Analysis of immune cell infiltration in white adipose tissue sites before and after bariatric surgery

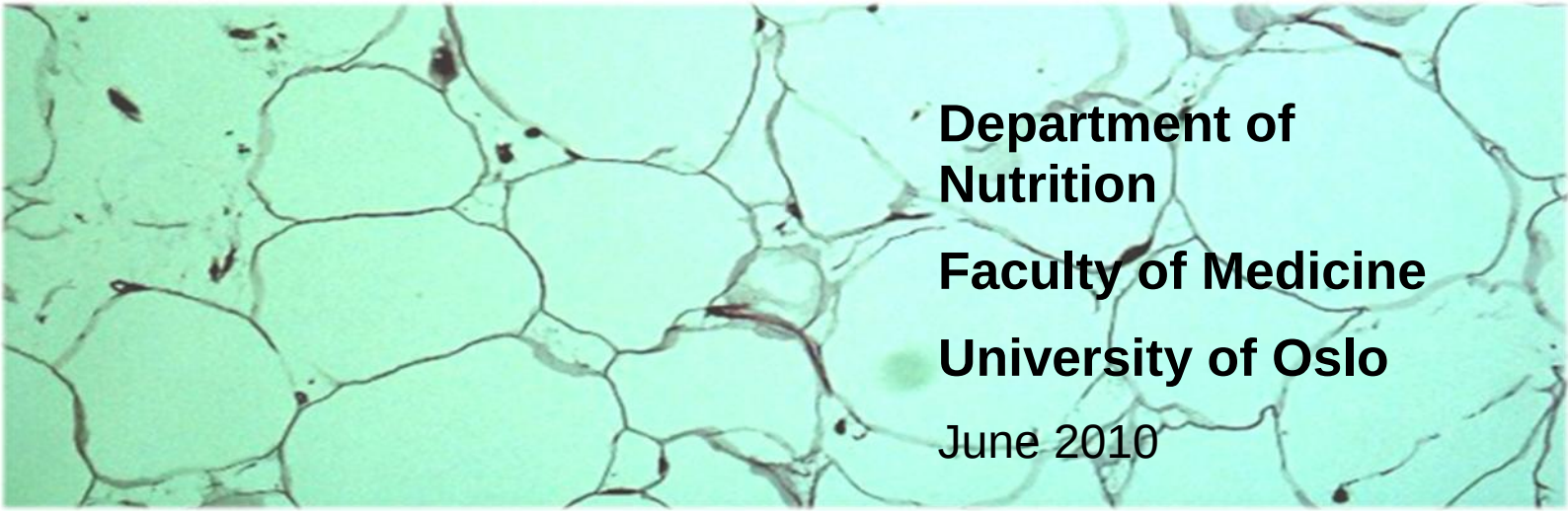
- Pilot study

Master thesis by

Kristine Møller

Supervisor

Bjørn Skålhegg



Department of Nutrition

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Summary

Obesity is becoming one of the most widespread non-communicable diseases in the world. Associated with obesity are various metabolic disorders, such as sleep apnea, non-alcoholic fatty disease and arthritis, all contributing to a decreased quality of life and great expanses on the health services world wide.

In all metabolic disorders identified, an increase in serum C-reactive protein (CRP) and low grade chronic inflammation is observed. This is thought to be one of the causes for the development of metabolic disorders, and involve macrophage infiltration of white adipose tissue (WAT), and the production of inflammatory markers such as Tumor necrosis factor- α (TNF- α), Interlukin-6 (IL-6), IL-1β, IL-8. When fat mass is reduced in obese subjects, the levels of these markers are reduced. Hence, low grade inflammation has been associated with pathological levels of WAT.

In a pilot study we have investigated if WAT of obese patients were infiltrated by immune cells causing inflammation. Pre surgery adipose tissue from patients undergoing gastric bypass at Oslo University Hospital Aker (OUHA) were used to optimize a method for the isolation of immune cells of WAT in order to determine the identity of infiltrating cells by flow cytometry. Moreover, WAT from patients undergoing bariatric surgery at OUHA and Haukeland University Hospital (HUH) were also analyzed histological and by immune histochemistry. Together our results demonstrated that immune cell infiltration in general was low both in subcutaneous as well as omental WAT. Interestingly, immune cell infiltration appeared more pronounced one year post- compared to pre surgery. Infiltration was observed as low number of B and T cells as well as macrophage aggregates described as “crown-like structures” (CLS). CLS are structures surrounding necrotic adipocytes. Infiltrates were only observed post surgery whereas we did not observe this phenomenon in pre surgery WAT. Our findings were unexpected since CLS´ have only been documented prior to substantial weight loss. Our results propose that immune cells infiltration also takes place post surgery in WAT.

