SciLifeLab

Discovery of novel biomarkers for cardiovascular disease, focus on endothelial specific proteins

A SCAPIS pilot study

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Master thesis

LUND UNIVERSITY

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Abstract

SCAPIS is a national project in Sweden and stands for Swedish CardioPulmonary bioImage Study. The objective of SCAPIS is to find biomarkers in order to improve the risk prediction and lower the death rate of Cardiovascular disease (CVD). [1] CVD is a big health problem and is one of the main causes of death in many industrial countries. [2] It is usually related to atherosclerosis, which is plaque buildup in the arteries and can result in a heart attack or stroke. [3] This study is a part of SCAPIS pilot study and focuses on analyzing endothelial specific proteins by a single binder assay in order to compare the relative protein levels within different known risk groups for CVD; smoking, BMI, hypertension, diabetes and coronary artery calcification score (CACS).

Endothelial cells line the blood vessels and are involved in many important processes, such regulating the vessel's structure and function, regulation of blood pressure, inflammation and hemostasis. Proteins involved in these functions tend to be overexpressed in endothelial cells, hence endothelial specific proteins. [4] Several studies have determined that dysregulation of endothelial function can lead to atherosclerosis and cardiovascular disorders. [5] [6] [7]

Based on the results from a discovery screening, several endothelial specific proteins were selected and analyzed by a single binder assay. Some interesting proteins showed a large separation between the cases and controls in one or several of the known risk groups for CVD, for example between smokers and non-smokers. In this study, the proteins that are affected by (p-value<0.01) two or more risk groups are of higher interest, since this could indicate that the protein might be affected by or involved in the development of CVD. However, these proteins have to be studied further, for example with a dual binder assay, in order to determine if some of them could be used as a biomarker for CVD.

Acknowledgments

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Abbreviations

- SCAPIS - Swedish CardioPulmonary bioImage Study
- CVD - Cardiovascular diseases
- TriGl - Triglycerides
- Chol - Cholesterol
- LDL - Low-density lipoproteins
- HDL - High-density lipoprotein (HDL
- CACS - Coronary artery calcification score
- CRP - C-reactive protein
- CTA - Coronary computed tomography angiogram
- MRI - magnetic resonance imaging
- BMI - Body mass index
- SA - Streptavidin
- PE - Phycoerythrin
- SBA – Suspension bead array

1. Introduction

Cardiovascular disease (CVD) is a general term for several conditions affecting the heart or blood vessels. [3] It is a common disease and it is one of the main causes of death in both Europe and the United states. [2] [8] The current diagnostic and prevention methods for CVD are outdated and 75% of fatal coronary events happen outside a hospital, due to failure to recognize vague and early symptoms of CVD [1] [9]. In fact, sudden death or acute coronary syndrome are often one of the first symptoms of coronary atherosclerosis. [1]

There are several known risk groups that have a higher risk for CVD such as smoking, obesity, diabetes and hypertension. However, there is a large proportion of the population suffering a heart attack or another cardio vascular disease without being classified in any known risk group. [10] It is therefore essential to develop better and more reliable diagnostic methods in order to evaluate the risk for CVD and prevent life threatening events and conditions. [1]

This project focuses on detecting biomarkers for CVD by analyzing several endothelial specific proteins by a single binder assay. By finding biomarkers for CVD more people can be properly diagnosed and it could also give rise to new medication and treatments for these types of diseases.

1.1 Aim

The aim of this project is to analyze potential biomarkers for CVD by measuring the relative levels of certain endothelial specific proteins by a single binder assay. In order to analyze which protein could be involved in or be affected by cardiovascular diseases several known risk groups for CVD will be used to compare the protein levels within the different groups.

1.2 Background

1.2.1 Cardiovascular disease

Cardiovascular disease is the leading cause of death in most industrial countries. It causes 3.9 million of deaths each year in Europe and approximately 730 000 Americans have a heart attack each year. In 2015 there were approximately 11.3 million new cases of CVD in Europe. [2]

A large proportion of CVD are related to atherosclerosis, which is a term when fatty deposits, plaques, are build up in the wall of the arteries, causing them to become thick and stiff and making it harder for the blood to flow through. Atherosclerosis can occur throughout the whole body and progress over a period of time. When this happens in the arteries of the heart, it is called coronary artery disease and when it occurs in the legs it is peripheral arterial disease. Atherosclerosis and plaque buildup can cause a blood clot and stop the blood flow. If this blood clot blocks the blood supply to a part of the heart, a heart attack can occur. Furthermore, a stroke happens when the blood flow to a part of the brain is blocked. [3]

1.2.1.1 Risk factors and diagnostic methods for CVD

Lifestyle and other diseases have a large impact on the occurrence of CVD. Several studies have determined known risk groups for CVD, which include patients with high blood pressure (hypertension), diabetes and dyslipidemia, which is elevated levels of triglycerides (TriGl), cholesterol (Chol) and low-density lipoproteins (LDL) or a low level of high-density lipoprotein (HDL). [2] [11] [12] Smoking and obesity also increases the risk for CVD. [2] [11]

In addition, coronary artery calcification (CAC), which is calcium build-up in the heart's arteries, have shown to be highly correlated to patient with coronary heart disease and can be considered an indication of CVD. Studies have determined that calcification is associated with arterial stiffness, which increases the risk for cardiovascular events. Also, the extent of CAC is strongly connected to the rate of future cardiac events and with the degree of atherosclerosis. In order to detect calcified lesions a coronary computed tomographic angiography (CTA) can be used to detect fat deposits and blockages in the coronary arteries and around organs. The coronary artery calcification score (CACS) can be calculated, which can be used to determine the risk for cardiovascular events and to compare in between patients. [13] [1] [14]

Another indication of CVD can be the degree of inflammation in the body. Inflammation occurs in response to an injury, infection or lipid peroxidation. The oxidization of low-density-lipoprotein (LDL) cholesterol can be amplified by known risk factors for CVD such as, hypertension, smoking and diabetes, resulting in a chronic inflammation. As a result, vulnerable plaques can be created, which are prone to rapture and can cause thrombosis and harmful cardiovascular event. The degree of inflammation can be detected by measuring inflammatory markers. The currently most used is called high-sensitivity C-reactive protein (CRP). This protein amplifies the inflammatory and the procoagulant responses. However, this protein is not specific since it is also elevated in normal processes such as infection and trauma. [15]

Both CTA and CRP analysis can be made in order to identify the risk for CVD. Alternatively, MRI (magnetic resonance imaging) can also be used to identify early atherosclerotic disease in several vascular beds. [1]

1.2.2 Endothelial cells and endothelial specific proteins

All blood vessels are lined with a single layer of endothelial cells, the endothelium, which controls the passage of material and white blood cells between the bloodstream and the surrounding tissues. *Figure 1* shows were the endothelial cells are located. The endothelial cells are essential for regulating the vessel structure and function and several other important processes such as regulation of blood pressure, inflammation, hemostasis and organize the growth of connective tissue. Proteins highly involved in these functions tend to be greatly expressed in endothelial cells, hence endothelial specific proteins. [4]

