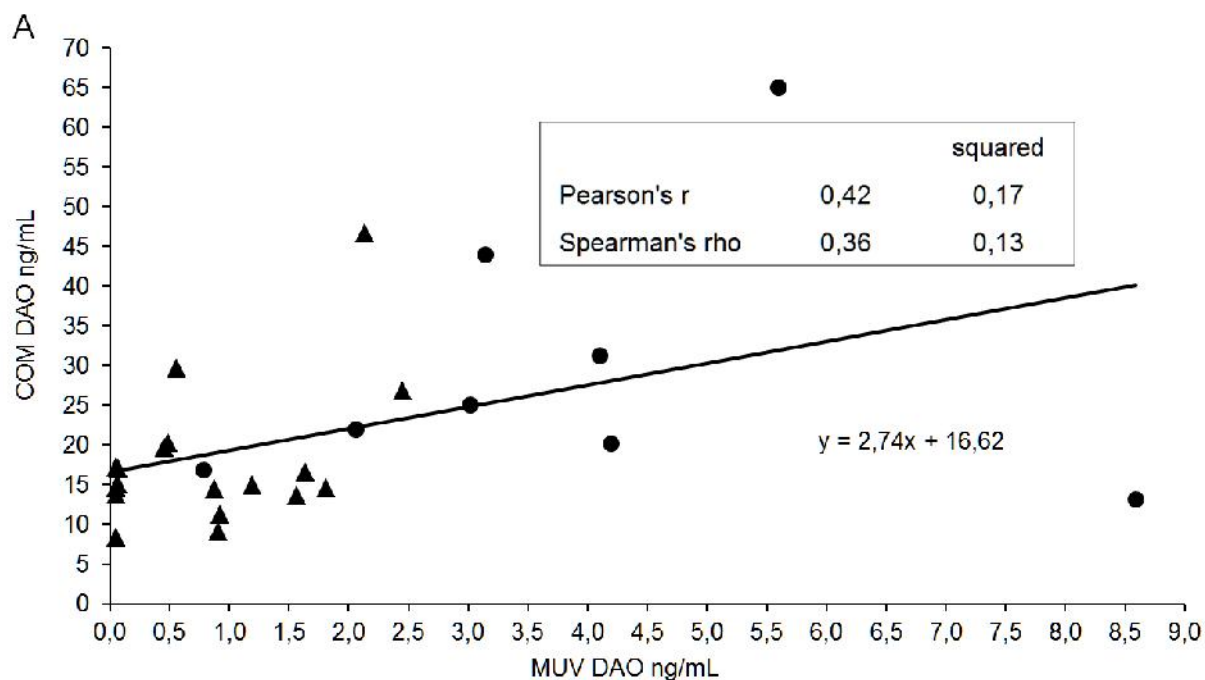
It was possible to obtain a DAO signal far above the eLoQ in 7 out of 8 human urine samples from healthy volunteers after a “rather cumbersome” sample preparation protocol including dilution, concentration and extensive dialyzation. The mean/median DAO concentration of all eight samples was 8.6 and 10.8 ng/mL urine respectively. These data are in agreement with published Western Blotting results [5]. Nevertheless just dialyzation of urine did not allow accurate DAO quantification (data not shown). Recovery rates after spiking of DAO were not adequate. A modified sample preparation protocol must be developed before DAO concentrations can be reliably determined in urine samples.