Contents

List of abbreviations

ATM	Adipose tissue macrophages
BMI	Body mass index
BSA	Bovine serum albumin
CD	Cluster differentiation
CLS	Crown like structures
CRP	C-reactive protein
CVD	Cardio vascular disease
F	Female
FBS	Fetal bovine serum
FFA	Free fatty acids
FITC	Fluorescein isothiocynate
GB	Gastric Bypass
HBSS	Hank's buffered salt solution
HDL	High density lipoprotein
HE	Hematoxylin
hsCRP	High sensitive CRP
HT	Hypertension
HUH	Haukeland University Hospital
iCAM	Intracellular adhesion molecule-1
IHC	Immune histochemistry

IL	Interleukin
IR	Insulin resistance
Kol	Cholesterol
LB3	Liberase Blendzyme 3
LBTM	Liberase blendzyme medium thermolysin
LDL	Low density lipoprotein
M	Male
Ma Sup	Matrix Supernatant
MCP-1	Monocyte chemo attractant-1
NK	Natural killer cell
OUHA	Oslo University Hospital Aker
PAI-1	Plasminogen activator inhibitor-1
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
RBC	Red blood cells
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SAT	Subcutaneous adipose tissue
SVF	Stromal vascular fraction
T2DM	Type 2 diabetes mellitus

TG	Triglycerides
TNFα	Tumor necrosis factor-α
UiB	University of Bergen
UiO	University of Oslo
WAT	White adipose tissue

1 Introduction

1.1 Overweight and obesity: a global perspective

The attitudes towards overweight and obesity have changed throughout the history. Hippocrates (460 BC-ca 370 BC) early described obesity as a disease, whereas overweight and obesity represented wealth and prosperity in the 17th century. Social trends maintained the positive association towards overweight and obesity until the beginning of the 20th century, where the negative health risks associated to overweight and obesity were revealed [1, 2]. Research continues to show how overweight and obesity are strong contributors to the development of chronic diseases, such as type 2 diabetes mellitus (T2DM), hypertension (HT), cardiovascular diseases (CVD) and dyslipidemia, and increases the risk of some cancers. Overweight and obesity also negatively affect the quality of life and increase the economical expenses in terms of health costs and social services in our society and healthcare system [3-5]. In addition to findings from research, the general opinion on overweight and obesity is becoming similar to the one Hippocrates had; it is a disease.

Developed countries have over the last decades experienced an increase in obesity among the populations about to reach epidemic measures, and recent observations indicate that developing countries are undergoing similar trend, mainly due to the rapid industrialization occurring [6]. During the last 10 years (1992-2002) the prevalence of the overweight and obese population in China, defined by the World Health Organization (WHO) standard (Overweight and obesity (kg/m^2): body mass index (BMI) $\geq$ 24 kg/m^2), has increased from 14.6- 21.8% (Wang, Mi et al. 2007), whereas 66.3 % of the American population is either overweight or obese (BMI > 25 kg/m^2) [7]. Of this 66.3%, 35.1 % of the adults are considered obese, defined by a BMI above 30 kg/m^2 [8]. And this development does not appear to slow down by any measures.

Table 1.1. The International Classification of adult normal weight, overweight and obesity according to BMI. From Sheipe 2006, http://who.int [9, 10]

Classification	BMI cut-off point (kg/m^2)
Normal range	**18.5 - 24.9**
Overweight	**25 - 29.9**
Obesity	**30 - 39.9**
Obese class I	30 – 34.9
Obese class II	35 – 39.9
Morbid obesity (Obese class III)	40 – 49.9
Super obesity	> 50

Seeing as the trend is comparable all over the globe in respect to the sharp increase of adverse obesity related complications and diseases on population level and economical loss in terms of lost labor, higher rates of sick leaves, reduced life expectancy [5], there have never been greater incentives for developing treatments, projects, pharmaceutical products, surgical methods etc. in order to combat obesity.

1.2 Obesity linked to low grade chronic inflammation

1.2.1 Adipose tissue in obesity

The adipose tissue is now recognized as the largest endocrine organ in the body, rather than just energy storage and thermal and mechanical insulation [11-13]. The adipose tissue consists of adipocytes; and the stromal and vascular cells (SV cells or non-fat cells), which include fibroblasts, mast cells, vascular endothelial cells, resident macrophages, leukocytes, and other inflammatory cells. The stromal vascular fraction (SVF) is recognized to be essential for adipose tissue inflammation [14-17].

The adipose tissue secretes inflammatory factors, called adipokines, which are contributors to metabolic disease [18] (**Figure 1.1**). It is thought that visceral adipose tissue of obese subjects has a higher metabolic activity than subcutaneous adipose tissue, with a greater production and secretion of inflammatory factors [19, 20].

1.2.2 Proinflammation

A functioning immune system differentiates between foreign infectious agents and self, it this way it have the ability to successfully combat infectious agents. In normal situations this occurs without causing undue harm to the surrounding tissues, however if this control is lost, inflammation develops, and unless the immune response is turned off, the inflammation becomes chronic. Proinflammation or low grade chronic inflammations, which have been thought to be beneficial to the host, are also a sequel to a variety of infectious diseases. In fact proinflammation is a causal factor in the development of diabetes and atherosclerosis, which are hallmarks of metabolic syndrome (**Figure 1.1**) [20].

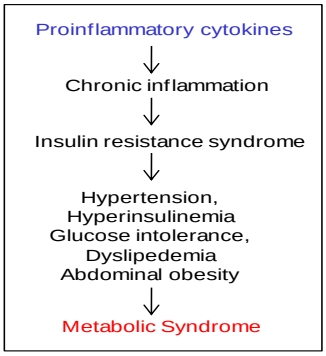


Figure 1.1. Overview of the development of metabolic syndrome initiated by proinflammatory cytokines. From Cancello and Clement 2006 [20].

