Detection function of cartilage VHH on synovial fluid antigen detection for OA diagnosis

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ABSTRACT

Osteoarthritis (OA) is a degenerative disease of the joint, most common in the knee. OA occurs in more than 500 million patients worldwide, and is one of the leading causes of pain and disability in the elderly. Currently, OA can not be cured, and the treatment that does exist has not been shown to modify the disease, at best providing pain relief at the cost of severe side effects. Diagnosis is often too late, at which point irreversible damage has already been done. Biochemical methods using biomarkers to predict, diagnose, or monitor disease have long been a point of interest of OA researchers. Biomarkers may be able to diagnose OA in a very early stage. However, no such biomarker is currently accepted as characteristic of OA. VHH antibodies may play a large role in OA biomarker discovery. In this study, ELISA is performed on synovial fluid samples using VHHs selected from phage-display biopanning on decellularized cartilage, in order to validate whether or not these cartilage specific VHH can also accurately detect antigens in synovial fluid. Furthermore, the synovial fluid samples donors have been treated with Celecoxib or Naproxen, or received no treatment prior to knee removal surgery, and sample retrieval.

Keywords: osteoarthritis, biomarker, VHH, diagnosis, synovial fluid

INTRODUCTION

Osteoarthritis

Osteoarthritis (OA) is a degenerative disease of the joint, which causes pain, disability, and in severe cases even loss of function. The most commonly inflicted joint is the knee joint, severely impairing patients' movement. This chronic disorder is most prevalent in the elderly, especially women, but can manifest itself at any age. Over the last 3 decades, occurrence of OA has more than doubled. From 247,51 million cases in 1990 to 527,81 million cases worldwide in 2019, accounting for 7% of the global population [1]. This rapid increase of OA in humans has had a significant economical impact. It is estimated that the average annual cost for a person with OA in treatment is between $700-$15.600 in 2019 [2].

OA, much like obesity, is speculated to be an 'evolutionary mismatch disease', resulting from environmental changes outpacing human genome adaptation [3]. Due to increased dependency on machines and computers, humans in the current age live a life far more sedentary than their ancestors. This sedentary lifestyle is not expected to decline anytime soon. Along with the increased life expectancy of the modern era, it seems the number of OA cases will increase even further in the future.

While OA is not directly lethal like many other chronic diseases like cancer or cardiovascular

disease, OA is suspected to contribute to a shorter lifespan, possibly resulting in premature death [4].

OA is commoonly recognized as a heterogeneous condition, indicating multiple underlying causes (etiologies) that lead to varying symptoms. [5]. These different etiologies result in differing endotypes of OA, all requiring a specialised treatment. For example, an endotype typically associated with senescence might be remedied by drugs targeting senescent cell in the joint whereas an inflammatory endotype would require cytokine-targeting drugs instead. This is possible reason why most clinical trials for Disease-Modifying OsteoArthritis Drugs (DMOADs) end in failure [6].

Popular Treatment Options
Popular drugs for pain relief, are Celecoxib and Naproxen [7]. While these Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are not classified as DMOADs, they can be utilized for treating pain caused by OA. Unfortunately, utility of NSAIDs is often limited due to significant side effects such as gastrointestinal ulceration and bleeding, or blood platelet inhibition. NSAIDs conventionally inhibit COX-1 and COX-2 non-specifically, 2 catalytic enzymes for biosynthesis of arachidonic acid into prostaglandins. Some NSAIDs can specifically inhibit only COX-2, greatly reducing risk of side effects.

Diagnosis
Along with inaccessibility of the joint organ, the limited understanding of its chronological progression also makes reasearch challenging. OA is most commonly diagnosed using biomedical imaging [8] in addition to a physical examination. Unfortunately, medical imaging is unable to distinguish the different endotypes of OA, which makes treatment less effective. It is evident that OA treatment is quite difficult due to the complex nature of OA. Therefore, it is of utmost importance that OA needs to be detected as early as possible [9]. Due to the lengthy progression of OA, an opportunity to apprehend the development of OA in an early stage could possibly result in the prevention of irreversible joint damage. However, conventional diagnostic methods such as X-rays often fail to detect early-stage OA, leading to delayed diagnosis until irreversible damage has already occurred [8]. There will already be irreversible damage to the joint. MRI scans offer superior sensitivity in detecting early OA, but their widespread use is limited by cost and accessibility. Arthroscopy, although providing direct visualisation of the joint, is infrequently utilized for diagnosis due to its invasive nature [8].

In addition to early OA, pre-OA is a preceding stage which differs from any other stage of OA [8]. Pre-OA does not show symptoms or signs of OA. However, pre-OA can evolve into full blown OA when it becomes symptomatic, possibly resulting in irreversible joint damage. It is possible to detect pre-OA, currently diagnosed by MRI or Arthroscopy. Detecting pre-OA might give medical practitioners the upper hand when or if it evolves into symptomatic OA, steering the progression of the disease away from irreversible joint damaging, greatly increasing wellbeing of the afflicted patient.

It is clear that there is a demand for a novel approach to OA diagnosis, in order to be able to capture the disease at its earliest stage, preventing the worst symptoms of OA.

Biomarkers
Despite using physical tools, biochemical methods are also widely used to diagnose diseases, with biomarkers serving as key elements in biochemical approaches. Biomarkers are roughly defined as a biochemical indicator of biological and/or pathogenic processes, or responses to an exposure or

intervention [10]. Biomarkers can be used to diagnose or even predict disease, PSA is a common example used for the diagnosis and monitoring of prostate cancer. Most importantly, biomarkers might present opportunities to be able to detect or even predict pre-OA and early OA, in addition to identifying the type of OA if the disease has progressed [8]. Biomarkers should be used in conjunction with biomedical imaging in OA diagnosis and treatment, as biomarkers are unable to give a clear insight into information as the number of joints inflicted, or the bone density of the patient.

Before detection of biomarkers can give us insight in biological processes occurring in patients, these biomarkers have to be discovered and validated to be specific for a certain disease and/or endotype, to ensure their reliability. While the concept of biomarkers appears straightforward. their discovery and validation present significant challenges. After an antigen, functioning as a biomarker, has been discovered, it is necessary for it to be detected accurately and specifically, further adding to the challenge of biomarker discovery. Throughout the years, countless of biomarkers have been suggested as 'OA biomarkers', but none are truly characteristic for OA as of yet [8].