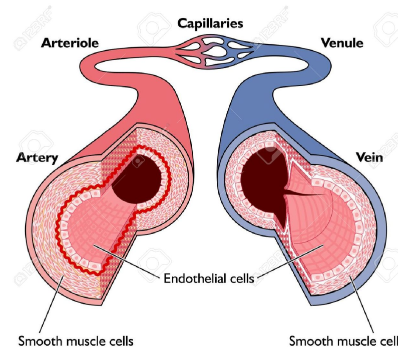

Figure 1. The endothelial cells line the blood vessels and separates the smooth muscle cells and the blood stream. [16]

Dysregulation of endothelial function could indicate vascular damage, atherosclerosis and cardiovascular disorders. [5] [6] [7] Additionally, the endothelial lining creates a barrier that prevents platelet adhesion and generates a natural resistance to thrombosis. However, when the endothelial layer is frequently disrupted or damaged, several processes are activated in order to repair the defects. Platelets have an essential role in the repair of vascular damage and adhere to the injured site. However, the direct link between endothelial damage and diseases such as venous thrombosis is unclear. [17] [18] Although, if the natural blockage of platelets is weakened, abnormal clotting can occur which have a direct correlation to CVD. [18]

Furthermore, a study associated with the Mayo Clinic has shown that local coronary endothelial dysfunction is associated with a large necrotic core, which is one of the characteristics of vulnerable plaques, prone to cause atherosclerosis. [19] Another study has displayed that endothelial dysfunction in the coronary artery has a correlation with the abundance of lipids in the vessel wall, which also are a distinction of vulnerable plaques. The research of endothelial cells and endothelial specific protein are very important in order to understand CVD even more and to lower the mortality rate. [7]

1.2.3. SCAPIS

SCAPIS is a national project in Sweden and stands for Swedish CardioPulmonary bioImage Study. It is a collaboration between the heart and lung foundation and university hospitals in different parts of Sweden. The objective of this project is to reduce the mortality and morbidity from CVD and to find better biomarkers in order to improve the risk prediction for CVD, than the now known risk groups, for example smoking, hypertension and obesity. Better biomarkers could also give rise to better medication and treatment methods. [1] [20] SCAPIS started in 2012 in Gothenburg south of Sweden and 1 100 people were tested in the pilot study. The study included both males and females in the ages of 50 to 64. All had an extensive examination, both medical such as measuring BMI, calcification, hypertension, amount of plaques and a questionnaire where the patients answered questions about lifestyle, food habits, smoking, medication and so on. The long-term goal of SCAPIS is to examine and analyze 30 000 randomly chosen people in Stockholm, Gothenburg, Malmö, Linköping, Uppsala and Umeå. [1] [21]

1.2.4. Single binder assay

Affinity proteomics have been applied to this study as a single binder assay, where one antibody is used to detect the level of a certain protein in plasma samples. The plasma samples are first biotinylated, and the antibodies are coupled to magnetic beads with a bead ID, which indicates what antibody it is and subsequently what protein is has bound to. Furthermore, Streptavidin, connected to the fluorescent dye phycoerythrin (PE), are added and binds to the biotin. Thereafter, the amount or level of the protein is measured with a Luminex machine. The fluorescent signal from PE will indicate the relative amount of protein in the sample. If several proteins are studied, several different antibodies can be used with different bead IDs. *Figure 2* show a schematic picture of the bindings between the different components in a single binder assay.

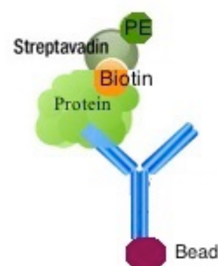

Figure 2. A schematic figure of the binding in a single binder assay. The antibody is connected to an internal color-coded bead, the protein is biotinylated, and Streptavidin conjugated to the fluorescent dye PE binds to the biotin.

2. Material and method

2.1 Plasma samples and the discovery phase

The plasma samples used in this study are from the SCAPIS pilot study (n=1068), which included both men and female in the ages of 50-64. All patients were examined at Sahlgrenska hospital in Gothenburg, the samples were thereafter stored in two different biobanks, the Karolinska institute in Stockholm or Sahlgrenska hospital. An extensive medical examination was done, which included patients' blood values (cholesterol, HDL, triglycerides, LDL, plasma glucose, high-sensitive C-reactive protein), diagnosis of diabetes, blood pressure, analyzing the lung function and calculation of the body-mass-index (BMI). Ultrasound and MRI examination were done to analyze intima-media thickness, plaque size and number of plaques. Images of heart, lungs and fat depots were done with a CT scanner and the calcium content in the coronary arteries were measured with a CTA and summarized to a CAC score. [1]

A questionnaire was also filled out with questions about self-reported health, family, work, smoking, medication, environmental exposure, psychosocial well-being, socio-economic status and overall lifestyle. [1] *Table 1* summarizes the division from some of the groups and the overall results from the medical examination.

Table 1. Summary of the SCAPIS study. The division between the different groups; where the patient lives (the centers), sex, age, pervious CVD, if a CTA analysis were done, the CACS groups, atherosclerosis plaque (Athero plaque), smoking, BMI (divided into underweight, normal weight, overweight and obese), diabetes, weight, height, hip, waist and tests from the lab such as HDL (high-density lipoprotein), LDL (low-density lipoprotein), CHOL (cholesterol), TriGl (triglycerides), CRP sensitive (C-reactive protein, measurement of inflammation).

<table>
<tr><td>Center</td><td colspan="2">Nordost</td><td>526</td><td colspan="2">Väster</td><td>542</td></tr>
<tr><td>Sex</td><td colspan="2">Female</td><td>446</td><td colspan="2">Male</td><td>622</td></tr>
<tr><td>Age</td><td colspan="2">Mean</td><td>59.05</td><td colspan="2">Standard deviation age</td><td>4.39</td></tr>
<tr><td>Age groups</td><td colspan="2">50-54 = 336</td><td colspan="2">55-59 = 362</td><td colspan="2">60-64 = 370</td></tr>
<tr><td>CVD (Prev.)</td><td colspan="2">Yes</td><td>32</td><td colspan="2">No</td><td>1036</td></tr>
<tr><td>CTA analysis</td><td colspan="2">Yes</td><td>1016</td><td colspan="2">No</td><td>52</td></tr>
<tr><td>CACS groups</td><td>0 = 600</td><td>>0-100 = 282</td><td>>100-400= 101</td><td>>400-1000=50</td><td>>1000</td><td>CVD & NV 35</td></tr>
<tr><td>Athero plaque</td><td colspan="2">Yes</td><td>593</td><td colspan="2">No</td><td>475</td></tr>
<tr><td>Smoking</td><td colspan="2">Yes</td><td>157</td><td colspan="2">No</td><td>905</td></tr>
<tr><td>BMI</td><td colspan="2">UW = 6</td><td>NW = 331</td><td colspan="2">OW= 501</td><td>OB= 230</td></tr>
<tr><td>Diabetes</td><td colspan="2">Yes</td><td>82</td><td colspan="2">No</td><td>986</td></tr>
<tr><td>Hypertension</td><td colspan="2">Yes</td><td>339</td><td colspan="2">No</td><td>722</td></tr>
<tr><td>Weight</td><td colspan="2">Mean</td><td>80.57</td><td colspan="2">Range (min-max)</td><td>39.8 – 158.8</td></tr>
<tr><td>Height</td><td colspan="2">Mean</td><td>171.7</td><td colspan="2">Range (min-max)</td><td>148-202</td></tr>
<tr><td>Hip</td><td colspan="2">Mean</td><td>102</td><td colspan="2">Range (min-max)</td><td>57-158</td></tr>
<tr><td>Waist</td><td colspan="2">Mean</td><td>95.39</td><td colspan="2">Range (min-max)</td><td>62-146</td></tr>
<tr><td>Lab HDL</td><td colspan="2">Mean</td><td>1.685</td><td colspan="2">Range (min-max)</td><td>0.38-4.3</td></tr>
<tr><td>Lab LDL</td><td colspan="2">Mean</td><td>5.763</td><td colspan="2">Range (min-max)</td><td>1-7.8</td></tr>
<tr><td>Lab CHOL</td><td colspan="2">Mean</td><td>5.63</td><td colspan="2">Range (min-max)</td><td>2.8-10</td></tr>
<tr><td>Lab TriGl</td><td colspan="2">Mean</td><td>1.351</td><td colspan="2">Range (min-max)</td><td>0.35-8.9</td></tr>
<tr><td>Lab CRP sensitive</td><td colspan="2">Mean</td><td>2.27</td><td colspan="2">Range (min-max)</td><td>0-9.62</td></tr>
</table>