1.2.3 The adipokines and the inflammatory cytokines

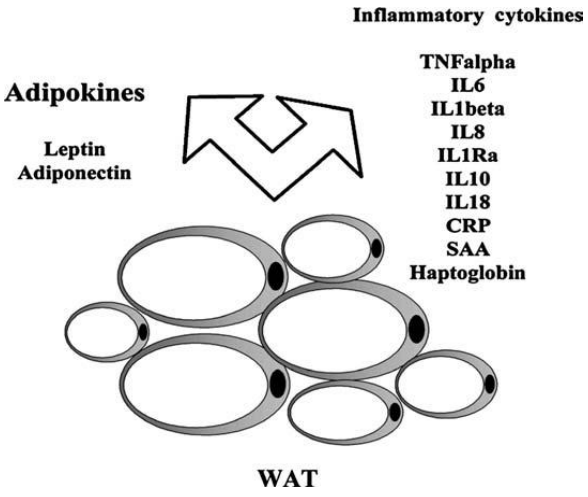


Figure 1.2. Adipokines and inflammatory cytokines specifically expressed and secreted by WAT. From Cancello and Clement 2006 [20].

Findings show that the amount of adipokines and leptin secreted from the adipose tissue is positively correlated to an increase in fat mass, adipocyte volume, BMI and with degree of insulin resistance, whereas the opposite apply for adiponectin [18, 19, 21].

The majority of the adipokines are secreted from the non-fat cells (from the SVF) and elevated in obesity, and these include TNF- α, IL-8, IL-10, IL-6 and IL-1 β. Leptin is secreted

exclusively from adipocytes, whereas adiponectin is secreted from both the adipocytes and the non-fat cells [15]. Monocyte chemo attractant-1 (MCP-1), intracellular adhesion molecule-1 (ICAM-1), plasminogen activator inhibitor-1 (PAI-1) and free fatty acids (FFA) are examples of other adipokines secreted from the adipose tissue [15, 18, 21, 22].

TNF-α is a pro-inflammatory factor which is extensively produced by the adipose tissue in obesity. It causes insulin resistance as it prevents insulin receptor signaling [23]. An improvement in insulin sensitivity has been observed by the use of TNF- α in obese animals and decrease in TNF- α secretion has been observed after weight loss [20, 24]. The pro-inflammatory IL-1β is thought to together with TNF- α up regulate the expression of IL-6 and IL-8. IL-6 is in turn positively correlated with C-reactive protein (CRP) [15, 25]. CRP is an acute-phase protein reflecting inflammatory processes which are found to be elevated in obese individuals when compared to non-obese individuals [12, 26]. Macrophages are the main target of MCP-1. MCP-1 recruit monocytes and has been found to be elevated in subcutaneous adipose tissue of obese women [27].

1.2.4 Obesity as an inflammation

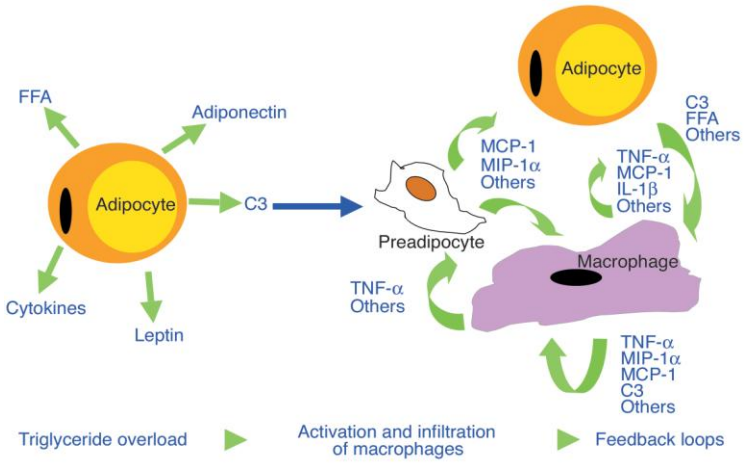


Figure 1.3. Chronic inflammation and adipocytes IR theoretically presented. From Xu, Barnes et al. 2003 [28]

Upon the gradual expansion of visceral adipose tissue when the body is in constant positive caloric balance, the mature adipocytes experience an enlargement, called hypertrophy. Whether preadipocytes undergo adipogenesis to become mature adipocytes in order to store the excess energy (hyperplasia) or the adipocytes expand (hypertrophy), is genetically determined. Research has shown that hyperplasia is less pathogenic and hypertrophy is

thought to result in adipocytes dysfunction, and one of the factors contributing to the increase in the inflammatory factors and the development of metabolic diseases [19].

When tissues are expanding the need for blood supply emerges, including adipose tissue. However when angiogenesis is delayed, hypoxia occurs. Hypoxia is well known to induce macrophage recruitment and proinflammatory response [19, 20]. What it is specifically that initiate the immune response seen in obesity, whether it is the hypertrophy, the hypoxia or the elevated cytokine secretion, is unknown. But what is acknowledged is that the proinflammatory response is initiated and macrophages are activated, where the resident macrophages secrete cytokines that can impair insulin sensitivity locally in the adipocytes. This further stimulates the infiltration of peripheral monocytes and macrophages into the fat, which is thought to surround the adipocytes in a CLS. TNF-α secretion stimulates cytokine release (MCP-1, IL-1β, FFA) from preadipocytes, enhancing the macrophage infiltration. T lymphocytes appear in chronic inflammation and have been associated in obesity, thought to be activated by the adipokines [29-31]. This is amplifying cascade of events causes lipolysis as the insulin resistance (IR) is enhanced and in which eventually can result in systemic IR, development of T2DM, HT and the other metabolic disorders associated with obesity [19, 20, 22, 27, 28, 32, 33].