Mass spectrometry is a widely used method for biomarker discovery due to its high accuracy and specificity [10]. However, it is less effective in detecting biomarkers present in low concentrations, which may be predictive for diseases like OA. This limitation makes mass spectrometry less ideal for researchers aiming to identify predictive biomarkers. Moreover, the equipment required for mass spectrometry is expensive, limiting its accessibility for routine use.

Currently, of all the state of the art discovery methods, immunological screening using an antibody library shows a fair amount of promise. For example, phage-display biopanning is able to select specific antibodies that bind to certain antigens, or even biological fluids such as blood, or synovial fluid [11]. The antibody library used can easily be genetically sequenced using Next Generation Sequencing (NGS) [12], in order to be able to replicate certain antibodies if phage-display panning shows affinity for a certain substance. Phage-display biopanning using antibodies is commonly run using antibodies of human or other mammalian origin.

VHH

Antibodies, or immunoglobulins (Ig) can be classified into several isotypes (IgG, IgM, IgA, IgD and IgE) [13]. The antibodies play an important role in the immune system. When an antigen is detected in the body, antibodies are released which in turn either neutralize or destroy the antigen.
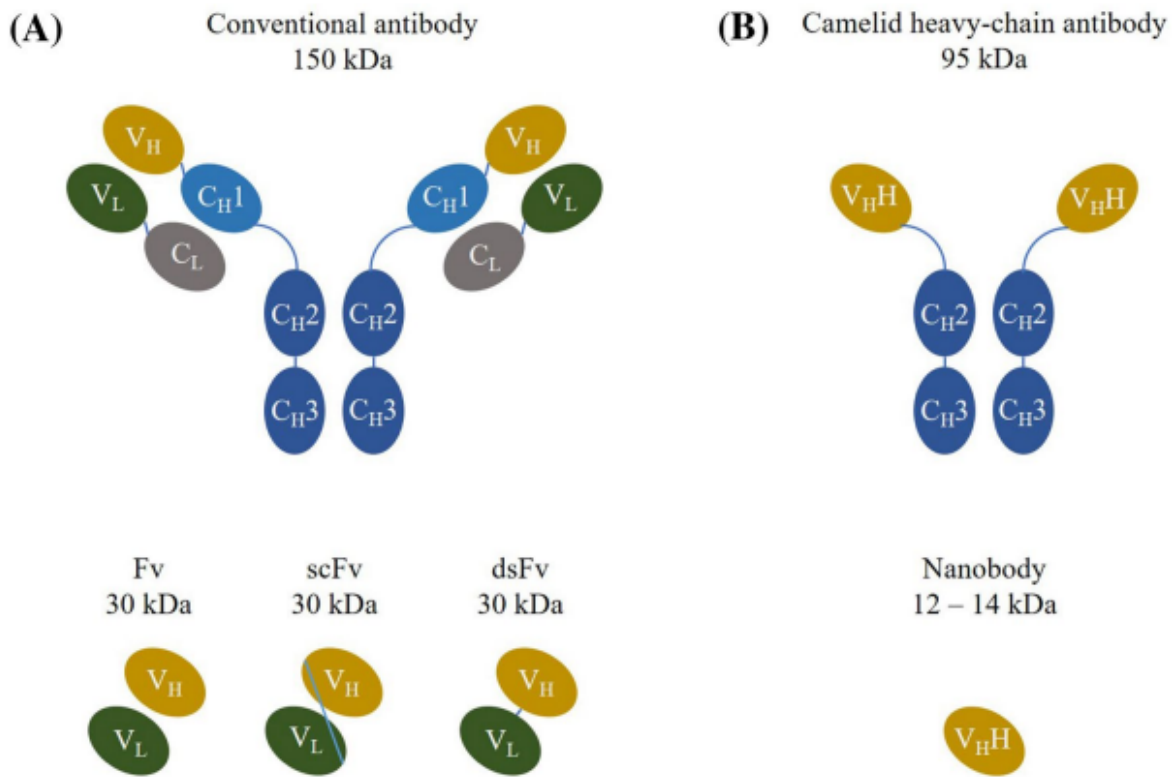
Figure 1. Structural overview of (A) conventional antibodies as found in humans, and (B) the VHH (nanobody) [13]

Antibodies consist of 2 identical heavy chains and 2 identical light chains which are connected to the heavy chains by disulfide bonds. The heavy and light chains are divided into a variable and constant heavy domain, and a variable and constant light domain. The constant domain determines which isotype an antibody is. The heavy and light variable domain contain the antigen binding site, and is a point of interest when researching biomarkers using antibodies. An antibody can only recognize a specific antigen due to the complex chemical structure in the variable domain [13]. Therefore, in order to find a biomarker using immunological screening, the right antibody has to be discovered first.

Despite the potential of immunological screening using conventional antibodies, there are certain factors limiting success. For example, these antibodies are quite large (ca. 150 kDa)[13] which makes production quite expensive. It has long been hypothesized that the variable heavy chain on itself might replace antibodies as a whole, due to the large contribution of the variable heavy chain to antigen binding [14]. In recent years, a new type of antibody has been discovered which is far better suited for immunological screening than human antibodies. These newly discovered Single Variable Domain on a Heavy Chain (VHH) antibodies originating in Camelids, often called nanobodies (Nb) have greatly improved stability, solubility and lower production costs than conventional antibodies leads to nanobodies being favored over conventional antibodies [13].

Aim of this study

In order to find a novel biomarker capable of detecting the different endotypes of OA, possibly in early stages, llamas were immunized with synovial fluid originating from a joint inflicted with OA. After the immune response of the llamas introduces VHH into their peripheral bloodstream, blood was drawn from the llamas.