The discovery phase of the SCAPIS pilot study at SciLifeLab, was performed by Larissa Kruse. The objective of Kruse's study was to analyze the relative levels of proteins in plasma samples of individuals in the SCAPIS study by a single binder assay. [22] Thereafter, the results were analyzed by comparing the protein levels within the selected groups; age, gender, BMI, present atherosclerosis plaques, smoking, diabetes, calcification score and hypertension. The discovery phase resulted in a lot of data such as a p-value for every protein within every group. The p-value indicates the separation between the subgroups within the risk groups, for example the separation between smokers and non-smokers. If the p-value is lower than 0.05 the separation is significant. The results were adjusted by age, gender and biobank in order to remove the effect of those groups.

2.2 Selection of proteins and Single binder assay

As a result of Kruse's discovery screening a selection of candidates was done. All proteins that had a p-value lower that 0.01 in the discovery phase were selected to be of interest. Since the present study focused on endothelial enriched proteins, the endothelial specificity was checked by looking at an internal list of endothelial enriched score, previously compiled by Lynn Butler at SciLifeLab. The list was compiled by using an integrative transcriptomics and antibody-based profiling method in order to determine a correlation score between a protein and three known endothelial specific proteins. If the correlation score between the protein and the endothelial specific proteins was higher than 0.5 the protein could be seen as endothelial specific. [23]

The discovery phase included approximately 377 proteins and 221 proteins of them were selected to be analyzed further in this study, the replication phase. Antibodies to the chosen proteins were selected, in most cases there were several antibodies for one protein in order to test which one gave the best signal.

A single binder assay was preformed once again with the proteins of interest in order to analyze the separation between cases and controls within different known risk groups for CVD and to confirm the results from the discovery phase. However, this study do not include cases, in the original sense, since the people involved in the study do not have a specific disease. Nevertheless, in order to make the distinction clear between for example smokers and non-smokers, diabetics and non-diabetics, people that are obese and people that are normal weight and so on, the description cases and controls will still be used throughout the report.

First of all, the protein samples were randomized and biotinylated, the method together with the evaluation of the biotinylation of plasma samples are presented in *Appendix I*. Second, the antibodies were coupled to magnetic beads and the suspension bead array (SBA) was created, the method together with the coupling test are presented in *Appendix II*. Thereafter, the immunoassay was preformed, and the plasma samples were transferred to 384-well plates and the antibodies, the SBA, were added to all the wells. The method for the single binder assay is presented in *Appendix III*.

2.3 Data analysis

Afterwards, the results from the Luminex machine were analyzed in the program R. [24] The plates included some wells with only buffer and some with pools, which contained several different plasma samples, these samples were removed from the dataset. Outliers were also identified and removed. Thereafter, the results were normalized and the result from the Luminex were combined with the information from SCAPIS including the values within the different groups. The risk groups selected to be analyzed in this project were hypertension, diabetes, smoking and BMI. The calcification score (CACS), which is considered to be an indication of CVD, was also analyzed.

The groups hypertension, diabetes and smoking were only divided into two groups; yes or no. However, CACS and BMI were divided into several groups. The CACS groups are presented in *Table 2* below. 15 people in the study did not do a CTA analysis. *Table 3* presents the BMI groups; underweight (UW), Normal weight (NW), overweight (OW) and obese (OB). 6 people in the study were underweight and these BMI samples were excluded from the study, since this has not shown any relation to CVD. The number of people that were in every group, based on the medical examination and the questioner, can be seen in *Table 1* above.

The data were adjusted by gender and age in order to remove the effect of these groups. The results from the Luminex were analyzed in R and the separation between the cases and controls within every group for every antibody was calculated.

Table 2. The CACS groups. Group 1 (CACS: 0). Group 2 (CACS: >0-100), group 3 (CACS: >100-400), group 4 (CACS: >400).

Group	**CACS**
Group 1	0
Group 2	>0-100
Group 3	>100- 400
Group 4	>400

Table 3. The BMI groups. Underweight (BMI: <18.5), normal weight (BMI: 18.5-24.9), overweight (BMI: 25.0-29.9), obese (BMI: >30).

Group	**BMI**
Underweight	<18.5
Normal weight	18.5-24.9
Overweight	25.0-29.9
Obese	>30

3. Result

3.1 Quality control

The biotinylation of the plasma samples and the coupling of the antibodies to magnetic beads were evaluated by sample test and coupling test. The coupling test were executed by adding PE labeled anti-mouse IgG, anti-rabbit IgG, anti-rat IgG and anti-goat IgG, which bound to the antibodies from the same host. Thereafter, the mean fluorescent intensity (MFI) was measured by a Luminex machine. The graphs are presented in *Figure 3* to *Figure 6* below in the following order; anti-rabbit IgG, anti-mouse IgG, anti-rat IgG and anti-goat IgG. The MFI is plotted against the analytes (antibodies). All coupling tests were done with replicated (Blue and grey in the graphs).

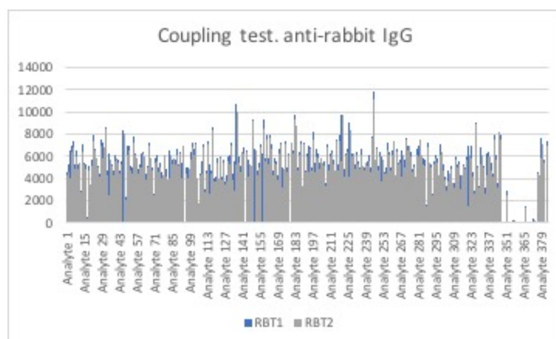

Figure 3. Antibody coupling test with PE labeled anti-rabbit IgG. The MFI are plotted against the analytes (antibodies).

