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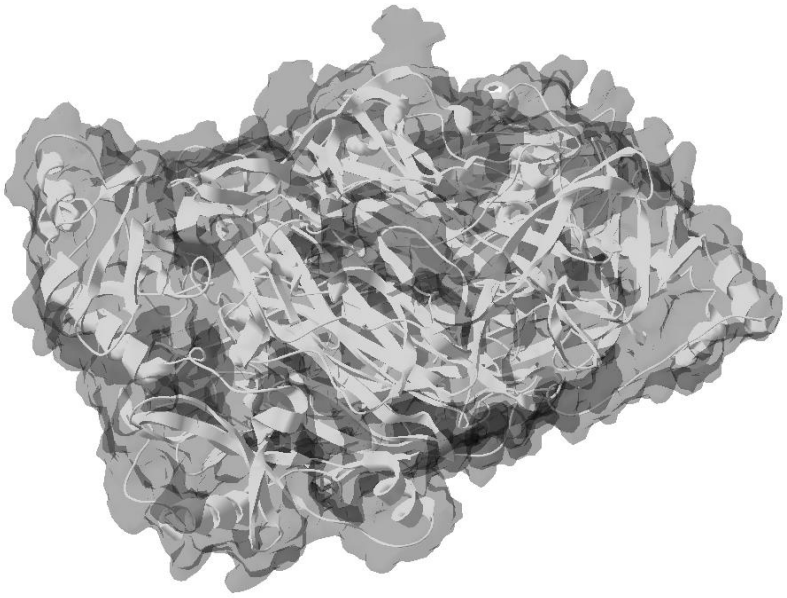
Radioenzymatic Assay for measurement of SSAO-activity in Human Plasma



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Summary

Cardiovascular diseases and type-2 diabetes mellitus are both strongly connected to obesity and lack of physical activity, and are thought to increase in frequency in the Western world due to our modern life style. Several investigations show a correlation between cardiovascular diseases and a high activity of the enzyme Semicarbazide-sensitive amine oxidase (SSAO) in blood plasma among humans, and also in rodents. In order to determine the reliability of SSAO as a biomarker for type-2 diabetes mellitus and cardiovascular diseases, SSAO enzymatic activity measurements using a radioenzymatic assay can be performed. In this paper a series of experiments designed and conducted to optimize the method are described. It is shown that several factors in the laboratory procedure affect the results.

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1. Background

In order to understand human biology and disease, it is important to investigate tissue-specific expression at both gene and protein level. In both patients with cardiovascular-related problems and in patients with diabetes an elevated activity of the enzyme semicarbazide-sensitive amine oxidase (SSAO) has been observed in plasma. Additionally consistent observations have been done in the plasma of transgenic mice expressing human SSAO (Goktürk et al. 2004). Even higher levels of SSAO-activity have been found in mice and humans with both diabetes and cardiovascular-related problems (Boomsma et al. 2005).

Cardiovascular diseases are the major underlying cause of death worldwide, and include for example atherosclerosis, myocardial infarction and stroke. According to World Health Organisation (WHO), this kind of diseases in 2008 caused approximately 17.3 million cases of death. Of these deaths, an estimated 7.3 million was due to coronary heart disease and 6.2 million were due to stroke (WHO September 2011). In fact, in the 20th century, when general hygienic measures greatly reduced the toll from infectious diseases and allowed people to live considerably longer, we realized the enormous impact of atherosclerosis on general health (Zaret 1992).

The causes of cardiovascular diseases are both genetic and environmental, but different lines of evidence indicate that environmental factors – such as life style and diet – are the most important (Willett 2002). This fact suggests the possibility of lowering the increasing number of patients with cardiovascular problems by life-style changes. Diabetes is a disease characterized by an inability to decrease plasma glucose, caused by defects in either the cell use of insulin or in the insulin production mechanism which entail a disability to decrease blood glucose levels (Pettersson 2012). Two different types of diabetes exist: type-1 and type-2. Type-2 is the most frequent, the cause of 90-95% of all diabetes cases in the western world, and is strongly connected to life style and obesity.

Today the life-style in the western world is characterized by an almost unlimited food supply and lack of physical activity, which together contribute to obesity. The WHO estimated that 1.5 billion adults suffered from obesity or overweight in 2008 (WHO March 2011). Obesity is a risk factor for cardiovascular diseases, and also for type-2 diabetes (Freedman et al. 1999). It is characterized by an unhealthy amount of adipose tissue on the body, either with an increased number (hyperplasia) and/or size (hypertrophy) of adipose cells. The latter case is more common, since the number of adipose cells normally is relatively stable in adults (Spalding et al. 2008).

It has been shown that transgenic mice expressing human SSAO have increased body mass index (BMI) and subcutaneous abdominal fat pad weights, when the SSAO-level is elevated, in an independent manner from food consumption. In the same study, the mice that both were expressing human SSAO and had increased SSAO substrate availability, showed an enhanced glucose uptake in an SSAO-dependent manner. Additionally, during biological stresses, such as diabetes and fasting, endothelial cells can contribute to increased activity levels of SSAO in blood plasma. The transgenic mice showed advanced glycation end product formation, elevated blood pressure, altered atherosclerosis progression, and nephropathy (Stolen et al. 2004).

An abnormal (stiffer) aorta phenotype, a lower blood pressure has been observed due to the loss of arterial elasticity, and it has been suggested to be caused by elevated SSAO-activity.

The aorta phenotype has been investigated in transgenic mice expressing human SSAO and with induced diabetes. The result was a straight and unfolded aorta phenotype. Moreover, the elastic fibres in the walls of the aorta were found to be irregularly arranged, compared to the ones among normal mice and transgenic mice without diabetes. A stiffer aorta morphology impaired the pulse- and blood pressure regulation mechanism, and it was found that smooth muscle cell's synthesis of elastic fibres in large arteries is affected by a high SSAO-activity (Gokturk et al. 2003 and Gokturk et al. 2004).

The increased activity of SSAO in patients with diabetes seems to not be a result of increased SSAO gene transcription. On the contrary, a negative correlation has been observed between increased enzymatic activity and mRNA expression. Furthermore, the two SSAO-inhibitors hydralazine and carbidopa do not affect this correlation (Nordquist et al. 2002). In diabetic rats, the SSAO-activity has been found unchanged in lung, aorta, and pancreas but increased in blood plasma (O'Sullivan et al. 2004).

Researchers have stated that identification and prevention of cardiovascular risk factors is the most promising tool in the work of reducing cardiovascular sequelae in diabetic patients (Kannel, W.B. & McGee, L. 1979). Since SSAO-activity is known to be higher in diabetic patient and also in patients with cardiovascular diseases, the enzyme has been suggested as a biomarker. Therefore it is of great importance to further investigate the correlation between SSAO-activity and the above-mentioned diseases.

2. Semicarbazide-sensitive Amine Oxidase (SSAO): molecular structure, catalytic functions, and origin

SSAO is an amine oxidase (AO) that, as the name implicates, is inhibited by carbazide. However, it is resistant to most monoamine oxidase (MAO) inhibitors. The cofactor of SSAO is the copper dependent molecule topa quinone (TPQ) (Figure 1 and 3). There are several other TPQ- and copper-containing amine oxidases in humans among which the most common ones are diamine oxidase (DAO), lysyl oxidase (LO) and retinal amine oxidase (RAO) (Nordquist et al. 2002). Characteristic for dimeric copper dependent amine oxidases in mammals is the oxidative deaminations of primary amines to aldehydes, in a reaction in which ammonia (NH_3) and hydrogen peroxide (H_2O_2) are also produced. This catalytic reaction mechanism has been thoroughly studied in bacteria and yeast (Elovaara et al. 2011).