Afterwards, mRNA is isolated and reverse transcribed using RT-PCR to get the cDNA resulting in large cDNA library. Then the phage-VHH library was used for biopanning with decellularized cartilage samples from OA patients. Cartilage was used as some extracellular matrix (ECM) and structural protein concentrations present in the cartilage can give a direct indication on the degree of tissue damage in the joint [15]. After 2 rounds of biopanning, the leftover phage-VHH display library was sequenced by NGS which allows all the VHHs to be subjected into differential display analysis These VHH were mass produced using prokaryotic expression in E.coli, by introducing the genetic sequences obtained using NGS into to E.coli.

These VHH are then mass produced using bacterial expression, by introducing the genetic sequences to bacteria.

Currently, 24 VHHs have been selected from a large library of NGS data. As we want to verify whether the antigens which these VHHs bind to also exist in synovial fluid samples, and find out the source of these antigens, we conducted the following research.

ELISA is an assay often used for antigen detection and/or quantification (using a standard curve). ELISA can also be used to detect if an antibody will bind to certain viruses or antigen.
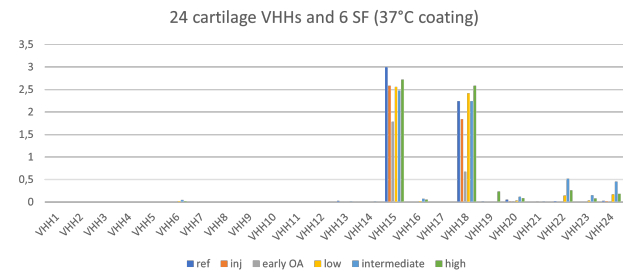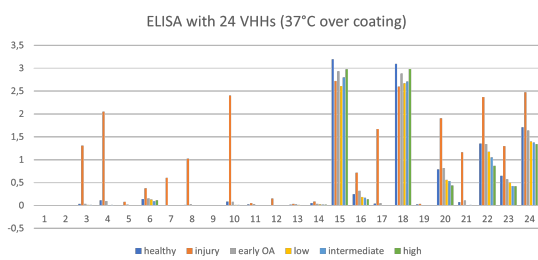

Figure 2. Coated 500µg/mL synovial fluid (ref, inj, early OA), 50µg/mL synovial fluid (low, inter-, high) and 0,5µM VHH. x-axis: Absorbance Units (AU), y axis: # of cartilage VHH tested

Figure 3. Coated 5µg/mL synovial fluid (ref, inj, early OA), 0,5µg/mL synovial fluid (low, inter-, high) and 0,5µM VHH. x-axis: Absorbance Units (AU), y axis: # of cartilage VHH tested

In this preliminary ELISA, it is evident that most of the pre-selected VHHs have at least some expression in injury specific OA, especially at higher coating concentrations like 500µg/mL for injury. VHH 10 and 24 show the largest response. For VHH 15 and 18, there is a lot of binding to antigens of all types, seen in figure 2, even when the concentrations is lowered significantly as seen in figure 3. For VHH 15, the largely the same between the 2 ELISA tests except for early OA synovial fluid. VHH 18 shows comparable intensity to VHH 15, but decreases further when synovial fluid concentration declines. VHH 22 and 24 show comparable results in both ELISAs. These comparable results could possibly indicate that these different VHH might bind to the same antigen, possibly with differing affinity.

The aim of this study is to further validate whether or not these cartilage specific VHH can also accurately detect antigens in synovial fluid, in order to find a VHH which could be suited for OA detection. In this study, VHH 10, 15, 18, 22, and 24 will be further investigated on synovial fluid

samples from 80 different patients using ELISA.

MATERIALS AND METHODS

Materials
All chemicals were purchased from Fisher Scientific (NL) if not otherwise stated. VHH and His-Tag antibodies were purchased from Fisher Scientific (NL). For ELISA plates, the Nunc Maxisorp 96-well plates were used, purchased from Fisher Scientific (NL). The synovial fluid (SF) samples used were kindly provided by Utrecht University.

For plate reading, a Multiskan GO plate reader was used.

Synovial Fluid Samples
Cartilage VHH's were tested on 80 samples synovial fluid, from different patients. Samples were retrieved after knee replacement surgery, as all patients had end-stage OA. Patients were divided into 4 treatment groups for celecoxib and/or naproxen for 4 weeks prior to surgery. Group A served as a control group, receiving no drugs prior to surgery. Group B received 2dd200mg celecoxib until surgery. Group C received 2dd200mg celecoxib until 3 days before surgery. Group D received 3dd250mg of naproxen until 3 days before surgery, due to the platelet-inhibiting effect of naproxen. At the point of experimentation, it was unknown which sample belonged to which treatment group in order to rule out any bias. After gathering of data, the data was linked.

Methods
BCA
A standard curve of Albumin was prepared for the following concentrations: 2000, 1500, 1000, 750, 500, 250, 125, 25, and 0 μg/mL using PBS as a solvent. Then the BCA Working Reagent was prepared in a ratio of A:B, 50:1. Afterwards, 80 SF samples were diluted 100x. 25μL of each standard or unknown was pipetted into each microplate well, in duplicate for the standard. 200μL of BCA Working Reagent was added to each well and mixed on a plate shaker for 30 seconds. The plate was then wrapped in aluminum foil and incubated at 37°C for 30 minutes. After incubation, the plates were cooled to room temperature after which absorbance was measured at 562nm using a plate reader.

ELISA
Prior to the ELISA itself, the ELISA plates (Nucn Maxisorp 96-well) were coated using 50μL per well of each of the 80 SF samples with differing concentrations, diluted using the same dilution factor of 54 in PBS. 2 negative controls were introduced; 1 well without SF, and 1 well without VHH. ELISA plates are then coated overnight without lid in a 37°C incubator, in order to let all liquids evaporate.

The next morning, ELISA plates are blocked using 200 μL 4% skimmed milk (milk powder in PBS) for 1 hour on an orbital shaker, making sure to cover the plates to prevent evaporation. After blocking, plates are washed 3x using a PBS-0,05%Tween-20 (PBS-T) washing buffer.
After the first washing phase, 50 μL 0,5M dissolved in 1% skimmed milk, of the desired VHH was added to each well except for well 11B containing a negative control. Pure 1% skimmed milk was added to well 11B instead. The plates were covered and left on the orbital shaker for 1 hour.
After incubation of the VHH, the plates are washed 3x with PBS-T. 50 μL of μg/mL dissolved in 1% skimmed milk, of the secondary antibody His-Tag was added to each well. The plates were covered and left on an orbital shaker for 1 hour.