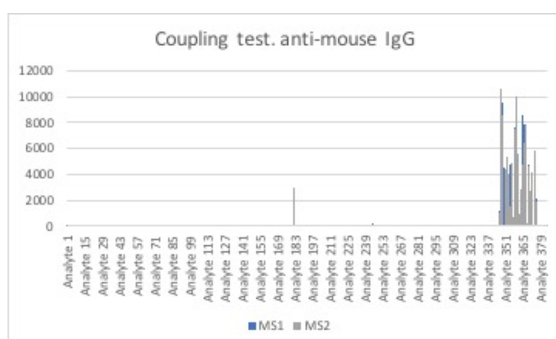

Figure 4. Antibody coupling test with PE labeled anti-mouse IgG. The MFI are plotted against the analytes (antibodies).

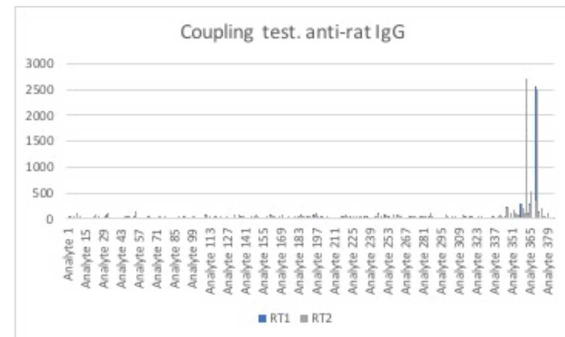

Figure 5. Antibody coupling test with PE labeled anti-rat IgG. The MFI are plotted against the analytes (antibodies).

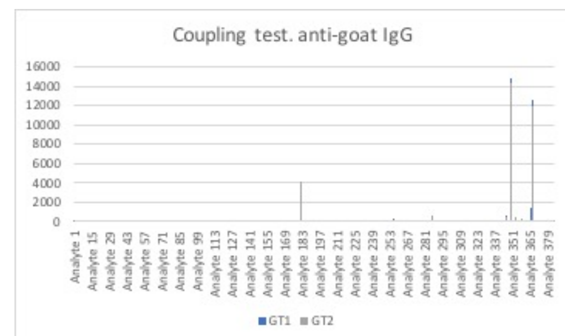

Figure 6. Antibody coupling test with PE labeled anti-goat IgG. The MFI are plotted against the analytes (antibodies).

In order to test the biotinylation of the samples, four internal controls were added in the SBA; mouse IgG, rabbit IgG, anti-human IgG, bare beads, and anti-Albumin. The signals from the blanks should be low and the signals from the plasma pools and the plasma samples should be similar to each other. *Figure 7* to *Figure 11* presents the graphs for the biotinylation test in the following order; mouse IgG, rabbit IgG, anti-human IgG, bare beads, and anti-Albumin.

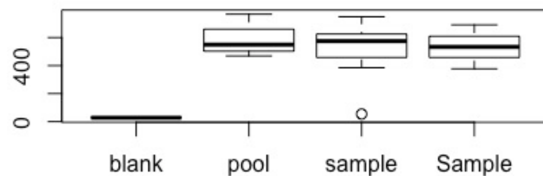

Figure 7. Biotinylation test for one blank, one plasma pool and two plasma samples, with the internal control mouse IgG.

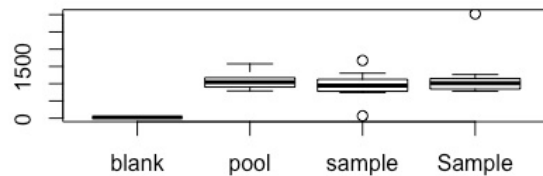

Figure 8. Biotinylation test for one blank, one plasma pool and two plasma samples, with the internal control rabbit IgG.

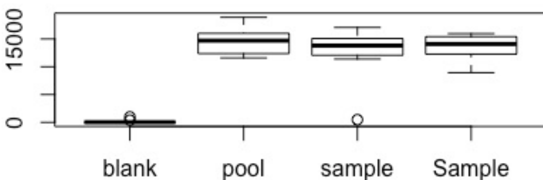

Figure 9. Biotinylation test for one blank, one plasma pool and two plasma samples, with the internal control anti-human IgG.

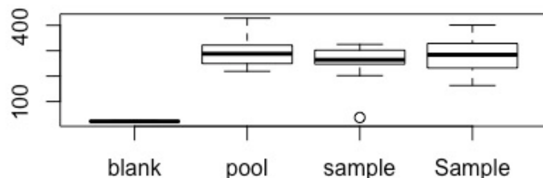

Figure 10. Biotinylation test for one blank, one plasma pool and two plasma samples, with the internal control bare beads.

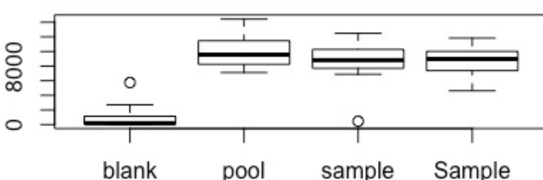

Figure 11. Biotinylation test for one blank, one plasma pool and two plasma samples, with the internal control anti-Albumin.

The results from both the coupling test and the biotinylation test were good, and the samples and antibodies could be used in the single binder assay.

The single binder assay was done as planned and resulted in three 384-well plates. However, in plate 2 and 3 too little amount of streptavidin connected to the fluorescent dye PE (SAPE) was added to the wells. This was noticed when only one row in plate 2 had be analyzed in the Luminex. Therefore, more SAPE was added and the plates were incubated once more. The fluorescent intensity from SAPE in plate 2 and 3 showed no greater difference compared to plate 1 and could therefore be used in the analysis.

After the three plates had been measured in the Luminex machine the results were analyzed in the program R studio. *Figure 12* presents an overview of the results, the mean fluorescent intensity (MFI), from plate 1, the results marked in red corresponds to only buffer and the blue are plasma pools, which were prepared with several plasma samples. *Figure 13* show the result from plate 3. Plate 2 appeared to have a systematic error and the analysis of plate 2 were repeated. The new overview of the results from plate 2 is presented in *Figure 14* below.

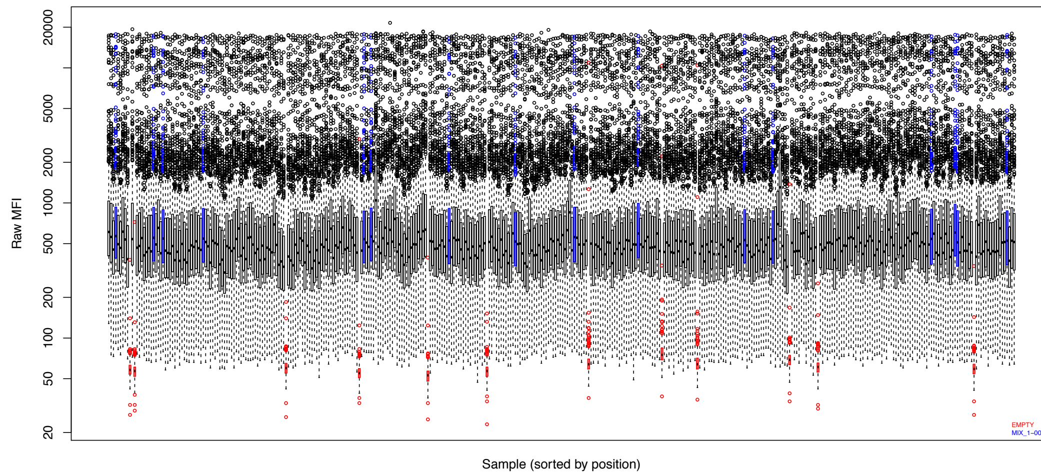

Figure 12. Overview of the mean fluorescent intensity (MFI) in plate 1. The results that are marked with red corresponds to only buffer and the blue are plasma pools, which were prepared with several plasma samples.