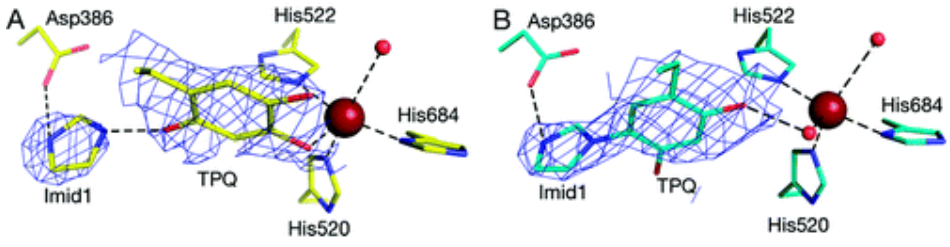

Figure 1 *The cofactor TPQ extracted from human plasma binding to SSAO. A) Inactive on-copper conformation of TPQ. B) Active off-copper conformation of TPQ (Elovaara et al. 2011).*

The catalytic function of SSAO is exerted in oxidation reactions of the primary amines methylamine and amineacetone, which are metabolized into formaldehyde and methylglyoxal respectively (Figure 2 and Reaction 1 and 2) (Nordquist et al. 2002). The reactions also

produce the cytotoxic compounds hydrogen peroxide and ammonia (Olivieri and Tipton 2011).

$$\text{Methylamine} + O_2 + H_2O + \text{SSAO} \longrightarrow \text{Formaldehyde} + NH_3 + H_2O_2 \quad (1)$$
$$\text{Amineacetone} + O_2 + H_2O + \text{SSAO} \longrightarrow \text{Methylglyoxal} + NH_3 + H_2O_2 \quad (2)$$

Figure 2 describes the enzymatic oxidation mechanism. On an oxidised enzyme, the amine substrate binds to TPQ cofactor, and a substrate Schiff base is formed. After the Schiff base is hydrolyzed and the aldehyde product released from the enzyme, the enzyme is reduced. This reduced form of SSAO is inactive and for reactivation, an oxidation reaction of the enzyme by molecular oxygen is due together with release of hydrogen peroxide and ammonia (Elovaara et al. 2011).

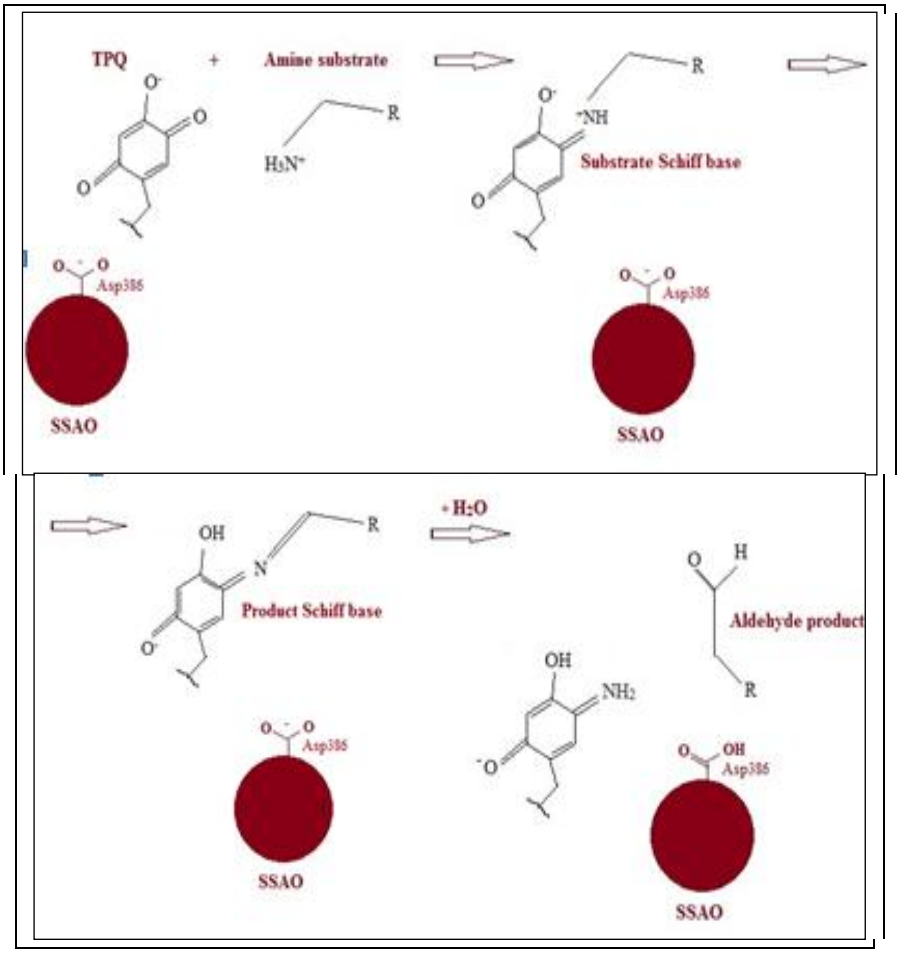

Figure 2. *Oxidation reaction catalyzed by SSAO, forming an aldehyde from an amine with the cofactor TPQ.*

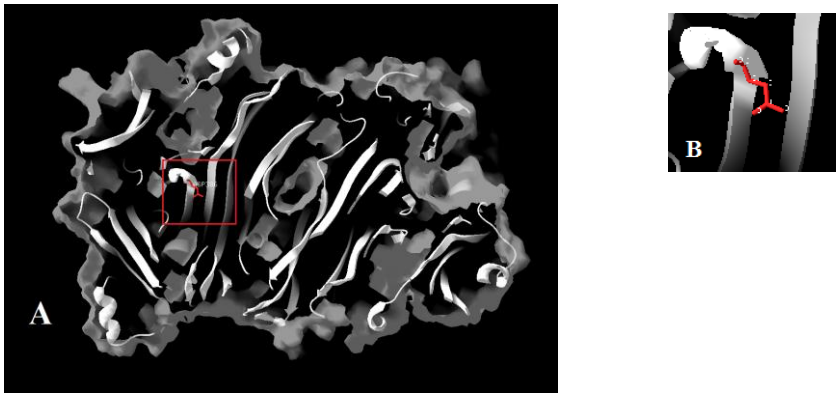

Figure 3. *Shape of the SSAO enzyme and the general base (Asp386) that abstracts one of the carbons from the substrate, forming the Schiff base. A. The shape of SSAO-dimer Asp386 marked in red. B. Close-up on Asp386*

To lighten the structural difference between the soluble form and the membrane bound form of the enzyme, the former is called pSSAO (plasma SSAO) (Olivieri and Tipton 2011). pSSAO is a result of proteolytic cleavage of membrane-bound SSAO at the N-terminus (Gokturk et al. 2004, Olivieri and Tipton 2011). It has been indicated that this cleavage can be derived either from liver cell membranes, heart cell membranes, or adipocyte cell membranes (Boomsma et al. 2005, Iffiú-Soltész, Prévot and Carpéné 2009).

In mammals SSAO mainly works as an ectoenzyme, meaning that it works outside the cell. SSAO has mainly tissue specific functions and it is mostly present as membrane-bound form in adipose tissue cells, vascular visceral smooth muscle cells and in endothelial cells (Gokturk et al. 2004). According to Human Protein Atlas (HPA), the most significant amount of SSAO in humans is in lung tissue - especially in the pneumocytes- and smooth muscle cells. A significant amount of the enzyme is also present in the adrenal gland and in the glandular cells of the digestive tract.

3. The Biological Functions of SSAO in Mammals

Evidence suggest that SSAO in mammals is involved in 1) adhesion of lymphocytes at inflammatory sites; 2) differentiation of proadipocytes to mature adipocytes; and 3) cell signalling (Nordquist, Göktürk and Oreland 2002). The adhesion function explains the other name of SSAO: vascular adhesion protein 1 (VAP-1).