After incubation of His-Tag, the plates are washed 3x with PBS-T and 1x with normal PBS. 100 μL of a 1:1 solution of Color Reagent A and Color Reagent B was added to each well. Plates are covered and wrapped in aluminium foil and left in a 37C incubator for 10 to 20 minutes. 50 μL per well of Stop Solution was used to stop color from developing further.

ELISA plates were read using a Multiskan GO Plate Reader. Plates were shaken on medium. Absorbance Spectra of 450 and 650 nm were measured.
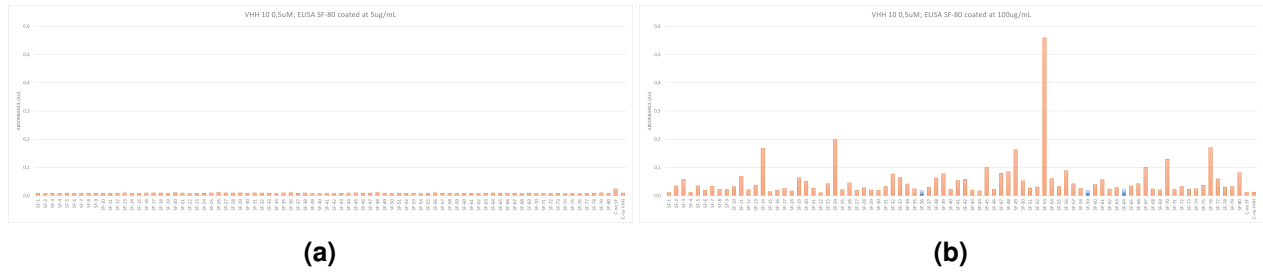
RESULTS

(a)   **(b)**

Figure 4. Results of VHH 10 at 5μM on 80 SF samples coated at 5μ/mL **(a)** and 100μg/mL **(b)**

For the entirety of the experiment, VHH 10 rarely showed any result when measured at the same SF concentration as other VHHs. Only when increasing the concentration drastically, VHH 10 was only able to significantly detect antigens in some samples. The most significant one is SF-53 at a reading around 0,6 Au, which is still a weak result considering the concentration used.
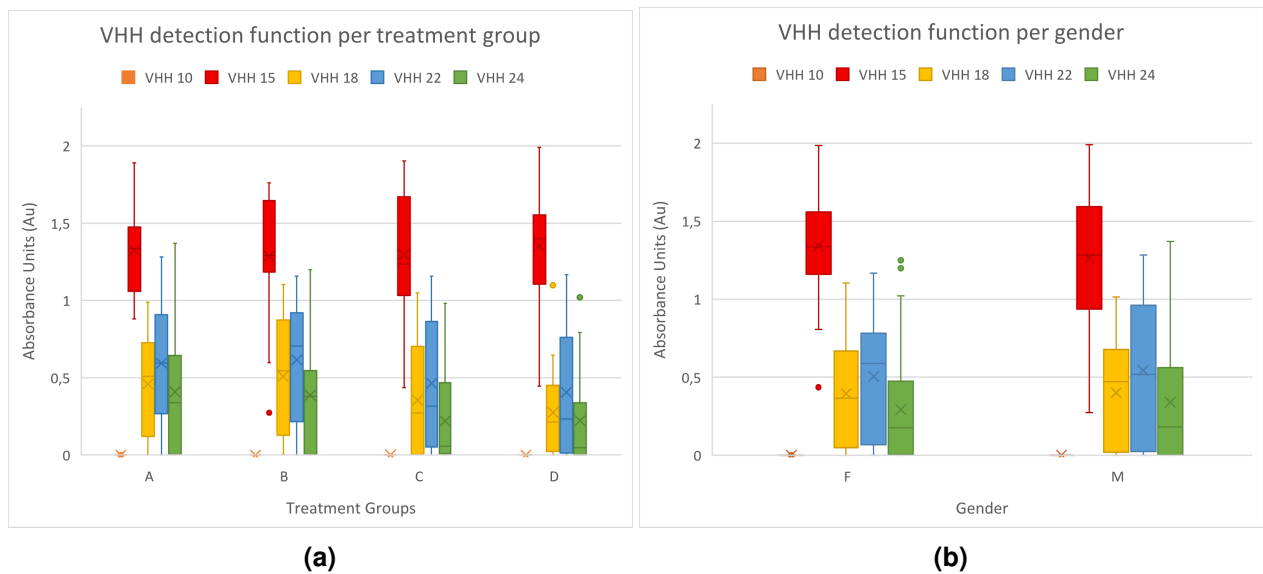

(a)   **(b)**

Figure 5. Box plots showing the results of all experiments per treatment group **(a)** and patient gender **(b)** (F: n=53 ,and M: n=27). Group A: control (n=21), group B: celecoxib-full (n=18), group C: celecoxib-3days (n=21), group D: naproxen-3days (n=20). All synovial fluid samples were diluted 5400x. VHH at 5μM

Treatment group A shows relatively high maximum readings for VHH 15, 22, and 24. The mean of VHH 18, 22, and 24 is comparable between groups A and B. For groups C and D the mean of VHH 18, 22, and 24 is slightly lower with group D having the lowest means for these VHHs, along with lower medians and quartiles compared to other treatment groups. The mean of VHH 15 stayed relatively constant in between all groups. Interestingly, group A shows the largest minimum for VHH 15, while showing relatively high maximum values for VHH 18, 22, and 24.

For gender-grouped results, VHH 24 boasts a larger 3rd and 4th quartile in female patients compared to male patients, while the mean stays the same and the median is relatively low. The

same is mostly true for VHH 18 and 22 as well, except for a smaller difference, and a lower average compared to male patients. VHH 15 shows little significant differences between genders.