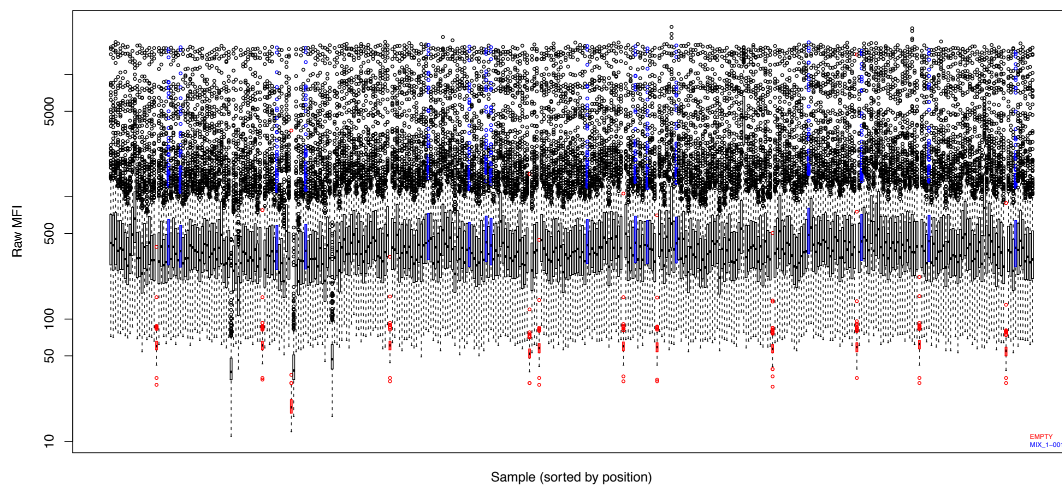

Figure 13. Overview of the mean fluorescent intensity (MFI) in plate 3. The results that are marked with red corresponds to only buffer and the blue are plasma pools, which were prepared with several plasma samples.

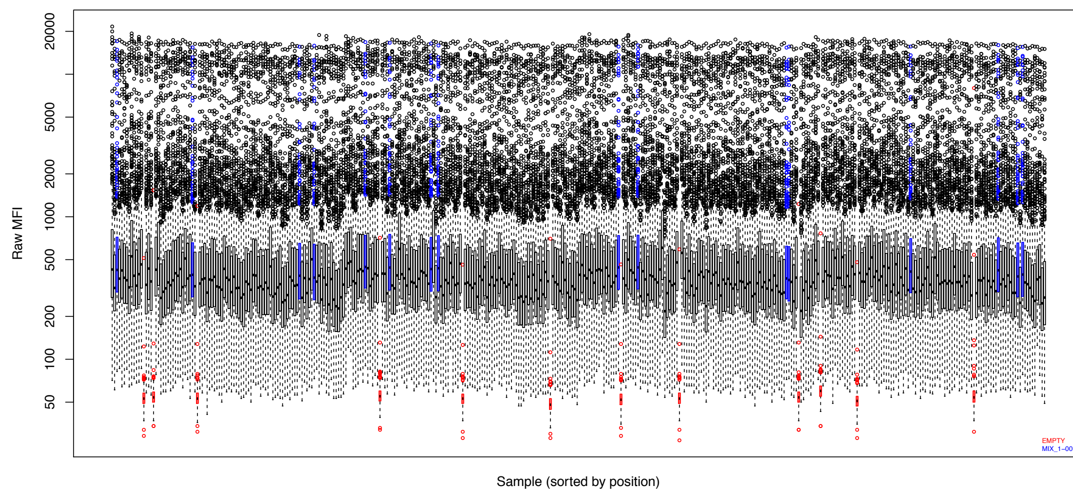

Figure 14. The new overview of the mean fluorescent intensity (MFI) in plate 2 after rerun. The results that are marked with red corresponds to only buffer and the blue are plasma pools, which were prepared with several plasma samples.

In a single binder assay, the coefficient of variation (CV) is usually calculated and indicates inconsistencies in the results. The CV should be below 10% (0.1), which it was for all the plates in this project. The CV plots are presented in *Figure 15* below. The CV values are calculated from the pools of different plasma samples in the three 384-well plates, which includes four 96-well plates. The black line is the CV-value for each 96-well plate and the blue line is all plates together. *Figure 15* A illustrates the CV-values for plate 1 (384-well plate), B is plate 2 and C is plate 3.

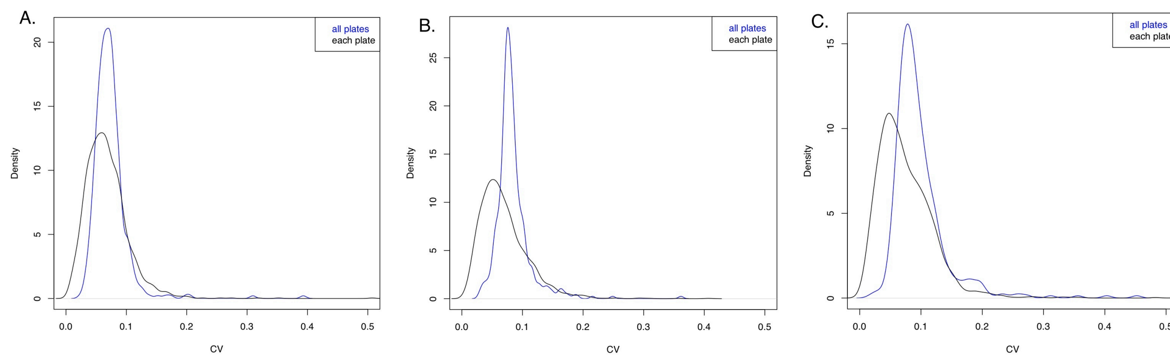

Figure 15. The coefficient of variation (CV) for the three 384-well plates. In the order plate 1 (A), plate 2 (B) and plate 3 (C). The CV value are calculated from the plasma pools (containing several plasma samples) in the three 384-well plates, which includes four 96-well plates. The black line is the CV-value for each 96-well plate and the blue line is all the plates together.

3.2 Protein analysis

The data from the Luminex machine were analyzed and a linear model were applied. Comparison of different groups resulted in p-values for every antibody. If the p-value is lower than 0.05 the separation is significant. In this project, only the proteins with a p-value lower than 0.01 were of interest. *Table 4* present how many proteins in the different groups (Diabetes, smoking, hypertension, BMI and CACS), have a significant separation (p-value<0.01) between the cases and controls within the groups. The table also presents how many proteins have a significant separation when using Bonferroni correction instead, which represents a more stringent cutoff of the p-values. Bonferroni is calculated by dividing 0.05 by the number of comparisons, in this case the number of antibodies which were 375. Thus, the Bonferroni correction is $0.05/375= 1.33*10^{-4}$. The data are adjusted by age and gender.