The SSAO adhesion function on lymphocytes is fascinating since it provides a link for the connection between SSAO-activity and obesity. Obesity is namely associated with a low-grade, general inflammation in the body, especially manifested in white adipose tissue, and increases the risk for insulin resistance and type-2 diabetes (Dandona, Aljada and Bandyopadhyay 2004). In endothelial cells, SSAO mediates adhesion of lymphocytes at sites of inflammation (Göktürk et al. 2003).

The enzyme is necessary for adipose tissue formation, and in the adipose tissue SSAO functions as a late marker for adipogenesis, thus it is involved in adipocyte homeostasis (Göktürk et al. 2003). However, the factors that might regulate the expression of in adipose tissue are still poorly defined. In fat depots, adipocytes are not only involved in the metabolic functions regulating lipid storage, namely lipolysis and lipogenesis, but are also known to

accomplish paracrine and endocrine functions that participate in body weight regulation, lipid handling or even glucose homeostasis, mainly by the secretion of adipokines and diverse other factors (Iffiú-Soltész et al. 2009).

Adipocyte SSAO activity in rats appears to be unvaried during 10 days of starvation. Thus, it is not shown that a weight loss decreases the activity of the enzyme. However, clear increase in SSAO activity was detected in intra-abdominal adipose tissue in the rats. Such increased SSAO activity is likely due to an enhanced expression of SSAO in the mature adipocytes which contain higher activity and higher mRNAs expression of the AOC3 gene encoding for SSAO/VAP-1 than the other cell types found in the adipose tissue (Iffiú-Soltész, Prévot and Carpéné 2009).

Since hydrogen peroxide exerts insulin-like actions on adipocytes, it has been demonstrated that substrates of SSAO activate glucose uptake and inhibit lipolysis in adipocytes, even in the absence of insulin (Iffiú-Soltész et al. 2009). Another function of pSSAO is to stimulate transportation of glucose into adipocytes via glutamate transporter 1 (GLUT1) and into smooth muscle cells via GLUT4 (Göktürk et al. 2003). Studies on SSAO and glucose transport have suggested that glucose transport can be enhanced by giving specific SSAO substrates either alone or in some cases together with low doses of vanadate, which in higher doses stimulates glucose uptake (Stolen et al. 2004).

Mature adipose cells produce cytokines, which are small proteins mediating signalling between cells. Adipocytes also regulate appetite and metabolism (Iffiú-Soltész et al. 2009).
Since lymphocytes are presumed to initiate neovascularisation, SSAO is suggested to be of importance for the mechanism of diabetic retinopathy as well (Garpenstrand et al. 1999).

4. Differences in pSSAO-Activity Level

Evidence strongly suggests that SSAO activity in blood plasma (pSSAO) is significantly higher in mice with diabetes. As previously mentioned, this correlation is even stronger among mice with both diabetes and cardiovascular complications. What causes this correlation is still not known. One hypothesis is that the cytotoxic compounds produced by the SSAO-catalyzed reactions (Gokturk et al. 2004). Human studies have shown that elevations in pSSAO-activity reflect the severity of cardiovascular-related problems, as shown by the correlation between pSSAO-activity and survival (Boomsma et al. 2005). Furthermore, SSAO-activity is higher among obese patients with cardiovascular-related problems, than in patients only suffering from obesity or cardiovascular disease alone. Also, the SSAO activity level is elevated in patients with chronic liver disease (Göktürk et al. 2003).

Decreasing pSSAO-activity rates are less commonly found but some studies suggest that thyroid hormone plays a moderate role in pSSAO-regulation. The fact that patients with hyperthyroidism have increased pSSAO-activity level, and after treatment show lower pSSAO-activity indicates that it is possible to control SSAO activity, and thereby hopefully find ways to reduce damage caused by an abnormal activity. The opposite was also shown in a similar mice experiment, using mice with hypothyroidism and with relatively low pSSAO-activity from the beginning (Iffiú-Soltész, Z. et al. 2009).

5. Medical Applications on SSAO-Research

A protective effect of Cu-containing amine oxidases purified from pea seedling on hearts of guinea pigs in which cardiac anaphylaxis, and the sudden release of histamine in the coronary effluent that comes with it, has been observed (Mondovì et al. 2003). Due to SSAO's ability to reduce the concentration of released histamine, it has been suggested that SSAO can be used therapeutically, to protect from cardiac anaphylaxis (Nocera et al. 2003).

It seems that plasma SSAO activity might be a useful clinical marker in the prognostic evaluation of diabetic vascular complications. Additionally, a deeper knowledge of the relationship between elevated SSAO activity and vascular complications may lead to development of new strategies for the prevention of vascular complications in cases of diabetes mellitus (Mészáros et al. 1999). In 2005, researchers suggested that a selective SSAO-inhibitor could provide clinical benefit for the treatment of stroke, and it was indicated that a small SSAO-inhibitor molecule can reduce neurological damage in rats (Xu et al. 2006).

Considering the relation between a high pSSAO-activity, diabetes and cardiovascular diseases, it is indeed a highly relevant question to ask if elevated pSSAO activities can be brought down to normal levels once elevated. It has been reported that in human patients with liver diseases and an elevated pSSAO-activity, a normal enzymatic level could be obtained after transplantation. Even though this suggests that the diseased liver could be the source of the high pSSAO activity levels, an alternative explanation is that an unknown substance from the diseased liver induces SSAO-production. During the last years, researchers have investigated if surgical correction of atherosclerotic lesions influences pSSAO activity. So far the results point in the direction that this is the case, thus it seems possible to lower elevated pSSAO activity by surgical intervention for coronary artery disease. This indicates that the heart can also be a source of pSSAO (Boomsma et al. 2005).

6. Radioenzymatic Assay For Measurement of pSSAO-activity

Figure 4. *Human blood plasma samples thawing on ice.*

In this project, pSSAO-activity in human plasma samples (Figure 4) was measured with a radioenzymatic assay (REA). The final aim of the project is to investigate correlations between the SSAO enzymatic activity and the patient's glucose level, presence of cardiovascular problems, and diabetes.

There are several ways to measure SSAO enzymatic activity. In a REA the substrate is first marked with a radioactive isotope, in this particular case ^{14}Carbon (^{14}C). Second, the radioactive decay in each tube is measured with a scintillation counter. The result is given in counts per minute (CPM), a number of beta-emitting nucleotides in the sample.

When the enzymatic reaction (Reaction 3) had been given a certain amount of time, there will be ^{14}C-benzaldehyde (product) in the tube as well as ^{14}C-Benzylamine (substrate), oxygen (O_2), water (H_2O), ammonia (NH_3) and hydrogen peroxide (H_20_2). Especially important in this experiment is to ensure that the limiting factor for the enzymatic reaction is not the amount of substrate.

The specific REA that was used in this experiment is liquid scintillation counting (LSC). The samples are suspended in a liquid scintillation cocktail containing an aromatic solvent and scintillators. A scintillator molecule is excited by ionizing radiation and reemits the absorbed energy in the form of light, which can be measured by a scintillation counting machine.

$$^{14}\text{C-Benzylamine (substrate)} + O_2 + H_2O + \text{SSAO} \rightarrow {}^{14}\text{C-Benzaldehyde (product)} + NH_3 + H_20_2 \quad (3)$$

REA has been a commonly used and is a well-established method for enzymatic measurements in blood plasma, urine and gastric juice. It was first used in the late 1960's and is relatively sensitive and appropriate for rapid determination of several samples (Rothenberg 1965). One disadvantage of the radiometric approach is its high cost compared to fluorimetric and ion exchange methods (Mészáros et al. 1999). The use of radioactive substances should also been taken in consideration, even though only small amounts of ^{14}C are used in this particular case. No doubt, the method is about to be replaced by a similar but improved method, a radioimmunoenzymatic assay.