VHH 10 had very little significant readings across all samples.
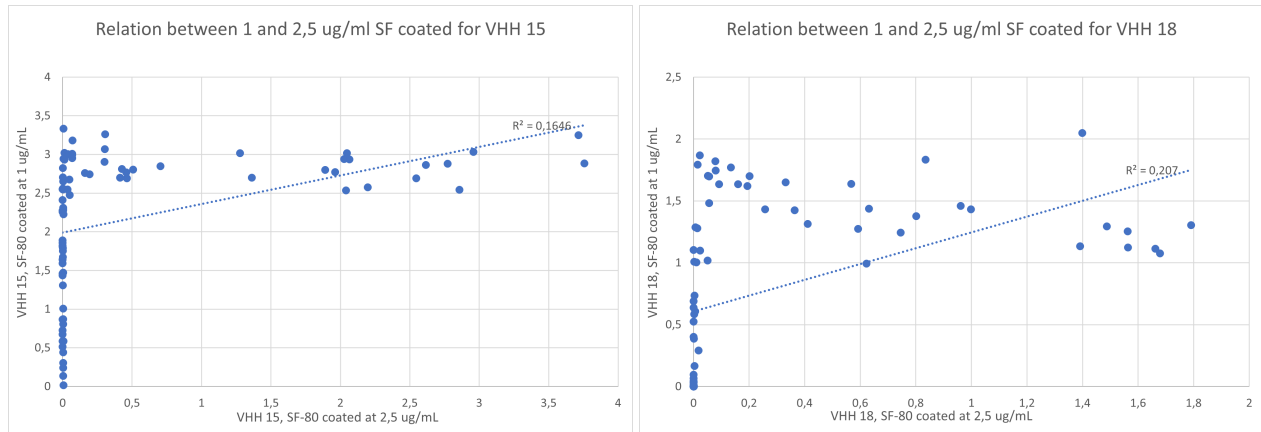
(a) Concentration of VHH 15 at 5μM, 2 different concentrations of 80 SF plotted against each other. 80 SF at 1μg/mL on the y-axis and 80 SF at $2,5\mu$g/mL on the x-axis

(b) VHH 18 at 5μM, 2 different concentrations of 80 SF plotted against each other. 80 SF at 1μg/mL on the y-axis and 80 SF at $2,5\mu$g/mL on the x-axis

Figure 6. Scattergraph of VHH 15 **(a)** and VHH 18 **(b)**

In figure 6 it is clear that the results of the 1 μg/mL and 2,5 μg/mL coated ELISAs are not in proportion to each other. Note that readings above 3 au should be considered inconclusive, as this is outside the linear range of the Multiskan GO. These measurements were only made for VHH 15 and 18.
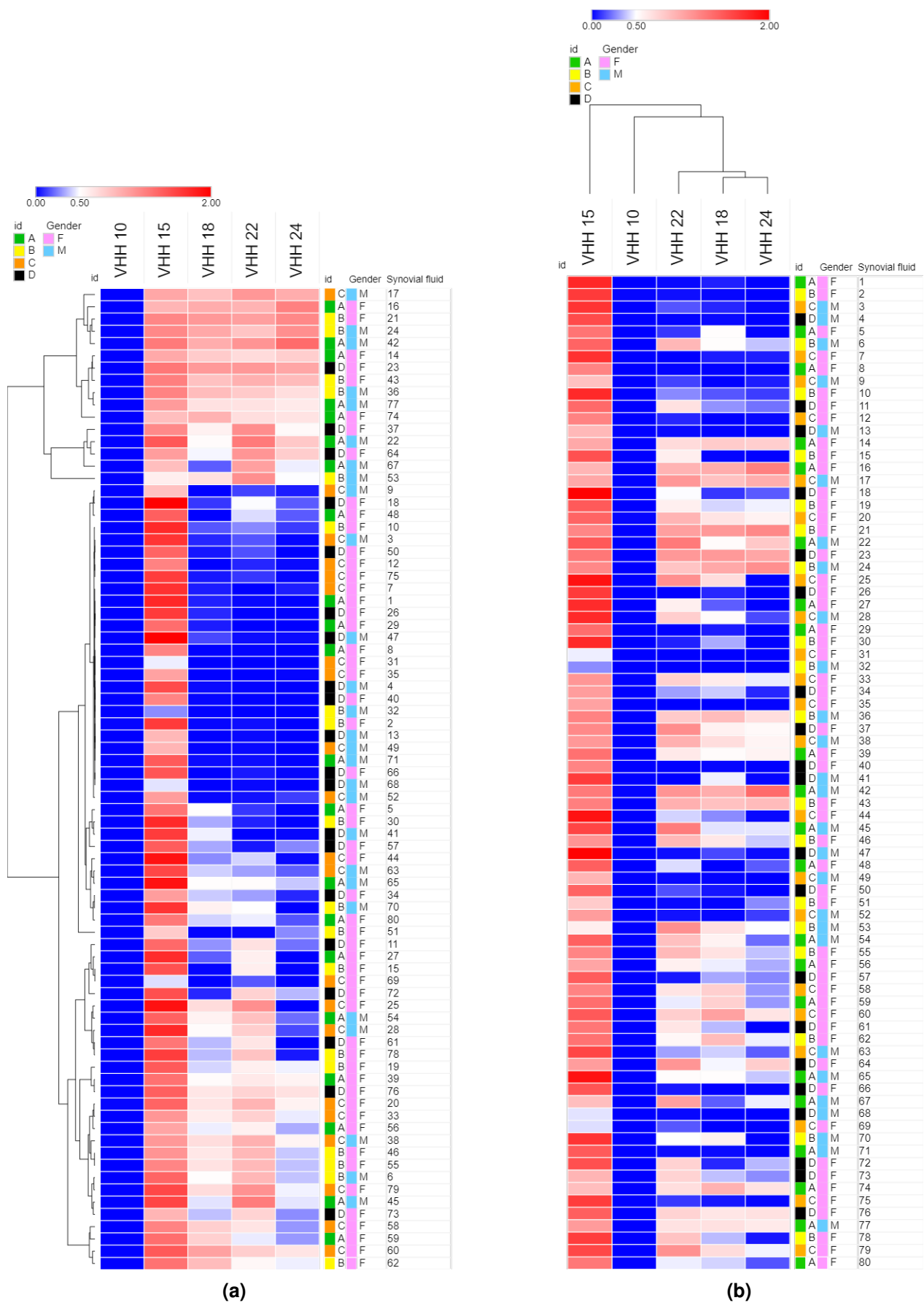
Figure 7. Heatmap rows clusteranalysed **(a)** and columns clusteranalysed **(b)**