With in mind that the visceral adipose tissue is regarded as more metabolic active than the subcutaneous adipose tissue, it is understandable that location of adipose tissue accumulation plays a role in terms of the co-morbidities associated to obesity. Excess central adiposity (visceral fat) is found to be one of the risk factors for T2DM, HT, dyslipidemia and CVD [19, 20]. Visceral adipose tissue is also the fat depot in which is in close proximity of the hepatic portal circulation, a factor that might explain the link between visceral fat and metabolic disturbances [15, 18]. The two adipose tissue sites were investigated in this thesis in order to possibly detect a difference in terms of infiltrating cells.

1.2.5 Identification of inflammatory factors

In order to identify infiltrating immune cells in selected adipose tissue of obese patients, various markers of T-, B lymphocytes and macrophages, as well as for natural killer (NK) cells were investigated since these cells are important in the immune response and thought to be involved in the low grade inflammation occurring in obesity. Previous studies have quantitatively investigated the heterogeneous cell population found in adipose tissue using

flow cytometry and immunohistochemistry (IHC), hence were the chosen methods for this master thesis [16, 21, 22, 27, 34].

1.3 Treatments of obesity

As the prevalence of overweight and obesity is increasing, so are the demands for treatment methods where sustainable weight loss and lifestyle change are achieved. Fad diets have been around for decades, such as the Atkins Diet, the South Beach Diet, the Blood Type Diet, and health "gurus", such as Grete Roede and Fedon Lindberg, have promoted their ideas to combat overweight. However, for a person who is morbidly obese, more comprehensive help and serious alternatives are needed.

1.3.1 Conventional therapy

Ebeltoft Kurcenter in Denmark was established in 2001 and was perhaps the first conventional treatment program available for morbidly obese in the Nordic countries. The leading focus here was lifestyle change and weight loss through exercise and strict diet. The stay was of a length of 15 weeks and a study has shown that the participants experienced an improvement in oral glucose tolerance tests and in the blood lipid levels. The weight loss was similar to what can be seen after surgical treatments [35]. Similar treatment centers have been developed in Norway the past years, such as Evjeklinikken, Røros Rehabilitation Center, Tonsåsen Rehabilitation and Alm Center (Molde), where weight loss is accomplished through exercise, healthy diet and behavioral therapy. The length of stay varies depending on the centre and most of these are connected to the leading hospitals in Norway.

Personal experience has been acquired at Røros Rehabilitation Centre by working there as a dietitian on the obesity team. This team consisted of physical activity therapists, nurses and a dietitian, working closely with a psychologist. One of the main reflections from my weeks of involvement was that the participants were not at Røros Rehabilitation Centre solely due to a sedentary lifestyle and unhealthy eating habits. The weight gain was rather an outcome of past events in which had contributed to psychological difficulties, therefore the competence of the psychologist was indispensible. The participants' degree of success throughout the stays at the center was not defined by weight loss, but rather their physical and mental state, their openness to change and the integration of the newly introduced habits for their home stay.

Although not resolved, the participants experienced an improvement in metabolic disorders such as T2DM and HT, steady weight loss and improved physical and mental health. This conventional management of obesity is a valuable alternative to surgical treatments of obesity.

1.3.2 Bariatric surgery

Oslo University Hospital Aker, Vestfold Hospital, Voss Hospital and Nordland Hospital Bodø are some of the several leading hospitals in Norway that perform surgical treatments of obesity. Bariatric surgery, the term used collectively for surgeries which have the intention of treating severe obesity in patients who have failed other attempts of weight loss and who are at serious risk of developing a number of diseases related to the obesity [36, 37]. When undergoing bariatric surgery, the obese patient is closely followed-up pre- and post surgery by an interdisciplinary team in order to achieve success and maintained weight loss.

Of the bariatric surgeries, the one performed the most in Norway is gastric bypass [38]. Findings in the literature show that 68.2% of the excess body weight was lost after a gastric bypass surgery, whereas 25% of the total body weight is lost 10 years after the surgery [39, 40]. The bariatric surgeries of interest for this thesis were biliopancreatic diversion with duodenal switch surgery and laparoscopic gastric bypass.