Moreover, highly sensitive analytical electrochemical and fluorimetry chromatography techniques are about to supersede both of these two above-mentioned methods. The development of high-performance liquid chromatography (HPLC) has been of importance for more sensitive measurements of catecholamines and metabolites in plasma and urine (Peaston and Weinkove 2004).

The activity of the soluble form of the enzyme (pSSAO) in human blood plasma has been measured in the present study with the above described REA method, also to compare the results with previously done measurements.

Since there is an overlap between SSAO and the mitochondrial amine oxidase MAO B, because they partly use the same substrate and they are both present in human blood (Boomsma et al. 2005), it is necessary to inactivate the enzymatic activity of MAO B to obtain correct results. MAO B is found in the platelets, and should not be found in completely purified plasma. However, to be sure the selective inhibitor L-deprenyl was added.

After radioactivity measurement with the scintillation machine, the following formula was applied to calculate the correct enzymatic activity:

$$\frac{\textbf{(cpm-blank) * 2 * 10 nmol}}{\textbf{(standard – blank) * 0,33 * 0,05 mL}}$$

The SSAO activity final value is given in nmol product/mL plasma/hour. The product concentration in the vials is measured in CPM, after blank activity sample reduction. Because of the extraction step, where only 500 µL of the 1 mL added toluene:ethyl acetate were transferred to each scintillation-vial, the compensation factor 2 is needed.

50 µL of 0.2 mmol/L substrate contain 10 nmol of substance (^{14}C-benzylamine), this is why the second compensation factor 10 is necessary. Since the reaction is ongoing for 20 minutes, the factor 0.33 is needed to transform the answer in hours. Finally 0.05 mL is the volume of the plasma sample that was used in each sample.

Hydrogen chloride (HCl) is added to stop the enzymatic activity. To note, when working with blood plasma, 1M HCl is more commonly used than 3M HCl. 3M HCl is recommended for

experiments with tissue samples. However, in order to lower the blank values, researches in this lab started to use 3M HCl for plasma samples when measuring pSSAO-activity. The HCl-concentration affects the pH which could contribute to an altered function of the enzyme.

6.1 Blank Value Problematic

The investigation of correlation between pSSAO-activity and cardiovascular abnormalities, with the method described above has been going on for about 5 years in this lab. Undoubtedly, the blank values have been too high during the last year/experiments. The change has been gradual and the values are, at the time this investigation started, about four times higher than one year ago (Appendix 1 and Diagram 1). The first three months of 2011, no samples with control plasma were measured. During the summer, the CPM-values of samples with patient plasma were 42% higher than the values of samples with control plasma. In December, the CPM-values of samples with patient plasma were in average 50% lower than the values of samples with control plasma.

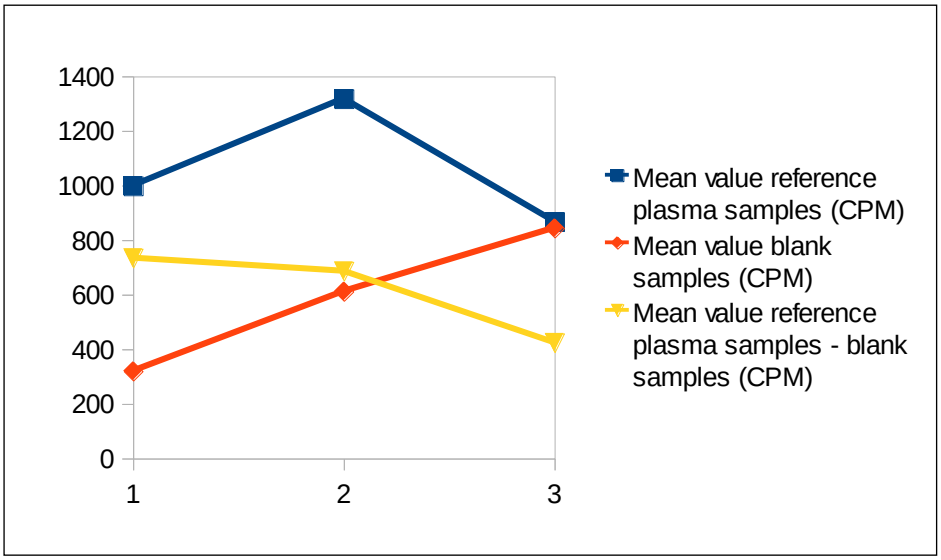

Figure 5. *Average CPM-values in 2011. On the x-axis is presented the time: 1 is the first 4 months, 2 the second 4 months, and 3 the last 4 months of 2011*

The enzymatic activity measurements have not showed any significant increase in reference- or patient plasma. Thus it looks like the enzymatic activity decreases, since this increase only in blank sample's radioactivity. CPM-values of the standard samples seems to have decreased but are now stable. The results indicates that something is not as it used to be, and it has been of great importance to figure out what is the cause of the altered resultsbefore further measurements of pSSAO-activity are done.

7. Aim

The aim of the present project has been to optimize the method described above, but also to investigate on why the blank values increased during the last year. In order to accomplish this, the following experiments have been done: 1) comparison between two Liquid Scintillation Counting machines (LSC-machines), 2) comparison of the enzymatic activity between samples that have been thawed 1, 2 and 3 times, 3) investigation of the effect of time between

the preparation of the samples and the scintillation counting, 4) a comparison between two different scintillation cocktails, 5) preparation of new labelled substrate, 6) comparison between samples with different concentrations and amount of HCl added, 7) measurement of radioactivity in samples containing water or buffer instead of plasma, and 8) measurement of monoamine oxidase-B-activity (MAO-B-activity). The main goal was to obtain lower CPM-values of the blank samples approximately to <500.

8. Materials and methods

For all tests the same pipettes were used: one for reference plasma, one for L-deprenyl, one for ^{14}C-benzylamine, one for HCl, one for toluene:ethyl acetate, and one for the final transfer step from tubes to scintillation vials. The plasma was kept in -20°C in aliquots of 600-2000 µL in glass or plastic tubes. Glass tubes have been always used for the experiment, if not otherwise indicated. Labelled substrate, ^{14}C-benzylamine, was diluted in NaK-buffer (pH 7.8) to a concentration of 0.2 M, and kept in -20°C. MAO B-inhibitor, L-deprenyl, was eluted to a concentration of 0.1 mM with NaK-buffer (pH 7.8), and kept at the same temperature as the labelled substrate and plasma. 3M hydrochloride (HCl) was diluted from 37% solution using distilled ion free water and kept in room temperature (RT).

The toluene:ethyl acetate used was protected from light and kept in room temperature in the form of toluene:ethyl acetate (1:1). To prepare the mixture, equal amounts of toluene and ethyl acetate were mixed with half as much of distilled water. In order to obtain complete separation of water and the organic phase, the bottle was allowed to stand for at least 24 hours before use. Scintillation cocktails were not exposed to light and kept in room temperature.

All reagents were thawed on ice before use and when not otherwise mentioned, the tubes were kept on ice during the experiment. After every substance was added to the tubes, the tubes were vortexed for approximately 10 seconds. The reagents used in the experiments are described in Table 1.

Table 1. *Reagents used in the experiment.*

REAGENT	BRAND
^{14}C-Benzylamine	Sigma Chemicals, Germany
Benzylamine	Sigma Chemicals, Germany
0.1 M β-phenylethylamine from peas	Sigma Chemicals, Germany
L-deprenyl	Sigma Chemicals, Germany
37% HCl for preparation of 3M	Scharlau Chemie S.A., Barcelona, Spain
1 M HCl	Fluka® Analytica, Sigma-Aldrich Co., Germany
α -Natriumhydrogenphosphate (for NaK-buffer)	Merck, Germany
Kalciumhydrogenphosphate (for NaK-buffer)	Merck, Germany
Liquid scintillation fluid Aquasafe 300 Plus	Zinsser Analytic, Frankfurt/M, Germany
Ultima Gold™ Scintillation Cocktail	PerkinElmer, USA
Toluene	Sigma-Aldrich Co., Germany
Ethyl acetate	Sigma-Aldrich Co., Germany

For all measurements in the scintillation counting machine, Zinsser Polyvials® (Zinsser Analytic, Frankfurt) were used. Except for the comparison between the two scintillation counting machines, PerkinElmer Liquid Scintillation Analyzer TriCarb® 2910TR was used for radioactivity measurement of the samples with the software QuantaSmart™.