In figure 7a all ELISA results are plotted on a heatmap clusteranalysed. The data is roughly clustered in 3 larger clusters, cluster 1 from SF-17 to SF-53, cluster 2 from SF-9 to SF-51 and SF-11 to SF-62, using the order of samples from figure 7a. While all treatment groups are present in each cluster, they are not divided equally. In the first cluster, showing the highest expression levels overall, treatment group C is present only once in the first major cluster. Furthermore, cluster 1 has a relatively high amount of treatment group A. For cluster 2 which has the lowest expression levels overall, treatment group B is relatively uncommon, while treatment group D occurs more frequently, compared to clusters 1 and 3. Finally, for cluster 3 which has midrange expression levels, treatment group B and D occurance is similar to cluster 1.

Figure 7b shows all ELISA results on a heatmap, and clusteranalysed for the different VHH used in the experiment. VHH 18 and 24 are most similar, with VHH 22 being the next similar. VHH 15 seems the least related to the rest of the tested VHHs.

DISCUSSION AND CONCLUSION

It is evident that treatment has little effect on the expression levels of VHHs for the 80 SF samples, except for treatment group A and C. Patients that received no treatment had relatively high expression levels compared to all other treatment groups with the exception of VHH 10, possibly suggesting Celecoxib and Naproxen inhibiting certain antigen expression. Inversely, patients that were treated with celecoxib until 3 days before surgery, had relatively low expression levels compared to other treatment groups. This may suggest inhibition of the antigens due to treatment. However, as the antigens that detected are unknown, it is uncertain whether or not this is coincidental or not.

VHH 15 almost always binds to the antigens present in the samples, regardless of treatment or gender. It is possible that VHH 15 binds to protein which are present in synovial fluid regularly, and is less suited for purposes such as monitoring the effect of certain treatments on a patient. However, as it is unknown which antigen VHH 15 binds to exactly, a formal conclusion can not be made.

VHH 10 rarely ever binded to a protein in the samples. However, in figure 4b there was one significant result compared to the rest of the samples, possibly indicating OA with similarties to injury based OA.

Based on cluster analysis VHH 18 and 24 are most similar, disproving what was expected from the preliminary ELISA.

Finally, the cartilage specific VHHs selected from the phage-display biopanning are able to successfully detect antigens in synovial fluid.

Outlook

Figure 6 shows a correlation between measurements which is different than expected. Ideally, Rsquared would be much closer to 1 for both VHH 15 and 18. This very likely indicates at least partially unreliable results, probably due to mixing or pipetting mistakes during the experiments. However, due to the absence of a standard curve as the antigen is unknown, it cannot be said for certain. Additionally, VHH 15 results often exceed 3 Au, falling outside the linear range of the

Multiskan GO and thus become unreliable. It is evident that the process of the SF ELISA needs to be optimised further, in order to obtain more accurate results.

Reflection on Methods

Contrary to the methods used in this study, most biomarker discovery research using immunoassays, an antigen is found first, after which an optimal antibody needs to be discovered. As the method used in this study uses antibodies first to discover potential biomarkers, there is a constant unknown variable in the form of antigen concentration in each well. It becomes difficult to call results accurate and reliable when it is unknown to what, and to how much protein an antibody binds. The presence of a standard curve would redeem unreliable results such as in figure 6, possibly reducing costs of research as repeat experiments are limited.

Furthermore, the current method prevents hard conclusions from being made, as OA is a complex disease and there is a very significant amount of different protein present in any sample. The results gathered can however be used in tandem with further research. For example, when the binded antigens are discovered, it might give new insights into data procured using the current method.

Lastly, synovial fluid is a very invasive sample to procure. In this case it was retrieved during knee replacement surgery, making it difficult substance to come by. Blood Serum could possibly replace synovial fluid as samples used in this study, however, further study into this is warranted.

ACKNOWLEDGEMENTS

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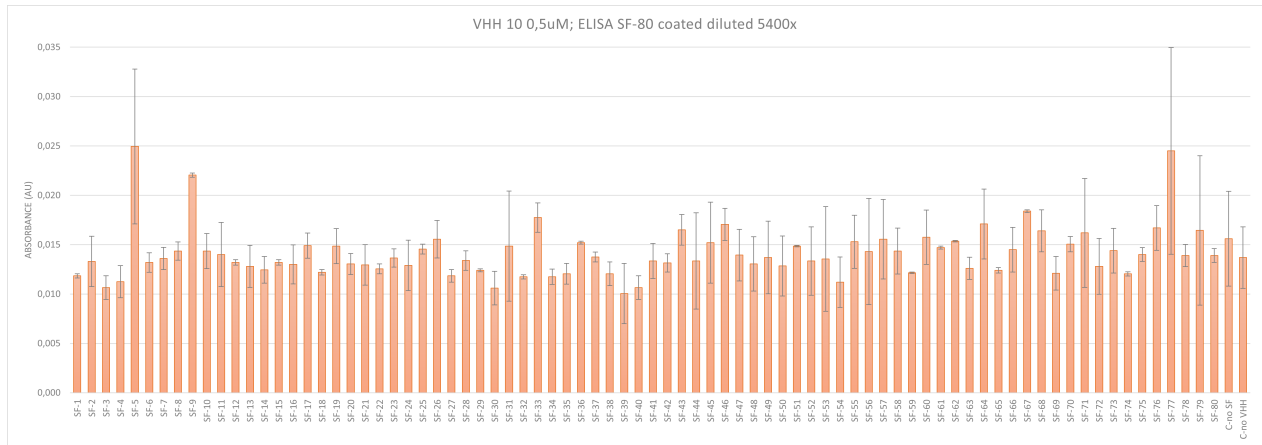
APPENDIX A: ELISA DATA