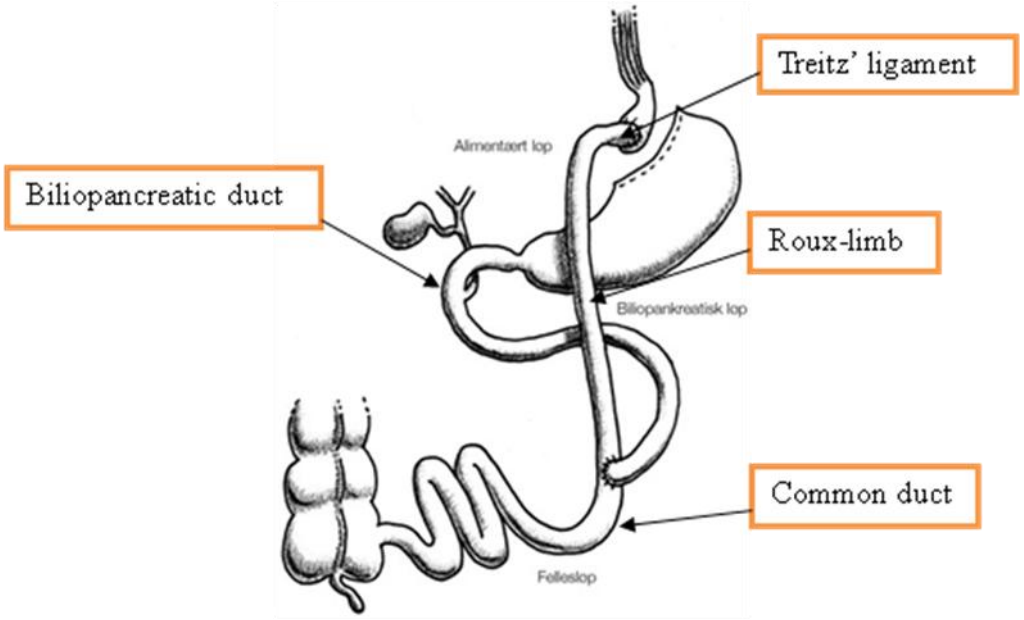


Figure 1.4. Diagram showing the outline of gastric bypass surgery. Figure taken from Aasheim, Mala et al. 2007 [38]. Gastric bypass is in short a 95% reduction of the stomach achieved by stapling the stomach which

becomes re-attached the lower part of the small intestine, called the Treitz' ligament. The lower part of the small intestine is from below the duodenum and is called the Roux-limb. The Treitz' ligament is the new opening of the gastric pouch to the rest of the intestines. The biliopancreatic duct is connected to the Roux- limb about 40 cm below the Treitz' ligament, creating the common duct, in which food and digestive juices rejoin before the digested food carries on into the large intestines.

The main aim for this operation is the significantly decreased stomach size and the shortened absorption length for the nutrients [38, 41].

Out of the various approaches developed in order to combat obesity, studies have shown that bariatric surgery is the only way in which T2DM [40], metabolic syndrome, HT and sleep apnea are resolved; all consequences linked to obesity [36, 38]. Bariatric surgery affects the immunological response, which explains the resolution and improvements of the metabolic disturbances due to the reduction in inflammatory factors, originating from the pathogenic adipose tissue [42]. No other weight loss method achieves the same reduction of metabolic disorders. In addition to this, a systematic review found that obesity surgery does not lead to significant psychological problems, but rather an improvement in psychological status [43]. Quality of life has also been found to be improved [44].

For an overweight or obese person taking the step towards a healthier life style in which includes altered eating habits and increased physical activity, whether it is a conventional method or bariatric surgery, professional health care workers are necessary in the path to success, with the emphasis on resolving psychological issues in order to achieve the desired weight loss [45-48].

2 Aims and objectives

Based on what has been described above it is commonly accepted that obese patients suffer from proinflammation and possibly infiltration of immune cells in the WAT. To determine if WAT from obese patients harbored infiltrated immune cells we initiated a pilot study. In this study we wanted to examine omental and subcutaneous fat of two populations of patients. For the first population (11 patients) we aimed at examining prior to and one year post bariatric surgery for cells infiltrating WAT. The other population (5 patients) we wanted to examine for infiltrating cells prior to bariatric surgery. For both populations we aimed at analyzing WAT for infiltrating cells by histology and IHC. For the latter population we also wanted to fractionate infiltrating cells followed by flow cytometry analysis. In order to perform fractionation we aimed at optimizing a method for gravimetric purification of cells infiltrating WAT.

3 Materials

Cell fractionation

Products	Supplier	Catalog number
Sterile Stainless Steel Surgical Blade	Paragon	4867-1023
Filter holder 25 mm Easy Pressure for syringe	PALL Life Sciences	PALL4320
O ring Viton	PALL Life Sciences	PALL83475
Spectra Mesh Nylon Filter 200µm pores, 125µm thickness	Spectrum Laboratories Inc.	SPEC 148116
Sterile Syringe (10 ml) Luer	BD Plastipak	REF 302188 Lot: 4196774
Liberase TM Research Grade	Roche	5401119001
HBSS	GIBCO	14025-050
BSA	US Biological (Biosite Norway)	A1311

Lysis

Products	Supplier	Catalog number
Lysis buffer	Sigma	R7757
HBSS	GIBCO	14025-050
BSA	US Biological (Biosite Norway)	A1311

Flow cytometry

Products	Supplier	Catalog number
PS- Micro plate. Sterile, V-shape	Greiner Bio-one	651161
PBS tablets	Sigma	P4417-100TAB
BSA	US Biological (Biosite Norway)	A1311
FITC Conjugated Anti-Human CD-56	eBioscience	11-0569-71
FITC Conjugated Anti-Human CD-14	eBioscience	11-0149-71
Monoclonal Mouse Antihuman CD4/FITC	DAKO	F0766
Monoclonal Mouse Antihuman CD8/FITC	DAKO	F0765
Mouse IgG/FITC	DAKO	X0927
CD19/FITC	BD-Becton Dickinson	345776

Sectioning of adipose tissue

Products	Supplier	Catalog number
Formalin Buffered 10%	Chemi-teknik	21154
Di-sodium hydrogen phosphate water free	Chemi-teknik	28026-292
Sodium hydrogen sulphate (monohydrate)	Chemi-teknik	28120-231
Distilled water	SIGMA	95284

Immunohistochemistry

Products	Supplier	Catalog number
Distilled water	SIGMA	95284
Citrate buffer	SIGMA	C2488
PT link (Target Retrieval Solution, pH 9 (10x), 3-in-1)	DAKO	S2375
Proteinkinase K Prediluted	DAKO	S3020
Wash buffer TBS	DAKO	S3006
Dilution Buffer	DAKO	S0809
HRP- Peroxidase Blocking	DAKO	S2023
Visualization system EnVison- DAB+	DAKO	K5007

Software

Software	Supplier
CellQuest for Macintosh	Becton Dickinson
Cytobank	Stanford University
Microsoft Office XP	Microsoft Corporation
PASW Statistics 18	SPSS Inc.
Endnote	Thomson Reuters

4 Methods

4.1 Ethics statement

After being informed about the study, its purpose and the subject's legal rights, each enrolled subject returned a written consent form. The study was approved by the Eastern Norway Regional Committee for Medical Research Ethics (REK).