Table 4. The number of proteins affected by the different known risk groups for CVD (diabetes, smoking, CACS, hypertension and BMI) by both the cutoff p-value<0.01 and the Bonferroni correction (p-value<$1.33*10^{-4}$). The data are adjusted by age and gender.

	Number of proteins affected (Adj. by age + gender)	
Groups	p-value<0.01	Bonferroni (p-value<$1.33*10^{-4}$)
Diabetes	19	5
Smoking	32	7
CACS group	9	1
Hypertension	19	4
BMI group	71	27

Figure 16 illustrates how many proteins that are affected, have a p-value lower than 0.01, within the groups diabetes, smoking and BMI and how many of those proteins are affected by several of the known risk groups for CVD. The data are adjusted by age and gender.

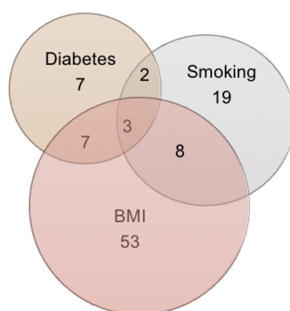

Figure 16. The figure illustrates how many proteins are affected (p-value<0.01) by diabetes, smoking and BMI and how many proteins are affected by several of these known risk groups for CVD. The data are adjusted by age and gender.

In order to illustrate the separation between cases and controls, *Figure 17* presents the separation with a boxplot for one of the proteins in the ADAMTS family in the risk group BMI. The logarithmic MFI is plotted against the BMI groups; normal weight (NW), overweight (OW) and obese (OB). The protein name is modified with XX.

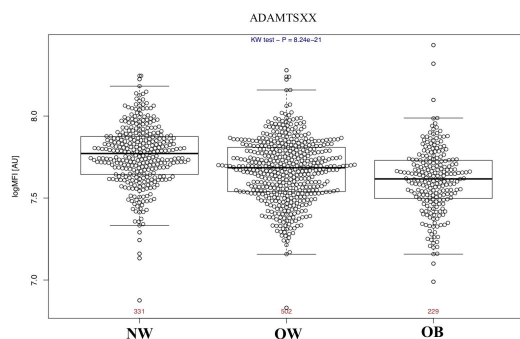

Figure 17. Boxplot of a protein in the ADAMTS family within the risk group BMI. The log MFI is plotted against the BMI groups; normal weight (NW), overweight (OW) and obese (OB).

Table 5 presents how many proteins are affected by two or more risk groups; smoking, hypertension, BMI, diabetes and how many also are affected by CACS, gender and biobank.

The significant separation is evaluated by both the cutoff p value<0.01 and the Bonferroni correction (p-value<$1.33*10^{-4}$).

Table 5. The table presents the number of proteins affected by two or more risk groups; smoking, hypertension, BMI and diabetes (adjusted by age and gender) and how many also are affected by CACS (adjusted by age and gender), gender (adjusted by age) and biobank. The significant separation is evaluated by both the cutoff p-value<0.01 and the Bonferroni correction (p-value<$1.33*10^{-4}$).

Number of proteins affected by:	p-value<0.01	Bonferroni. p-value<$1.33*10^{-4}$
2 or more risk groups; Smoking, hypertension, BMI, diabetes (Adj by age + gender).	28	6
2 or more risk groups; Smoking, hypertension, BMI, diabetes and CACS (Adj by age + gender)	4	1
2 or more risk groups; Smoking, hypertension, BMI, diabetes, CACS (Adj by age + gender) and gender (Adj by age)	2	1
2 or more risk groups; Smoking, hypertension, BMI, diabetes, CACS (Adj by age + gender), gender (Adj by age) and biobank	1	1

3.3 Supplementary table

Some results are compiled in a confidential supplementary table including:

- The antibodies used in this study and the discovery study.
- A gene ontology (GO) analysis to identify molecular function of the proteins. By Gene Ontology Consortium and with PANTHER classification.
- Proteins affected by two or more risk groups; smoking, BMI, hypertension diabetes and which proteins also are affected by CACS, gender and biobank.
- The antibodies correlation value between the discovery and the replication study.

4. Discussion and future studies

The results from the single binder assay show several proteins that displays a significant difference in protein levels between the cases and controls within the known risk groups for CVD, as displayed in *Table 4* and *Table 5*. However, the result from this project, the replication study, and the results from the single binder assay in the discovery phase were quite different. Although, this is not presented directly in the report due to confidentiality, and protein names cannot be presented. Therefore, some protein names are altered.

The reason for the different result in the replication and discovery study are difficult to establish, one explanation could be that the single binder assay is not as specific and selective as a dual binder assay (sandwich assay) and can result in unspecific bindings. Nevertheless, a single binder assay was used in this project, and many other similar studies, due to its ability to incorporate a large panel of proteins and antibodies in one analysis run.

Even though the two single binder assays gave different results for some of the proteins, it can still give an indication of interesting proteins to be studied further. One protein that is expected to have a large separation between cases and controls in several of the known risk groups is VWF (Von Willebrand factor), which it also had. The amount of VWF were higher in both smokers and people with diabetes, hypertension and higher BMI. VWF have an essential role in platelet aggregation and adhesion which is highly correlated to cardiovascular events. Additionally, VWF are a promising target for new antiplatelet medication. [25] [26]

Proteins that have a large separation between cases and control in several known risk groups are of more interest in this project since this could actually indicate an underlying or ongoing cardiovascular disease. The number of proteins affected by two or more risk groups are presented in *Table 5*. In contrast, if the protein levels of a certain protein are higher in people that are obese compared to people that are normal weight, this might just be an indication of the obesity and the higher BMI. However, since endothelial specific proteins are highly related to cardiovascular disease, this theoretical protein could be an interesting protein to study in order to evaluate the connection between obesity and CVD, but this is another scope for another study.

One protein that displayed a large separation between the cases and controls in several risk groups, both diabetes, CACS, BMI and hypertension, was the protein CLECXX. This protein had lower levels in the cases than the controls. CLECXX was not included in the discovery phase. Moreover, CLECXX has been studied in other project and has shown potential to be a biomarker for both atherosclerosis, cardiovascular disease and myocardial infarction which indicates that this protein could be of interest when continuing to investigate potential biomarkers for CVD. [27]

In addition, two proteins in the ADAMTS family displayed a significant difference in both diabetes, BMI and hypertension. The ADAMTS is a large family of 19 enzymes which all are involved in many important processes, such as, extracellular matrix (ECM) assembly and degradation, hemostasis and arthritis. The ECM provide structural support and regulating cell activity, including differentiation, proliferation and apoptosis. ADAMTS

ability to interact with ECM have been associated with vascular disease processes, including atherosclerosis, which makes them interesting proteins to study regarding CVD. Even though, only a few proteins in the ADAMTS family displays separations between cases and controls within known risk groups for CVD, all should be studies further in order to analyze if any of them could be used as a biomarker for CVD. [28]