For all samples, doublets were used. When measuring only the solutions (in the contamination test), 8 mL scintillation cocktail was added together with the same amount of the solution that was used for sample preparation (described below) in scintillation vials.

Sample preparation (Figure 5). The same method was used for preparation of patients' plasma samples, control plasma samples and reference plasma samples. First, 50 µL newly thawed human plasma was added to each tube. Second, 25 µL L-deprenyl was added, and incubated at room temperature (approximately 22°C) for 20 minutes. After incubation MAO B is inhibited, and the only enzyme in the plasma that can oxidise benzylamine is pSSAO.

Third, 50 µL ^{14}C-benzylamine was added to each tube, and the tubes were put in 37°C water bath for 20 minutes. During this time pSSAO catalyses the oxidation of the labelled substrate. To stop the reaction, 30 µL 3M HCl were added.

The product ^{14}C-benzaldehyde is soluble in toluene:ethyl acetate. Thus 1 mL toluene:ethyl acetate was added to each tube and the tubes were shaken for 45 seconds. Afterwards they were centrifuged with 2500 revolutions per minute (RPM) at 18°C for 5 minutes. This step is needed to separate the toluene:ethyl acetate (upper) layer containing the product, from the water (lower) layer containing the substrate.

Finally, 500 µL from each tube's upper (toluene:ethyl acetate) layer was transferred to scintillation vials, before 8 mL of scintillation cocktail. Measurement of the radioactivity was performed in a scintillation machine for 10 minutes per sample.

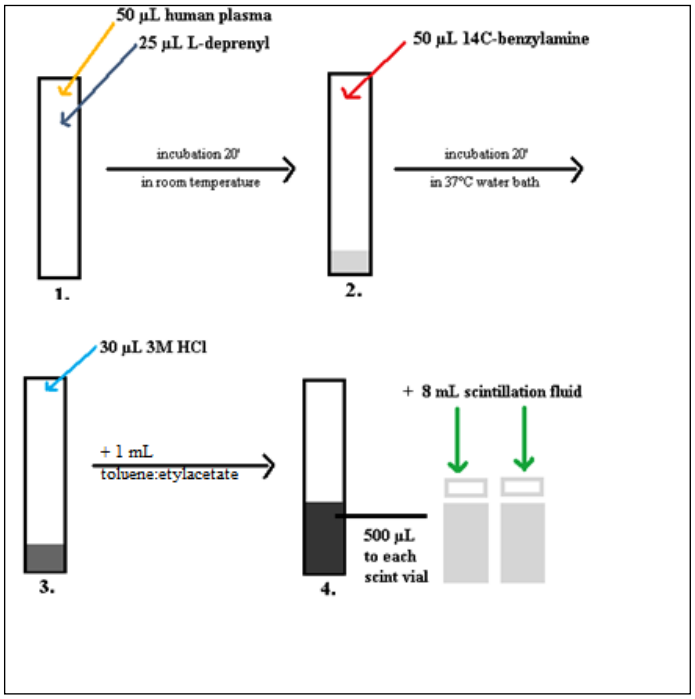

Figure 6. *Sample preparation.*

Blank sample preparation. The preparation of blank samples followed the same steps as for the sample preparation described above. The difference is that in the blank tubes, 30 µL 3M HCl was added before the adding of the substrate, and no HCl was added after the water bath, if not otherwise indicated. The purpose of adding HCl before the substrate is to inhibit

pSSAO before the substrate is added. Thus there will be no enzymatic activity that possible could produce ^{14}C-benzaldehyde from ^{14}C-benzylamine.

Standard sample preparation. 50 µL ^{14}C-benzylamine and 8 mL scintillation cocktail was added to a plastic scintillation vial. The vial was shaken by hand for a couple of seconds and then put in the scintillation counting machine.

Two different LSC-machines. The old measurements were done with Beckman Scintillation Counter LS6000IC, but the ones done in this experiment is done with PerkinElmer Liquid Scintillation Analyzer TriCarb® 2910TR. To be able to compare my results with previous, it is of importance to investigate if there are any differences in the two machines' measurements. The same measurement time (10 minutes/sample) and the same samples were used.

Contamination tests. To investigate if any used substances were contaminated, the same amount of each substrate that is used in sample preparation was added to scintillation vials. Then 8 mL scintillation cocktail were transferred to each scintillation vial. Before put into LSC-machine, the vials were shaken by hand for a couple of seconds.

The radioactive contamination test of glass tubes was done by adding toluene:ethyl acetate to 10 tubes and incubates in room temperature for 3 hours, before transferring 500 µL from each tube to scint vials. Control vials contained toluene:ethyl acetate direct from the bottle. To all vials 8 mL scintillation cocktail were added before measurement in LSC-machine.

Comparison between the two scintillation cocktails Ultima Gold™ and Aquasafe 300 Plus. The scintillation cocktail's efficiency for different kinds of samples differed. For that reason, two different cocktails were compared using the same laboratory procedure and the same amount of scintillation cocktails (8 mL).

Investigation of the effect of thawing plasma samples on the enzymatic activity. The samples were in this experiment prepared in the same way as described above (Sample preparation and Blank sample preparation). Thawing of plasma was done on ice and the plasma was out of the fridge for about 4 hours per experiment.

5 or 7 minutes centrifugation time. The separation of product from substrate is critical in order to not obtain misleading results, and the centrifugation step is in this experiment a big part of that. Therefore, CPM-values of 8 blank samples centrifuged for 5 minutes were compared to values of 8 samples with 7 minutes centrifugation time. The same plasma, centrifugation RPM and -temperature were used for both groups.

Different concentrations and amounts of hydrochloric acid. 1M HCl is commonly used for plasma samples. Therefore, to ensure that the higher HCl-concentration was not the cause of the high blank values, fabric made 1M HCl was tested as well as the 3M HCl prepared in our lab. Also, different amounts and time of adding for both the two concentrations were compared. Otherwise, the experiment followed the same steps as previously described.

MAO-B-activity measurement. The experiment was done using platelet rich plasma and reference plasma (containing no platelets), by adding 1M HCl before and after the adding of plasma. This test was done with both platelet-rich plasma and with reference plasma, and the results were compared.

1. **Preparation of platelet-rich blood plasma.** First, the blood was centrifuged at room temperature at 800G for 10 minutes. 2500 µL plasma was extracted and aliquoted in smaller tubes and frozen at -20°C until the next day.

2. **Sample preparation for determination of MAO-B-activity.** The platelet rich plasma as well as reference plasma were thawed on ice. First, 50 µL of the two different plasmas were added to 50 µL β-phenylethylamine in some tubes and to 50 µL 0.1mM 14C-tryptamine in other. The tubes were put into 37°C water bath for 4 minutes. They were during that time vortexed for 3 seconds, approximately once per minute.

   After 4 minutes in water bath, 30 µL of 1 M HCl was added to each tube to stop the reaction. Afterwards, 750 µ toluene:ethyl acetate was added to each tube and the tubes were shaken vigorously for 10 seconds. Centrifugation was done at room temperature at 1000G for 5 minutes. 500 µL of the upper (toluene:ethyl acetate, organic) phase in each tubes were transferred to scintillation vials, and before the measurement in scintillation counting machine was done, 8 mL scintillation cocktail was added as well.