4.2 Biochemical Analyses

4.2.1 Blood samples obtained from patients donating adipose tissue at HUH

At the commencement of the study "Switch from Stress Response to Homeobox Transcription Factors in Adipose Tissue after Profound Fat Loss" by Dankel et al. [49], blood samples and anthropometric measures were obtained from all participating patients, the obese and the controls. All blood samples were taken while fasting and before the surgery. For the obese patients, additional blood samples and anthropometric measures were obtained at follow-up one year after the surgery.

4.2.2 Blood samples obtained from patients undergoing gastric bypass at OUHA

A specific blood analysis was created for the included subjects in the Intrafat study (see appendix 1). This specific requisition included an extensive range of blood values, including glucose, insulin, HbA1C, triglycerides, total-,high density lipoprotein (HDL)- and low density lipoprotein (LDL)- cholesterol (parameters not included in the general blood test). Patients who qualify for gastric bypass, whether or not included in the study, have a general blood test taken two weeks before the surgery and a screening on the day of the surgery at OUHA. Intentionally the 5 included subjects were meant to have the Intrafat blood analysis done in addition to the screening on the day of surgery; however it was only conducted on 2 of them. This resulted in having to use the blood tests from two weeks prior to the surgery for three of the subjects when analyzing the results.

4.3 Isolation of cell fractions in adipose tissue

Fractionation of fat samples was performed according to the method developed and described in the result section.

4.3.1 Red blood cell lysis

The following steps were built upon: "The protocol for separation of lymphocytes from mouse spleen and lymph nodes". The tube with the SVF solution was centrifuged at 585 x g for 5 minutes and once the supernatant was removed the pellet was resuspended in 200 µl red blood cell (RBC) lysis buffer (ACT buffer, Sigma R7757) and incubated for 2 minutes at room temperature (RT). After incubation, the lysis was stopped by adding 800 µl Hank's buffered salt solution (HBSS) containing 5% bovine serum albumin (BSA) and the solution gently mixed. The tube was then centrifuged at 382 x g for 7 minutes and the pellet was resuspended with 500 µl HBSS 5% BSA.

4.3.2 Flow cytometry

The flow cytometry method was optimized on analysis of peripheral blood lymphocytes (PBL) (Ullevål University Hospital). The protocol was modified to be used for one layer staining. The flow cytometer was from Becton Dickinson and was a FACSCalibur; the software used was Cell Quest, which was also delivered by Becton Dickinson, as well as Stanford CytoBank developed by Stanford University.

In short the method was as followed:

1) The eppendorph tube containing the SVF solution was filled with cold phosphate buffered saline (PBS), ~ 1 ml

2) Centrifuged for 7 min at 478 x g

3) The cell pellet was resuspended in cold PBS, ~1 ml

4) Centrifuged for 7 min at 478 x g

5) The pellet was resuspended in dilution solution (PBS 1% BSA)

6) 5 µl one layer antibodies were added to each well of a 96 well micro plate

Table 4.1. Antibodies selected for the flow cytometry. The antibodies were primarily conjugated to fluorescein isothiocyanate (FITC).

Used Antibody for:	Type of marker:
Negative control	Mouse IgG1
CD4	T cells
CD8	T cells
CD14	Monocytes and macrophages
CD19	B cells
CD56	Natural killer cells

7) 45 µl of cells /0.5-1.0x10^6 mill cells /mL) were added to each well

8) The micro plate was placed in refrigerator for minimum 30 minutes

9) The wells were filled with cold PBS and centrifuged at 478 x g for 5 minutes.

10) The PBS was removed and resuspended gently using vortex on the tray

11) The wells were filled with cold PBS and centrifuged at 478 x g for 5 minutes

12) The PBS was removed

13) The cells resuspended in 50 µl dilution solution

The amounts of the antibody displaying immune cells were investigated using the flow cytometer and the software Cell Quest. The same software was used for analyzing the results, as well as the software CytoBank.

4.4 Histology

4.4.1 Adipose tissue biopsies from patients from HUH

Adipose tissue biopsies (approximately 1 gram) were received from HUH and stored on dry ice. This was tissue from a selection of obese patients and control persons who had been included in a study between 2003 and 2007. Biopsies received were all fixated in formaldehyde and were further treated at the Pathological Department at Rikshospitalet HF. Blood test results were also received for all patients.

The original patient identifications created for the biopsies from HUH remained when further working on the biopsies in Oslo.

4.4.2 Fixation of adipose tissue biopsies

The adipose tissue biopsies were individually fixed in 1.6 ml 4% paraformaldehyde at the laboratory, then further dehydrated, paraffin embedded and finely sectioned (0.2 µm) by the Pathological Department at Rikshospitalet HF. The sections were placed onto individual microscope slides, and the sections stained with hematoxylin (HE).