One protein in the ABCC family also showed separation between cases and controls with in both diabetes, hypertension and BMI. This protein is a type of ABC transporter and is involved in ATP-sensitive potassium channel. It also acts as a sensor for metabolic stress and hypoxia. Studies have associated mutations in ABCCX to CVD. Impaired potassium transportation is related to delayed metabolic cycle leading to changes in the electric coupling of cardiomyocytes, which will result in myocardial damage and loss of cardioprotective responses. [29] Furthermore, knockout mice lacking the ABCCX gene showed elevated blood pressure and coronary artery vasospasm, which is sudden narrowing of a blood vessel. [29] [30]

One protein that had the largest separation between cases and controls in the discovery phase was the protein ClaudinX, which was more abundant in smokers then no smokers. However, this did not show any separation in this study, but ClaudinX could still be an interesting protein. Smoking is a well-known risk factor for CVD and is one of the main causes of death from CVD. The chemicals in the cigarette smoke cause the endothelial cells to become inflamed and swollen leading to narrow blood vessels, rise in blood pressure and increase heart rate. This can lead to several life-threatening conditions such as atherosclerosis and stroke. [31] [32]

ClaudinX is essential in endothelial tight junctions, which control permeability through the paracellular space between endothelial cells. [33] [34] This is an important function to maintain endothelial integrity and if this barrier function is reduced, edema and blood lipids could easily penetrate into the vascular wall. When the endothelium is damaged inflammatory cells and monocytes infiltrate and platelets adhere and aggregate, which causes thrombosis to form and could lead to CVD. [34]

4.1 Future studies

Future studies have to be conducted in order to analyze the proteins further. The first step might be to analyze the most interesting proteins with a dual binder assay, which will result in more reliable outcome since two antibodies have to bind to the protein in order to give out a signal. The proteins of most interest in this phase might be the proteins that are affected by two or more risk groups, as discussed previously. Additionally, mass spectrometry can be used to make sure that the antibodies are binding to the selected protein. However, if the antibodies bind to some other protein, this protein could be of interest instead.

Moreover, there are other factors to considered, a study called the Northern Sweden Population Health Study, investigated the effect of some common medications have on protein levels. [35] This should be explored further in order to conclude that the protein level is affected by an underlying disease and not common medications. The patients' medical information was included in SCAPIS and could be a suggestion for additional analysis. Moreover, it would be very interesting to do a follow up examination for example 10 years later, on the people involved in SCAPIS in order to analyze the health and disease statistics on the SCAPIS participants.

Furthermore, a protein also has to be suited to be a biomarker in the clinic and this should also be investigated. The purpose of a biomarker is often to diagnose a certain disease by measuring the amount of a protein for example. But other factors also have to be considered and tested before introducing it into the clinic. First of all, the stability of the protein is of high importance, it should not be too sensitive to deviance in handling of the sample, since the test probably will be taken and handled by different people in different hospitals, stored and maybe transferred before it is analyzed. In addition, some proteins can easily be affected by outside factors, such as if the patient had worked out before the test, taken medication or had a special diet. This could be problematic when analyzing the test. [36] For example, in short term, exercise produces an inflammatory response, thus elevating the levels of inflammatory proteins. [37] For that reason, these types of proteins might not be a good biomarker to use in the clinic.

To summarize, more research has to be executed in order to discover novel biomarkers for CVD. Still, to further investigate the proteins that are affected by two or more risk groups might be the way to proceed after this study.

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Appendix I – Randomization and biotinylation of plasma samples

A. Randomization of plasma samples

- Thaw raw cohort samples in 4°C, spin down (3000 rpm, 2 min).
- Prepare worktable in TECAN:
  - Place the cohort sample plates on worktable.
  - Place new PCR 96 well plates or skirted PCR plates on worktable.
  - Place 50 µl or 200 µl DiTi TECAN tips in the holder (depending on the volume of sample to randomize).
- Run program.
- If sample pools are included in the randomized plate as replicated references, prepare a pool of cohort samples and add to these wells.
- Seal, vortex and centrifuge (2000 rpm, 1 min) plates containing the now randomized samples. The plates can be stored in -20°C over night if the labelling is preformed later.

B. Biotinylation of plasma samples (two 384 plates)

- Thaw samples in 4°C, spin down (3000 rpm, 2 min)
- Prepare a PCR plate containing 220 µl sterile filtered PBS/well.
- Transfer 22 µl sterile filtered PBS/well to PCR plates using SELMA.
- Transfer 3 µl randomized samples with SELMA.
- Fill up the empty wells by adding 3 µl filtered PBS into these wells.
- Seal the plates with randomized raw plasma and place in -20°C. When frozen transfer the box to -80°C.
- Fill two 15 ml falcon tubes with 1650 µl filtered PBS/each tube
- Dissolve 25 mg of biotin in 2500 µl DMSO.
- Add 1000 µl of biotin/DMSO to one of the PBS tubes and vortex.
- Fill a 96-well PCR plate with 22.5 µl/well. Keep plate on ice as much as possible.
- Use SELMA to transfer 5 µl/well to all diluted sample plates.
- Seal, vortex and centrifuge (2000 rpm, 1 min) the plates.
- Incubate 1 h in 4°C.
- Take out plates, vortex and centrifuge (2000 rpm, 1 min).
- Incubate another 1 h in 4°C.
- Stop reaction with Tris-HCl 0.5 M, pH 8.0. Pour Tris-HCl into a trough and distribute 120 µl/well into a PCR plate.
- Use SELMA to distribute 12.5 µl/well to all sample plates.
- Seal, vortex carefully and incubate plates in room temperature for 20 min on shaker (650 rpm).
- Store the newly labelled samples in -20°C.

C. Sample test

- Prepare assay buffer by mixing 1:10 rabbit IgG in PVXcas, 50 µl is needed for each well. Make extra for blanks (only assay buffer).
  3 replicates + 1 blank = 225µl PVXcas + 25 µl rabbit IgG = 250 µl (50 µl more then you need as a safety)
- Dilute your labelled test sample 1:50 in a tube. 4 µl + 196 µl assay buffer.
- Take a PCR plate and fill three wells with 50µl diluted sample.
- Fill a forth well with 50µl assay buffer (the blank).
- Heat-treat the plate in water bath, 56°C for 30 minutes and 23°C (room temperature) for 10 minutes.
- Dispense 5µl beadstock in 4 wells in an assay plate.
- Transfer 45µl heat-treated sample and blank from the PCR plate to the assay plate containing beadstock.
- Incubate for 1 h in room temperature, 650 rpm, dark.

- Wash the wells with PBS-T 0,05% 3x100µl on magnet.
- Prepare the fluorophore, SAPE. Make a 1:750 dilution - > 1µl SAPE + 749µl PBS-T 0,05%
- Dispense 50µl SAPE dilution in each well.
- Incubate 20 minutes in room temperature, on shaker (650 rpm), dark.
- Wash the wells with PBS-T (0,05%) 3x100µl on magnet.
- Add 100µl PBS-T (0,05%) to each well.
- Run the assay on FlexMap 3D (Luminex), make sure to use a protocol that matches your bead ID's.