2.5.Comparison of commercially (COM) available ELISA with our DAO ELISA

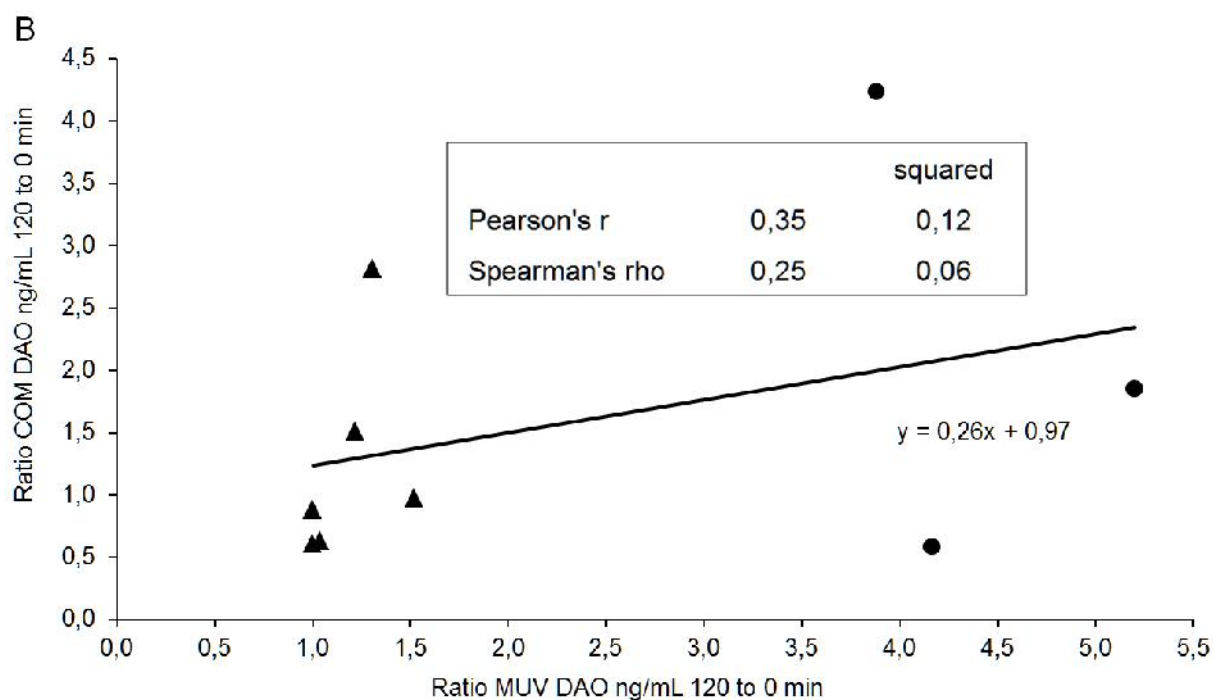
Histamine intolerance is a condition with largely unknown underlying pathophysiology, but supposedly with a high prevalence in the general population [6]. Nevertheless, the prevalence might be overestimated [7]. Considering that the DAO COM ELISA is proposed to be used for the diagnosis of histamine intolerance [6] we decided to test it using rhDAO and other samples, where we measured the DAO concentrations also with our DAO ELISA. In general, the dynamic range of this COM DAO ELISA as documented in the manual using the lowest and highest standard is only 27-fold. This ELISA does not seem suitable for DAO

quantification during pregnancy considering that DAO concentrations during pregnancy increase more than hundred-fold. The calculated DAO levels differed more than 10-fold, when we measured a pregnancy serum with this COM ELISA at different dilutions to fall within the DAO standard curve as suggested by the manufacturer (data not shown). Although our rhDAO standard was recognized by the ELISA, surprisingly the COM ELISA DAO standard was not recognized in our ELISA (data not shown). The company did not provide us with information about the nature of their DAO standard. If they use a truncated version of DAO, it is possible that our monoclonal capturing antibody is not able to recognize their DAO standard.