4.4.3 Adipose tissue biopsies from patients from OUHA

A subcutaneous and one abdominal biopsy (same location as the biopsies for the fractionation) were directly immersed in separate tubes of 15 ml formaldehyde when the tissue was removed from the patients, this for histology. These biopsies were treated identical to the tissue biopsies from HUH.

4.4.4 Immunohistochemistry (IHC)

IHC is a widely used procedure performed to detect various proteins present in biological tissue by the use of antibodies. The antibodies are conjugated to an enzyme that will elicit a type of excitation producing a color, visualizing antigen-antibody interaction.

The immunohistochemistry was performed at the Pathologic Department at HUH and they used antibodies conjugated to 3,3-diaminobenzidine (DAB), which is the chromomeric substrate for peroxidase. Peroxidase is an enzyme conjugate that will upon the localization of antibody-bound antigenic sites elicit a colored reaction. If an antibody reaction occurred, it was in this case stained brown [50, 51]. The markers for cluster differentiation 3 (CD3), CD20 and CD68 were used in this project.

Table 4.2. The markers selected for the immunohistochemistry. IHC was performed on the biopsies displaying infiltrating cells from the obese patients and the biopsies for the control patients received from HUH, the biopsies from the first two patients from the gastric bypass operations at OUHA, gastric bypass1 (GB1) and GB2.

Used Antibody for:	Type of cells:
CD3	T cells
CD20	B cells
CD68	Monocytes/macrophages

From the observations through the microscope, the biopsies showing infiltrating cells were sectioned onto super-frost slides. The biopsy blocks for the control patients were also included in the IHC. With ten consecutive samples (marked 1-10) of each biopsy it was possible to detect the depth of the infiltration and the position of the infiltration. The super-frost slides were packed back to back in aluminum foil and stored at -80 ºC until needed, and then thawed in RT before the IHC.

For each selected adipose tissue biopsy, three super-frost slides were used for each antibody, and seeing that three antibodies were used, a total nine slides were used per adipose tissue biopsy. Example: For 4 A2 super-frost slides 1-10, 1-3 were stained with CD3, 4-6 were stained with CD20 and 7-9 were stained with CD68, this for all biopsies.

The super-frost glasses were de-paraffinised, and processed for antigen retrieval in a microwave processing lab station.

IHC procedure

In short, these are the steps that were conducted when staining the slides with antibodies to the IHC that was performed at HUH.

1. The slides were incubated at 50 ºC overnight.

2. The slides were then either heated in a microwave or a PT-link (DAKO). If a microwave was used the steps were as follows: the slides were heated at 850W for 9.5 min, at 400W for 15 min, and rested in 15 min. For the PT-link the slides were inserted when the PT-link was heated to 65ºC and slides were kept in the PT-link for 20 min while the temperature was increased to 97 ºC, before temperature was reduced

to 65 ºC, cooling the slides. (For both methods, Envision FLEX Target Retrieval Solution is used).

3. The slides were rinsed in washing buffer (TBS 1:10)

4. Slides placed in the DAKO Autostainer machine

5. The selected antibodies were applied and were on for 30 min in RT, washed with buffer

6. Peroxidase blocking for 5 min, washed with buffer

7. Envision for 30 min, washed with buffer

8. DAB for 2x5 min, washed in distilled water

9. Standard staining with HE for 2 min.

10. Washed in distilled water for about 8 min, then dehydrated in alcohol, xylene.

11. Cover slides applied in the machine of Sacura Tissue Tek.

Samples ready for microscopic observation.

The slides were returned to the Department of Pathology Rikshospitalet HF and investigated semi-quantitatively through a microscope and photographed in order to detect CD3, CD20, or CD68 positive cells.

4.5 Statistical analyses

For the differences between the anthropometric- and biochemical data of the patient group and the control group independent samples T tests and paired samples T tests were performed. All statistical analysis was performed with PASW Statistics 18. All analysis were set to $P = < 0.05$ significance level.

5 Results

5.1 Patient inclusion

5.1.1 Adipose tissue biopsies of received from HUH

The inclusion criteria for the obese patients included in the Dankel et al. study [49] were BMI > 45 kg/m^2 (morbid obesity) and Norwegian Caucasian origin. The inclusion criteria for the controls were good health, BMI < 27 kg/m^2 and no history of drug abuse or disorders. For the obese patients, the adipose tissue was collected during the bariatric surgery, biliopancreatic diversion with duodenal switch, and for the controls, during hernia repair. In addition, of the adipose tissue, blood tests were also collected for all of the patients.

The anthropometric and the biochemical data for the obese patients and controls from Bergen included in the adipose tissue biopsy part of the project are shown in the tables below.

The patient information refers to the order of when the adipose tissue was taken, 1 being the first and so on. A1 refers to "Adipogen", the name of the project at HUH. Number 1 means the first round of biopsies taken, respectively from patients at Førde Hospital, A2 being tissue from patients at Haraldsplass Hospital.

Eleven obese patients and three controls were included in the histology part of the project.