Appendix II – Coupling of antibodies of magnetic beads

A. Dilution of antibodies

- Download and insert the .gwl file from LIMS into the TECAN script:
- Prepare worktable:
  - Put 200 µl LiHa tips and 50 µl LiHa tips on the worktable.
  - Fill 4x 10 ml tubes with 10 ml MES buffer and place them in tube holder on the TECAN worktable.
  - Place 4 PCR plates as destiny plates.
- Vortex and centrifuge Ab source plates (1 min at 2,000 rpm).
- Place the correct Ab source plates according to the layout. Check plate positioning, rotation and order.
- Run procedure. Change Ab source plates and tips when needed.
- Seal all Ab plates after they have been used.
- Return Ab plates to -20°C and store diluted antibodies in 4°C until coupling, max 24 hours.

B. Coupling of Antibodies to Beads

- Install the cassette for PBST/MES/AB buffer and wash with dH_2O 5 sec, EtOH 5 sec and dH_2O 5 sec, before priming with AB buffer 5 sec.
- Washing the beads in 80µl AB buffer on magnet.
- Add 50 µl AB buffer off magnet manually with automated multi-channel pipette.
- Prepare activation solution
  - Prepare NHS solution by adding the calculated volume of AB buffer to the tube. (min. 1200 µl).
  - Prepare EDC solution by adding the calculated volume of AB buffer to the tube. (min. 1200 µl).
  - Mix 1200 µl of each solution with 3600 µl AB → Total volume of 6000 µl.
- Add 50µl activation solution to the beads with automated multi-channel pipette, off magnet.
- Incubate 20 min on plate shaker, room temperature, 650 rpm, dark.
- Continue by repeating step 3-7 for plate 2-4.
- Wash BioTek cassette with dH_2O and EtOH as in step 2 and prime with MES buffer.
- When the incubation has finished, wash the beads in 2x100 µl MES buffer, on magnet.
- Vortex and centrifuge the diluted antibody plates (2000 rpm, 1 min) while the beads are being washed.
- Add controls to antibody plates:

Plate 1	*93*	*a-albumin*	*0.5 µl + 99.5 µl MES*
Plate 2	*190*	*rIgG*	*1 µl + 300 µl MES*
Plate 3	*287*	*a-hIgG*	*1 µl + 99 µl MES*
Plate 4	*384*	*bare bead*	*100 µl MES*

- Add diluted antibody (100 µl) with manual multi-channel pipette to all plates, off magnet.
- Seal the plates and vortex carefully before incubating for 2 hours in room temperature, 650 rpm, dark. The incubation can also be performed over night at 4°C.
- Thaw a frozen 5 ml aliquot of 10xBRE and add 45 ml milliQ water and 50 µl ProClin to create storage buffer.
- After incubation, wash the plates with PBS-T 0.05%.
- Add 50µl storage buffer to each well, off magnet, with automated multi-channel pipette.
- Seal the plates and vortex carefully before incubating the plates at 4°C over night or 2 hours in room temperature.

C. Pooling of beads

- Suspend the beads in the wells by pipetting up and down with a manual multi-channel pipette.
- Transfer the whole volume to a flat-bottomed tray (slide washers) on magnet.
- Wash the wells with an additional 50µl storage buffer manually and transfer to the same tray.

- Carefully remove ~7 ml storage buffer (from the side opposite the beads), on magnet. This should leave approximately 3 ml in the tray.
- Carefully remove the tray from the magnet and re-suspend beads in the remaining 3 ml storage buffer that is left in the tray by pipetting up and down.
- Transfer the beads to two regular tubes and one Starlab tub placed in a magnetic holder (approx. 1 ml/tube). Let the beads settle to the magnet ~30 s.
- Wash the tray 3 x 1 ml using the supernatant from the bead tubes.
- Let the beads settle to magnet and remove ~700 ml from each bead tube.
- Remove the tubes from the magnetic holder and re-suspend (vortex) the beads in the remaining 300 µl.
- Transfer the 3x 300 µl to the Starlab tube labelled with the correct SBA-ID and number of antibody coupling plate (ABC plate) in the magnetic holder and let the beads settle for ~30 s.
- Use the beads supernatant to wash the two regular tubes once each.
- Adjust the volume in the tube to 1.5 ml with storage buffer and store the tube in box in 4°C.
- Repeat for plate 2-4. Mark the tubes SBAXX ABC1-4.
- The beadstock is created when mixing the contents of ABC1-4 together into a 384-plex SBA.

D. Coupling efficiency test

- Prepare detection antibody by diluting α-rabbit IgG coupled to R-PE 1:2000 ->1 µl + 2 ml PBS-T 0.05%.
- Dispense 5 µl beadstock into three wells in an assay plate.
- Add 50 µl diluted detection antibody.
- Incubate 20 minutes in room temperature, 650 rpm, dark.
- Wash the wells with PBS-T 0,05% 3x100µl on magnet.
- Add 100µl PBS-T 0,05% to each well.
- Run the assay on FlexMap 3D (Luminex).

Appendix III – Plasma profiling assay run

A. Dilution and heat-treatment of labeled samples

- Transfer labelled samples from -20°C to 4°C to thaw.
- Spin down sample plates in centrifuge (2000 rpm, 1 min).
- Prepare assay buffer by mixing 22.5 ml PVXcas (90%) and 2.5 ml rabbit IgG (10%).
- Use a digital multi-channel pipette to fill a 96-well PCR plate with 220µl assay buffer/well. Seal the plate and spin down to avoid air bubbles.
- Use SELMA to transfer 2x25 µl buffer to 4 96-well PCR plates.
- Transfer 1 µl labelled sample to the buffer plates using SELMA.
- Seal all plates and return the labelled undiluted sample plates to -20°C.
- Heat-treat diluted samples in water bath 56°C for 30 min and then cooled to 23°C (room temperature) for 10 min.

- Prepare SBA-mix.
- Use a multi-channel pipette to aspirate 6µl beadstock in a Greiner 384-well assay plate.
- After heat-treatment, vortex and centrifuge (2000 rpm, 1 min) the diluted samples
- Use SELMA to transfer the heat-treated samples from 96-well plates to 384-well plate.
- Seal the plate and vortex carefully before incubating over night (16 hours) 650 rpm, room temperature, dark.

B. Assay run on FlexMap 3D

- Start BioTek and FlexMap 3D (Luminex machine).
- Prepare 30 ml PFA 0.4% in a falcon tube for each 384-plate.
  27 ml PBS-T 0.05% + 3 ml PFA 4%
- Centrifuge the assay plate (2000 rpm, 1 min).
- Use BioTek to wash the plate with 3x60 µl PBS-T 0.05%/well.
- Distribute 50µl PFA 0.4% /well.
- Seal the plate, vortex carefully and incubate 10 min, room temperature, 650 rpm, dark.
- Prepare 30 ml 1:750 SAPE in a falcon tube for each 384-plate.
  30 ml PBS-T 0.05% + 40µl SAPE.
- Wash the assay plate with 3x60 µl PBS-T 0.05%/well.
- Distribute 50µl SAPE/well.
- Seal the plate, vortex carefully and incubate 30 min, room temperature, 650 rpm, dark.
- Wash the assay plate with 3x60 µl PBS-T 0.05%/well.
- Add 60 µl PBS-T 0.05%/well.
- Run assay plate on FlexMap 3D.