Water or buffer instead of plasma. Distilled ion-free H_2O, or a NaK-buffer with the same pH as the blood plasma (pH 8.8) was added instead of blood plasma in 8 tubes (4 blank sample tubes with water, 4 with buffer + 4 sample tubes with water, and 4 with buffer). The tubes were then treated as blank or sample tubes. In tubes with 25 µL plasma, there was also 25 µL buffer added to obtain the same volume as in the tubes with 50 µL plasma. When 50 µL distilled water was added instead of plasma, no buffer was used.

β-phenylethylamine as pSSAO-substrate. Additionally 0.1M β-phenylethylamine from peas was in one experiment added instead of ^{14}C-benzylamine, in order to investigate if it was possible for pSSAO to use that as a substrate. 50 µL β-phenylethylamine was in that experiment added in each tube after incubation in room temperature, before the water bath incubation step.

9. Results

Contamination test of hydrochloric acid, toluene:ethyl acetate, L-deprenyl, glass tubes and two scintillation cocktails. The first thing to be investigated was if any of the solutions used in the sample preparation was contaminated, and thereby added radioactivity to the samples. This was not the case as shown by the CPM-values of toluene:ethyl acetate, 3M HCl, the old scintillation cocktail, and L-deprenyl. However, the Scintillation cocktail Ultima Gold seemed to contain radioactive molecules (Appendix 2).

Moreover, no difference could be seen in CPM-values between samples or blank samples that had been prepared in glass- and plastic tubes. Neither could any radioactive contamination be found in the glass tubes.

Comparison between two LSC-machines. When comparing the CPM-values between the same samples counted with two different scintillation counting machines (Beckman Scintillation Counter LS6000IC PerkinElmer Liquid Scintillation Analyzer TriCarb® 2910TR) no significant difference could be seen (Appendix 3). The difference in CPM-values between Beckman Scintillation Counter LS6000IC compared to PerkinElmer Liquid Scintillation Analyzer TriCarb® 2910TR was 3.7%.

Comparison between the two scintillation cocktails Ultima Gold™ and Aquasafe 300 Plus. When measuring the radioactivity in only the scintillation cocktails, it was shown that the CPM of Ultima Gold^{TM} was 131% higher than in the other scintillation cocktail (Appendix 4). This was present even though the standard samples with Ultima Gold^{TM} were 8% lower than the standard samples with the old cocktail.

Investigation of the effect of thawing plasma samples on the enzymatic activity. The results of measurements of pSSAO-activity in plasma that had been previously thawed once or twice indicated that the activity is highly affected by this treatment (Appendix 5). Therefore, results of so called re-runs of already measured plasma that has been preformed earlier in the project were probably not trustworthy. It is shown that 24% of the enzymatic activity is lost after one and 39% after two thawing steps, compared to the "real" CPM-value.

24 hours after preparation of scintillation vial comparison. No difference could be seen in any CPM-values between samples that were measured in the scintillation machine immediately after preparation and the CPM-values of the same samples measured 24 hours later (Appendix 6).

New ^{14}C-Benzylamine. A new substrate dilution was prepared in order to test if the substrate is oxidised when it is kept at -20°C, and thereby is found in the toluene:ethyl acetate layer after centrifugation. The same protocol that had been used in previous experiments was followed. This protocol was also the one used when the CPM-values were not increasing, before January 2011. However, no difference between samples containing the newly prepared and the older ^{14}C-benzylamine could be seen, not in reference plasma samples or in blank- and standard samples (Appendix 7).

When the samples were prepared as usual but no ^{14}C-Benzylamine was added, there was no radioactivity detected in the LSC-machine at all (30 CPM).

Different amounts and concentrations of hydrochloric acid. When the measurements of SSAO-activity in human blood plasma first started, 1M HCl was used. Then an increase in blank CPM values was observed and to exclude that the cause could be that the pSSAO-activity was not completely inhibited after the water-bath incubation, the molarity was changed to 3M HCl. Details are presented in Appendix 8.

The comparisons between 7 tubes with 3 M and 7 tubes with 1 M hydrochloric acid added showed only a 3% decrease in CPM-value in reference plasma samples. Moreover, the difference was almost non-existent in the CPM-values of blank samples (7 tubes with each concentration); 0.7% decrease.

When comparing CPM-values of blank samples to which HCl was added both before and after incubation in the water bath, with blank samples with HCl only added once (before water bath), the difference was -9%. The comparison was done between 12 samples with HCl added once and 14 samples with HCl added twice; half of the samples with 1 M and the other half with 3 M hydrochloric acid. The difference in CPM-values between 3 M HCl added twice and 1 M HCl added twice was -0.4%.

Measurement of the radioactivity in samples with water or buffer instead of plasma. No plasma, and thus no pSSAO should give a very low CPM value in the samples, since no product can be formed without the enzyme. When CPM-values in blank samples with half amount plasma (25 µL) were compared to the ones in samples with 50 µL plasma, no significant difference could be seen (Appendix 9). This suggests that there is no activity from any enzyme in the plasma that causes the high blank values. If that would had been the case, the CPM in the tubes with half plasma amount would also be 50% of the CPM compared with the tubes with usual amount of plasma. The buffer showed no radioactivity (22 CPM).
In the normal samples, the CPM-values in tubes with only 25 µL plasma were approximately half of the values in tubes with 50 µL. This indicates that there is an enzymatic activity in the plasma, and it also means that the enzyme is relatively equal distributed in the plasma.

5 or 7 minutes centrifugation time**.** **T**he mean value of the samples that was centrifuged for 5 minutes was 1010 CPM, while the same value of samples centrifuged two minutes longer was 952 CPM. The difference between the mean values is approximatively 6%.

The activity of MAO-B. MAO-B-activity measurement was done for investigation of another REA for enzymatic activity measurement and its blank samples' CPM-values, to see if these values were increasing as well. They were not. Moreover, it was confirmed that pSSAO can use β-phenylethylamine as substrate to some extent. The β-phenylethylamine standard samples' CPM-values were however much lower than the ones with ^{14}C-benzylamine. To investigate whether MAO-B-activity contributes to catalysis of the reaction with 14C-benzaldehyde as product, CPM-values were compared between blank samples with and without the MAO-B-inhibitor hydralazine. It was shown that the adding of hydralazine resulted in lower CPM-values. However, these values were not as low as the blank samples' in the beginning of 2011.

Patients' plasma samples. This investigation shows significant increases in control plasma samples' CPM-values, as well as the CPM-values of patients' plasma samples and reference plasma samples (Appendix 10). All of these values are significantly higher than the ones obtained in previous measurements. The CPM-values in samples with patient plasma and were in average 9% higher than the CPM-values of the samples with control plasma.

CPM-values from January to March 2012. The CPM-values of the reference plasma samples, with blank values subtracted, increased between the end of January 2012 and the first week of March 2012 (Figure 6).

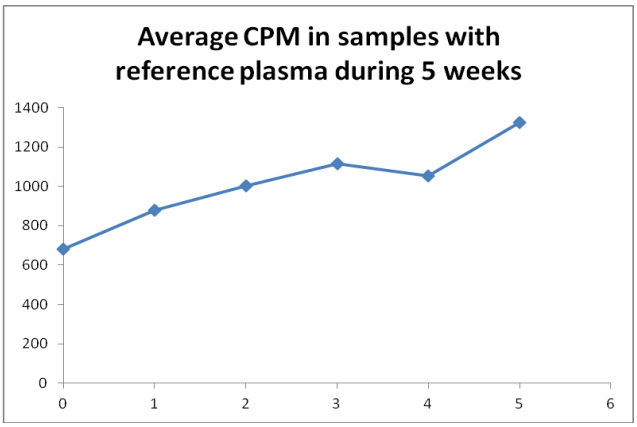

Figure 7. *Mean CPM-values of samples with reference plasma from January 2012 (week 1) to March 2012 (week 5).*

Regarding the blank values, no significant difference can be seen between the CPM-values in January and the ones in March. The mean blank sample CPM-value is 1021. This value is higher than the mean value for measurements done in December 2011. Also the CPM in standard samples during these weeks seem stable around 103 000.