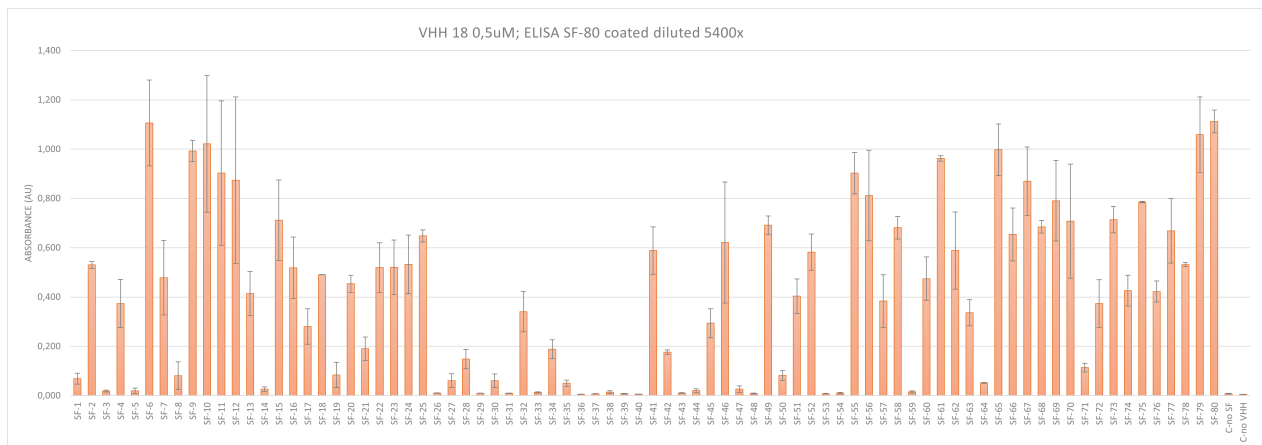**Figure 8.** VHH 10 at 5μM, 80 SF coated at 5400x dilution

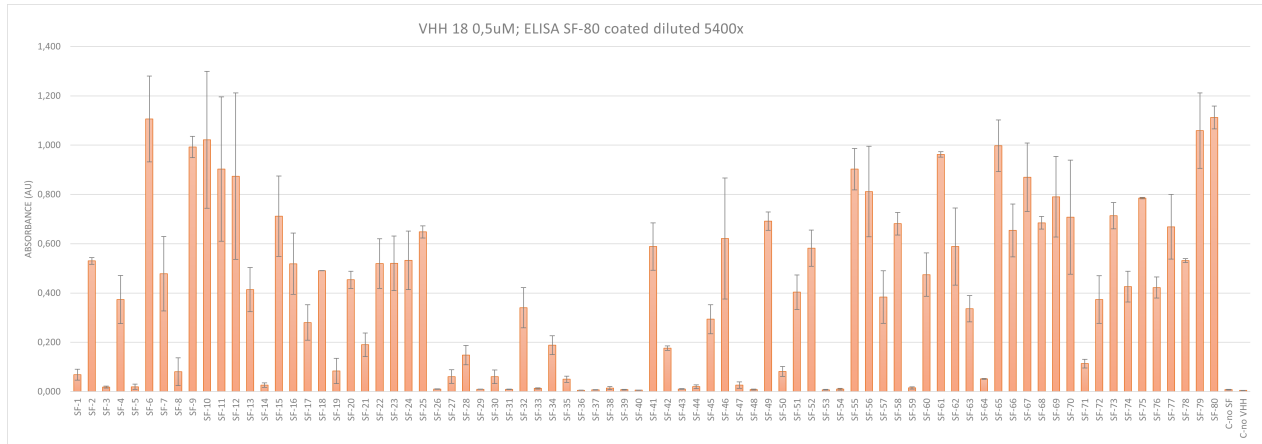**Figure 9.** VHH 15 at 5μM, 80 SF coated at 5400x dilution

Figure 10. VHH 18 at 5μM, 80 SF coated at 5400x dilution
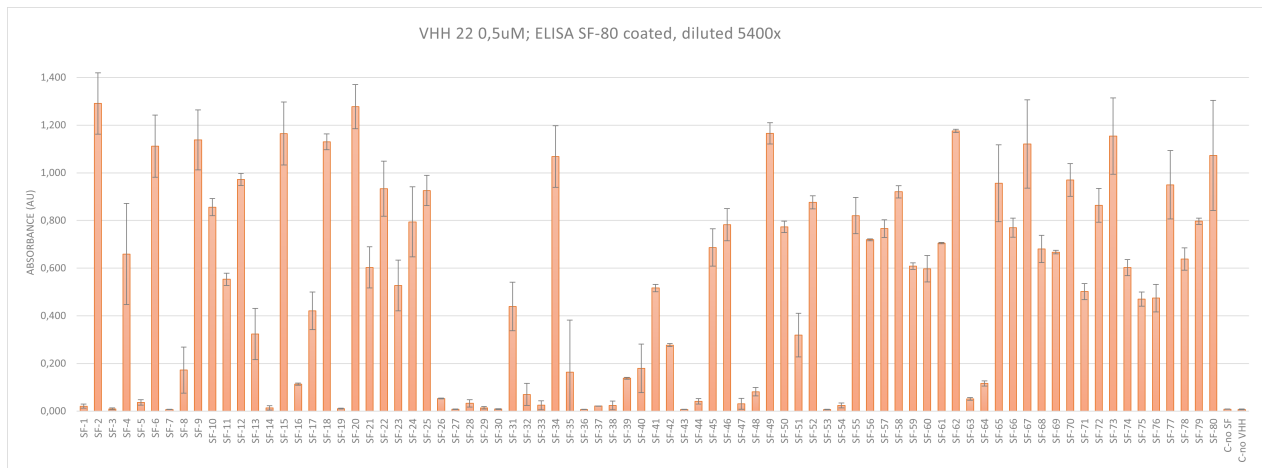