When we tested 26 samples from healthy volunteers (9 serum, 9 EDTA and 9 citrate samples from three healthy volunteers incubated for different time periods at RT) the Pearson and Spearman correlation coefficients were only 42 and 36% respectively (Figure S5A). The intercept of the regression line was 16.6 ng/mL. We measured a “DAO” concentration of 15.3 ng/mL with this COM DAO ELISA using just 1% HSA PBS. Contamination of commercial good manufacturing practice (GMP) HSA solutions with DAO can be excluded. Control DAO concentrations of 16.8 and 11 ng/mL were published using this ELISA, but we doubt that they measured DAO antigen considering the data presented above and that we measured between 8 and 18 ng/mL using this COM ELISA in samples where our ELISA was below the LoB or close to zero (Figure S5). References 10 to 15 in the Supplement Reference List used the Immundiagnostik K8500 DAO ELISA for quantification of DAO in serum or plasma. In Figure S5B we calculated and plotted the ratio of DAO measurements at baseline and 120 min from Figure S5A to test, whether this COM ELISA is able to relatively quantify DAO with precision. Nevertheless, also in this case the correlation coefficients are below 40% indicating weak performance (Insert Figure S5B). This commercially available ELISA is not suitable for absolute or relative DAO quantifications in non-pregnant, low DAO, or during pregnancy, high DAO, samples.



Supplement Figure S5A



Supplement Figure S5B

Supplement Figure S5: Absolute and relative comparison of commercial (COM) versus MUV ELISA DAO antigen measurements.

(A) Twenty six samples from three healthy volunteers (9 sera, 9 citrate and 9 EDTA plasma samples collected at the same time) were measured using both ELISAs. One serum sample

was an extreme outlier (MUV 12.2 versus COM 186.3 ng/mL DAO concentration) in both populations (Q3+3x IQR for MUV samples 10.7 and for COM samples 63.9 ng/mL) and not included. These samples are not independent and represent preparation of serum or plasma at 0 (rapid serum tubes containing thrombin), 30 and 120 min. Tubes were incubated at room temperature. The x-axis and y-axis show the DAO concentrations using the MUV and COM ELISA respectively. Absolute DAO concentrations were weakly correlated (Pearson's $r=42\%$; Spearman's $\rho=36\%$). The COM ELISA measured high DAO values, when the MUV ELISA shows values below the limit of detection (LoD, 0.48) or limit of blank (LoB, 0.27). Linear regression shows that the blank value of the COM ELISA is 16.6 ng/mL. Serum samples are shown as filled circles. (B) The ratios of the DAO concentrations at 0 to 120 min were compared for 3 subjects using serum, ethylenediaminetetraacetic acid (EDTA) and citrate samples. This is a relative comparison in contrast to an absolute comparison used in (A). The Pearson correlation coefficient is 35% and Spearman's ρ is 25%. DAO is released probably from neutrophils in serum samples during longer incubation times (data not shown). This explains the three ratios >4 in the MUV DAO ELISA samples. Serum sample ratios are shown as filled circles. MUV, Medical University of Vienna; COM, commercial; Q, quartile; IQR, interquartile range.

2.6.DAO radio extraction assay (DAO-REA) for measuring DAO activity

We also tested a commercially available DAO radio extraction assay (DAO REA, Sciotec, Tulln, Austria) using different rhDAO concentrations spiked into 0.1% HSA PBS and serum. In the assay description the units/mL might be converted to ng/mL using a factor of 1.25. The origin of this conversion factor is not detailed. In 0.1% HSA 82 units/mL or converted 102 ng/mL were measured twice using 10-fold different rhDAO concentrations (1 and 0.1 $\mu\text{g/mL}$). Spiking of 10 ng/mL DAO generated a signal of 7 units/mL. In serum spiking of

1, 0.1 and 0.01 $\mu\text{g/mL}$ rhDAO resulted in very similar units/mL concentrations (66, 70 and 53 units/mL). This lack of linearity over three orders of magnitude might be partially explained considering that the final putrescine concentration is only 0.7 nM according to information provided by the manufacturer. The putrescine substrate concentration is at least 10000-fold below the K_m [2; 8]. The endogenous putrescine concentration in serum is approximately 100 nM [9]. In the assay serum is diluted 2.5-fold and therefore the endogenous serum putrescine concentration is 40 nM or at least 40-fold above the substrate concentration used in the assay. Nevertheless, the total putrescine concentration is still far below the K_m . This assay is able to measure DAO activity but is not suitable for precise DAO quantification.

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