10.Discussion

In this experiment different factors that possibly could have caused the increasing CPM-values of blank samples have been excluded one by one. No radioactive contamination could be found in any of the substances used, or in the tubes. The results suggest that the cause of the rising CPM-values was the labelled substrate 14C-benzylamine, but no significant increase in radioactivity of only labelled substrate could be proved. On the contrary, standard samples' CPM-values were significantly decreasing during 2011, but have during this period been back at the same level as in the beginning of 2011.

Since there was no difference in values between the two LSC-machines, my results are comparable with previous measurements. Additionally, there was no contamination found in any of the substrates or tubes used, thus these factors can be excluded as contributing to increased blank sample CPM-values. This was also confirmed by the lack of radioactivity in the mixture containing all substances that are added to a sample, but no ^{14}C-benzylamine. When the time between sample preparation and CPM-measurement is less than 24 hours, no difference is seen.

The CPM-values in samples containing patients'- or reference plasma have increased as well as the blank samples' CPM-values. I would recommend an investigation on if there is a factor (plasma glucose- or cholesterol levels, age or gender of patients), that explains the big difference in CPM-values between the previous measurements, and mine or if the cause is methodological.

No doubt, one potential source of error has been the change of persons performing the experiment. However, this is probably not the only reason of the change in CPM-values. A striking result is the relatively large effect of thawing on the enzymatic activity. This indicates that previous results are not completely trustworthy, since some of the samples were measured more than one time and frozen between the experiments. The enzymatic activities are probably lower than the actual ones in these measurements.

When taken in consideration the difference in standard samples' CPM-values between ^{14}C-benzylamine and β-phenylethylamine, the latter seem to be a usable substrate for pSSAO-activity measurements as well. But a change of substrate would probably not solve the methodological problem of this method. However, the MAO-B-activity measurements gave us more information. It was shown that an amount of MAO-B-activity is present even in samples with 14C-benzylamine as substrate and with added MAO-B-inhibitor L-deprenyl. Since MAO-B is only present in the blood platelets, and not in the plasma, this indicates that the used plasma is not completely free from other blood contents.

HCl is added as a broad spectra enzymatic activity inhibitor, no processes should be able to proceed after HCl is added. The double amount or a lower concentration (1 M instead of 3 M) of HCl does not seem to affect the blank sample CPM-values, which indicates that the amount that has been used in this experiment before is enough for inhibition of pSSAO.

Interestingly, something else than the pSSAO-activity seems to affect the CPM-value, shown by the quite high values in samples containing buffer or water instead of plasma. Moreover, hydralazine added at the same time as L-deprenyl seems to lower the blank samples' CPM-value. The amount of L-deprenyl might not be enough to inhibit all MAO-B-activity. This is rather confusing since 3 M hydrochloride acid should inhibit MAO-B as well as pSSAO.

To ensure that there is nothing else in the plasma that contributes to the oxidation of ^{14}C-benzylamine, a gel chromatography for protein separation would be recommended if the problem with significantly changing CPM-values occurs again. This can be done by separating the plasma molecules according to size and charge, and afterwards investigate if there is another ^{14}C-benzaldehyde forming molecule present in the plasma, except from pSSAO and MAO-B.

The obtained results point out the effect of thawing, and it is of great interest to compare the number of re-runs in every group of previous measurements. To compare the number of re-runs between previous reported data groups would probably give a better picture of the average enzymatic activity in the samples and thus of the difference between blank sample CPM-values and sample CPM-values. If this difference has not changed, there is possibly to continue with the same substances and the same method as before.

The research considering the possibility to use pSSAO-activity as a biomarker for diabetes and cardiovascular diseases also supports the research on new pharmacological treatments for these diseases, and vice verse. The more that is known about an enzyme and the exact molecular function behind its catalytic pathways, the more reaction steps can be tested as targets for an inhibitor. Further investigations on the functions of SSAO in the forming of elasticity fibres, is one way to go in the research of prevention drugs for cardiovascular diseases.

Three quantitative factors direct contribute to the function of SSAO: number of molecules, availability of substrate, availability of copper and TPQ. What has previously been done, but I think would add strength to this study, is investigation of the number of pSSAO-monomers.

SSAO-monomers are not able to catalyse any reactions and therefore the measurement could be done for example by using enzyme-linked immunosorbent assay (ELISA). One hypothesis is that under certain physical conditions the monomers dimerizes and become active enzymes. The more monomers that are present in a soluble from in blood plasma, the more active enzyme can be formed. However, more enzyme molecules do not necessary imply a higher activity, and since the mRNA-expression is not higher in mice with higher pSSAO-activity, the number of monomers probably do not differ even though the enzymatic activity does.

Additionally, the concentration of TPQ in the investigated plasma can be measured, to see if the availability of co-factor is correlated to cardiovascular-related problems, diabetes, and loss of elasticity in arteries. Whether the substrate availability is higher in patients with mentioned kinds of diseases or not, is also possible to measure. It would in that case be necessary to analyse the blood, and not only the plasma.

To measure the enzymatic activity, it is also possible to measure amount of substrate left, or amount of the other two products of the reaction H_2O_2 or NH_3. As mentioned, radioimmunoassay is also an alternative.

SSAO is a fascinating enzyme with a lot of diverse functions and there are several reasons to believe that the distribution of time between its different tasks varies between species and individuals. This may also be a factor to investigate more deeply in order to fully understand what causes correlations between the enzymatic activity and the frequency and severity of complications.

The impact of life style on Type-2 diabetes and cardiovascular-related problems is as mentioned relatively large, and one of the easiest and inexpensive ways to decrease the frequency of these diseases is a change in diet and exercise habits. This in combination with new pharmaceutical treatments, biomarkers to discover who is at risk, and also a greater understanding of the genetics behind, will be helpful tools in the preventive work of the development of these diseases.

Conclusion

The data above indicates that it is not optimal to continue with this REA for measurement of pSSAO-activity. If the problem with significantly increasing CPM-values continues, there are several tests that can be done in order to find the source of error. Finally, it is of great importance to further investigate the functions of pSSAO and the correlation between the enzymatic activity and Type-2 diabetes and cardiovascular diseases in order to be able to develop new and effective preventive methods.

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APPENDIX 1

Previous measurement groups	**S1**	**S2**	**S3**
Mean value reference plasma samples (CPM)	999	1318	868
Mean value blank samples (CPM)	320	612	444
Mean value reference plasma samples - blank samples (CPM)	734	686	424
Mean value standard samples (CPM)	110743	78163	78945
Number of blank samples	9	16	7
Number of reference plasma samples	18	32	6
Number of standard samples	9	16	7

APPENDIX 2

Contamination tests

Solution	CPM
Toluene:ethyl acetate	44
Toluene:ethyl acetate	21
Toluene:ethyl acetate	21
Toluene:ethyl acetate	24
Toluene:ethyl acetate	23
Toluene:ethyl acetate	26
Toluene:ethyl acetate	28
Toluene:ethyl acetate	25
3M Hydrochloric acid	24
3M Hydrochloric acid	20
1M Hydrochloric acid	26
1M Hydrochloric acid	22
Old scintillation fluid	19
Old scintillation fluid	19
Old scintillation fluid	20
Old scintillation fluid	23
Old scintillation fluid	25
Old scintillation fluid	18
Old scintillation fluid	21
New scintillation fluid	470
New scintillation fluid	674
New scintillation fluid	449
New scintillation fluid	685
New scintillation fluid	244