Figure 11. VHH 22 at 5μM, 80 SF coated at 5400x dilution
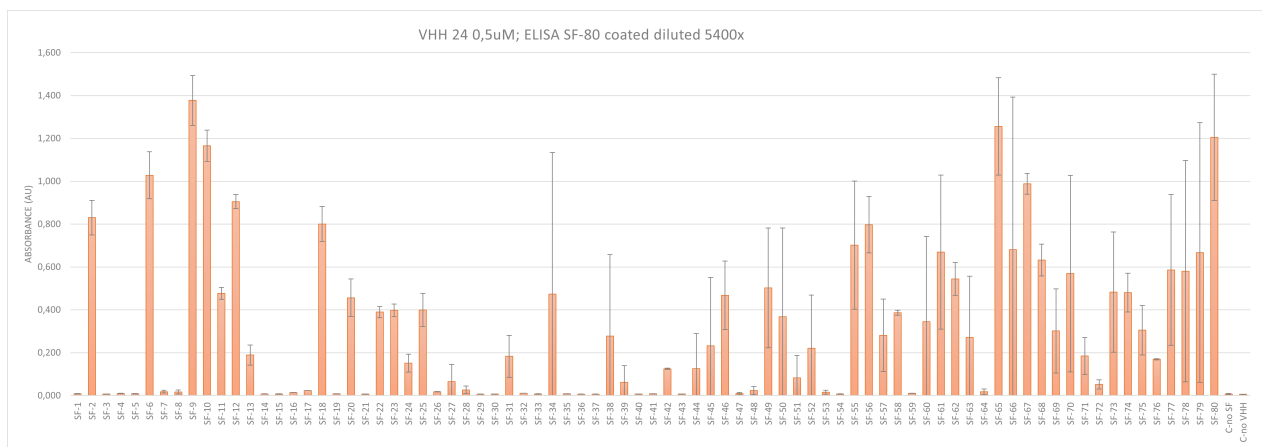

Figure 12. VHH 24 at 5μM, 80 SF coated at 5400x dilution

APPENDIX B: 80 SF SAMPLE INFORMATION

SF	Patient Number	Date of Birth	Sex	Treatment Group	Date of Collection
1	1	11-3-1943	F	A	21-1-2008
2	2	11-7-1951	F	B	29-1-2008
3	3	7-4-1944	M	C	22-1-2008
4	4	21-2-1947	M	D	5-2-2008
5	6	21-7-1946	F	A	24-1-2008
6	7	15-12-1932	M	B	29-5-2008
7	8	22-4-1949	F	C	19-2-2008
8	9	30-9-1934	F	A	19-2-2008
9	10	19-6-1942	M	C	10-6-2008
10	11	14-7-1939	F	B	17-3-2008
11	12	21-8-1925	F	D	7-3-2008
12	13	2-7-1939	F	C	11-3-2008
13	14	13-6-1940	M	D	5-3-2008
14	15	25-12-1919	F	A	14-3-2008
15	16	14-7-1946	F	B	18-3-2008
16	17	17-9-1940	F	A	8-4-2008
17	18	19-7-1928	M	C	2-4-2008
18	19	11-2-1936	F	D	21-3-2008
19	20	3-3-1926	F	B	11-4-2008
20	21	13-7-1933	F	C	4-4-2008
21	22	23-6-1944	F	B	1-4-2008
22	23	11-7-1947	M	A	2-4-2008
23	24	31-8-1936	F	D	21-4-2008
24	25	30-5-1948	M	B	10-4-2008
25	26	31-3-1956	F	C	8-5-2008
26	27	19-12-1929	F	D	17-4-2008
27	28	15-7-1937	F	A	18-4-2008
28	29	31-3-1932	M	C	28-4-2008
29	30	28-10-1941	F	A	29-4-2008
30	31	29-11-1950	F	B	23-4-2008
31	34	25-10-1942	F	C	19-5-2008
32	37	28-12-1931	M	B	22-4-2008
33	39	8-5-1932	F	C	3-6-2008
34	40	16-11-1945	F	D	8-5-2008
35	41	24-1-1949	F	C	10-6-2008
36	43	5-5-1935	M	B	16-5-2008
37	44	16-6-1955	F	D	21-5-2008
38	45	3-12-1951	M	C	12-6-2008
39	47	26-12-1942	F	A	27-5-2008
40	48	22-11-1936	F	D	19-5-2008
41	49	5-5-1944	M	D	6-6-2008

42	50	26-4-1947	M	A	29-5-2008
43	51	5-4-1942	F	B	3-7-2008
44	52	3-7-1937	F	C	14-7-2008
45	53	19-4-1933	M	A	11-6-2008
46	55	20-1-1939	F	B	10-6-2008
47	56	29-1-1940	M	D	20-6-2008
48	57	25-4-1921	F	A	11-6-2008
49	58	16-11-1928	M	C	8-7-2008
50	59	16-2-1947	F	D	11-7-2008
51	60	1-9-1940	F	B	19-6-2008
52	61	28-2-1954	M	C	27-6-2008
53	62	14-9-1945	M	B	24-6-2008
54	63	9-4-1952	M	A	20-6-2008
55	65	6-1-1939	F	B	21-7-2008
56	66	15-3-1930	F	A	16-7-2008
57	67	30-11-1955	F	D	28-7-2008
58	68	21-3-1945	F	C	29-7-2008
59	69	31-1-1933	F	A	12-8-2008
60	70	9-2-1931	F	C	11-8-2008
61	72	19-6-1938	F	D	30-9-2008
62	73	21-4-1947	F	B	16-9-2008
63	74	4-2-1939	M	C	15-10-2008
64	75	9-10-1940	F	D	14-10-2008
65	76	17-8-1947	M	A	25-9-2008
66	80	9-5-1930	F	D	10-10-2008
67	82	23-5-1946	M	A	16-10-2008
68	87	15-4-1940	M	D	28-10-2008
69	88	28-9-1946	F	C	9-10-2008
70	90	17-3-1949	M	B	28-10-2008
71	91	30-10-1944	M	A	17-11-2008
72	92	9-8-2027	F	D	4-12-2008
73	93	15-3-1932	F	D	13-11-2008
74	94	22-2-1941	F	A	24-11-2008
75	96	9-11-1940	F	C	7-11-2008
76	97	4-10-1937	F	D	22-10-2008
77	98	4-2-1937	M	A	15-11-2008
78	99	30-12-1932	F	B	18-11-2008
79	100	20-10-1937	F	C	25-11-2008
80	101	23-2-1947	F	A	29-1-2009

Table 1. All the information for the 80 SF samples used.