APPENDIX 3

Comparison between two LSC-machines

Content	**Scintillation Counting Machine (CPM)**	
	New	**Old**
Reference plasma	1620	1578
Reference plasma	1744	1673
Reference plasma	1647	1579
Reference plasma	1665	1584
Reference plasma	1730	1639
Reference plasma	1813	1734
Reference plasma	1753	1690
Reference plasma	1707	1607
Toluene:ethyl acetate	21	21
Toluene:ethyl acetate	24	23
Blank samples with reference plasma	1044	1002
Blank samples with reference plasma	1009	954
Standard	104572	99915
Standard	103644	101003
Average	*15999.5*	*15428.71429*
Difference in percent (new/old)	*3.70%*	

APPENDIX 4
Two different scintillation cocktails

	CPM
Mean value old scint fluid all tubes (cpm-blank)	997
Mean value gold scint fluid all tubes (cpm-blank)	1454
Difference new/old	*1.458375*
Mean value old scint fluid all tubes 2012-02-06	2235.375
Mean value gold scint fluid all tubes 2012-02-06	2692.375
Mean value old scint ref plasma 1	2191.5
Mean value old scint ref plasma 2	2279.25
Mean value gold scint ref plasma 1	2455.75
Mean value gold scint ref plasma 2	2929
Mean value old scint fluid all tubes (except standard) 2012-02-07	3649.625
Mean value gold scint all tubes (ex st) 2012-02-07	3109.5
Mean value ref plasma 3	4190.833
Mean value ref plasma 4	3879.333
Mean value old scint after 24h/mean value direct	1.084221
Mean value new scint after 24h/mean value direct	0.998231
Mean value old scint fluid all tubes (except standard) 2012-02-08	2109.1
Mean value new scint fluid all tubes (except standard) 2012-02-08	2325
Difference old/new	*0.91*

APPENDIX 5

The effect of thawing plasma

Plasma	**CPM not thawed before**	**CPM thawed once before**	**CPM thawed twice before**
Reference plasma 1.	1785	1633	1031
Reference plasma 1.	1716	1605	1267
Reference plasma 1.	1726	1613	1170
Reference plasma 1.	1738	1597	1352
Reference plasma 1.	1793	1647	1246
Reference plasma 2.	1523	1001	
Reference plasma 2.	1534	1071	
Reference plasma 2.	1577	1231	
Reference plasma 2.	3239	1920	
Reference plasma 2.	2566	1522	
Reference plasma 2.	2487	1909	
Reference plasma 2.	2179	1309	
Mean value	*1988,583333*	*1504,833333*	*1213,2*
Difference in percent from the first measurement		*-24%*	*-39%*

APPENDIX 6

CPM values after 24 hours

Plasma	**CPM direct**	**CPM after 24h**
Reference plasma 1. Thawed once before.	1620	1737
Reference plasma 1. Thawed once before.	1744	1651
Reference plasma 1. Thawed once before.	1647	1661
Reference plasma 1. Thawed once before.	1665	1633
Reference plasma 1. Thawed once before.	1730	1806
Reference plasma 1. Thawed once before.	1813	1723
Reference plasma 1. Thawed once before.	1753	1670
Reference plasma 1. Thawed once before.	1707	1744
Reference plasma 1. Thawed once before.	3710	3675
Reference plasma 1. Thawed once before.	1785	1751
Reference plasma 1. Thawed once before.	1716	1742
Reference plasma 1. Thawed once before.	1726	1679
Reference plasma 1. Thawed once before.	1738	1733
Reference plasma 1. Thawed once before.	1793	1787
Reference plasma 2.	1523	1529
Reference plasma 2.	1534	1483
Reference plasma 2.	1577	1596
Reference plasma 2.	1650	1588
Reference plasma 2.	1772	1725
Reference plasma 2.	1539	1509
Reference plasma 2.	3239	3199
Reference plasma 2.	2566	2583
Reference plasma 2.	2487	2479
Reference plasma 2.	2179	2170
Mean value	*1925,541667*	*1910,541667*
Difference after 24h in percent	*-1%*	

Standard Samples	**CPM direct**	**CPM after 24h**
^{14}C-Benzylamine standard sample	104572	104702
^{14}C-Benzylamine standard sample	103644	103920
^{14}C-Benzylamine standard sample	103171	103315
^{14}C-Benzylamine standard sample	105793	106175
^{14}C-Benzylamine standard sample	103244	103176
^{14}C-Benzylamine standard sample	103377	103457
Mean value	*103966,8333*	*104124,1667*
Difference after 24h in percent	*0*	

Blank Samples	**CPM direct**	**CPM after 24h**
Reference plasma 1	1044	1007
Reference plasma 1	1009	1043
Reference plasma 1	1143	1149
Reference plasma 1	1122	1125
Reference plasma 1	919	936
Reference plasma 1	1254	1157
Mean value	*1081,833333*	*1069,5*
Difference after 24h in percent	*-1%*	

APPENDIX 7

New 14C-benzylamine compared to the older one

	^{14}C-benzylamine	
	Prepared in February 2012 (CPM)	Prepared in August 2011 (CPM)
Reference plasma sample	2100	2025
Reference plasma sample	2125	1813
Reference plasma sample	1893	1501
Reference plasma sample	1990	2345
Reference plasma sample	2125	2350
Reference plasma sample	2392	1790
Reference plasma sample	1720	1893
Reference plasma sample	1835	1957
Standard sample	103200	102250
Standard sample	103235	103620
Standard sample	105230	104230
Standard sample	103298	980983
Standard sample	104585	105670
Blank sample	1201	1082
Blank sample	1183	1194

APPENDIX 8

1 M HCl and 3 M HCl added once or twice

HCl added once (before RT-incubation)	
Concentration HCl	**CPM**
3M HCl	1281
3M HCl	1337
3M HCl	1207
3M HCl	1338
3M HCl	1336
Mean value 3M HCl added once	*1299,8*
1M HCl	1284
1M HCl	1332
1M HCl	1249
1M HCl	1277
1M HCl	1364
1M HCl	1296
1M HCl	1240
Mean value 1M HCl added once	*1292*

Reference plasma samples	**CPM**
3M	1805
3M	2858
3M	2399
3M	2322
Mean value 3M HCl	*2346*
1M	2633
1M	2526
1M	2512
1M	2417
1M	2130
Mean value 1 M HCl	*2444*

APPENDIX 9

Water or buffer instead of reference plasma

Blank samples	**CPM**
50 µL plasma	1060
50 µL plasma	1104
25 µL plasma + 25 µL buffer	1207
25 µL plasma + 25 µL buffer	1121
50 µL buffer	824
50 µL buffer	1091
50 µL water	1003
50 µL water	1099
Standard	103254

Reference plasma samples	
50 µL plasma	3417
50 µL plasma	3736
25 µL plasma + 25 µL buffer	2630
25 µL plasma + 25 µL buffer	2578
Standard	105635

APPENDIX 10

Measurement of CPM-values in patient plasma samples.

Samples marked with red have been thawed one time before this experiment. Patients' plasma samples have numbers >1000 and control plasma samples have numbers <1000.

Nr	**CPM**	**CPM-Blank**	**Gender**	**Age**
Average blank	*1129*			
452	3520	2391	male	54
452	3856	2727	male	54
456	2928	1799	male	60
456	2912	1783	male	60
476	3284	2155	woman	54
476	3476	2347	woman	54
1575	1575	446	male	66
1575	1752	623	male	66
1576	3046	1917	woman	83
-	-	-	woman	83
1578	3148	2019	male	76
1578	3137	2008	male	76
1580	3126	1997	male	89
1580	3200	2071	male	89
Average Standard	*103570*	*102441*		
Average Reference	*2878*	*1749*		