Table 5.1. Anthropometric data for the obese patients at inclusion

Patient	**4A1**	**7A1**	**10A1**	**11A1**	**16A1**	**23A1**	**24A1**	**25A1**	**27A1**	**28A1**	**30A1**
Sex	F	F	F	M	M	F	F	F	M	F	F
Age	35	57	42	57	59	26	56	30	23	30	35
Weight (kg)	149	120	178	135	177	157	161	141	206	132	145
BMI (kg/m^2)	48	49	59	58	51	51	58	52	54	47	53

The average age of the eleven obese patients was 40.9 years old. The average body weight was 154.6 kg and the average BMI was 51.5 kg/m^2. The average BMI for the obese patients was above the BMI range for morbidly obese (> 40 kg/m^2) (Table 1.1). 22.3% of the obese patients were male, and 77.7% were female.

Table 5.2. Anthropometric data for the controls

Patient	**4A2**	**20A2**	**27A2**
Sex	F	F	M
Age	27	55	54
Weight (kg)	75	67.8	69.2
BMI (kg/m^2)	24.8	23.2	22.3

The controls had an average age of 45.3 years old, the average body weight was 70.7 kg and an average BMI was 23.4 kg/m^2. The average BMI was within the normal range < 24.9 kg/m^2. One of the three controls was a male.

Table 5.3. Biochemical data for obese patients at inclusion. Reference ranges from: Fürst 2008, Dietitian's Pocket Book 2006 [52, 53]

	ID:	4A1	7A1	10A1	11A1	16A1	23A1	24A1	25A1	27A1	28A1	30A1
Measure:	Reference:											
S-cholesterol	< 4.5 mmol/L	5.9	3.9	4	4	4.1	5.2	5.7	4.6	4.5	3.7	5
S-LDL	≤ 1.8 mmol/L	4.2	2.5	2.7	2.6	2.8	3.9	3.8	3.4	3	2.6	3.1
S-HDL	F: ≥ 1.2 , M: ≥ 1.0 mmol/L	1.4	1.1	1.1	1.1	1	0.8	0.9	0.9	0.9	0.8	1.5
S-TG	< 1.7 mmol/L	1.4	1.4	1.2	1.3	1.4	2.5	1.9	1.5	1.4	1.4	2.1
Fasting glucose	< 6.0 mmol/L	5	6.2	7.6	6	10.6	8.7	7.9	5.2	5.8	5.8	5.5
Insulin	< 11mU/L	11	5.8	57.5	15.5	17.2	29.3	33.5	18.2	20.2	64.6	12.8
Insulin C-peptide	0.3-1.2 nmol/L	0.8	0.2	0.3	1.4	0.5	2.3	1	1.8	1.6	2.2	1.1
CRP	< 5 ml	20	5	29	5	6	11	11	8	15	19	14

Table 5.4. Biochemical data for the controls at inclusion

		4 A2	20A2	27A2
S-cholesterol	< 4.5 mmol/L	3.9	5.1	5.9
S-LDL	≤ 1.8 mmol/L	2.7	3.3	3.8
S-HDL	F: ≥ 1.2, M: ≥ 1.0 mmol/L	1.2	1.4	1.8
S-TG	< 1.7 mmol/L	0.9	1.2	0.9
S-Insulin	< 11mU/L	3.1	6.7	
S-Insulin C-peptide	0.3-1.2 nmol/L	0.4	0.9	
S-CRP	< 5 ml	1	6	< 1

Comparison of the anthropometric and biochemical data of the obese patients and the controls from HUH

When comparing the anthropometric data for the obese patients to the controls at inclusion (**Table 5.1 and 5.2**), there were significant difference between the body weights (P= < 0.005), and hence the BMI (P= < 0.005).

When comparing the biochemical data for the obese patients to the controls at inclusion (**Table 5.3 and 5.4**), no significant difference in the total cholesterol level (P= 0.604), the LDL cholesterol (P= 0.759) or the HDL cholesterol (P= 0.124) were found. However, it was found that the obese patients had significantly higher average triglyceride (TG) level when compared to the controls (P= 0.004).

The fasting glucose level was not obtained for the controls. The average fasting glucose level for the obese patients was 6.8 mmol/L, this average value indicates impaired glucose tolerance [53].

The average insulin level for the controls was 4.9 mU/L and 25.9 mU/L for the obese patients. When comparing the insulin values, there was significant difference between the insulin level of the obese patients and the controls (P= 0.005). Whereas there was not found a significant difference in the insulin C-peptide between the two groups (P= 0.214).

The average CRP value for the controls was 2.7 mg/L and it was under the reference value which is < 5 ml [53]. The CRP values for the obese patients were considerably higher than the

controls, with an average of 13 ml. The obese patients had significant higher CRP levels compared to the controls (P= 0.004).

5.1.2 Anthropometric and biochemical data for bariatric surgery patients at inclusion and 1 year post surgery

Table 5.5. Anthropometric and clinical data for the obese patients pre and post surgery. Blood tests were obtained fasting, and post surgery blood tests were taken one year after the surgery

	ID:	4 A1		7 A1		10 A1		11 A1		16 A1		23 A1		24 A1		25 A1		27 A1		28 A1		30 A1	
Measure:	Reference:	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Sex (M/F)		F		F		F		M		M		F		F		F		M		F		F	
Age		35	36	57	58	42	43	57	58	59	60	26	27	56	57	30	31	23	24	30	31	35	36
Weight (kg)		149	103	120	64	178	103	135	73	177	143	157	109	161	107	141	69	206	122	132	75	145	96
BMI at inclusion (kg/m^2)	18.5-24.9 kg/m^2	48	35	49	37	59	36	45	24	51	41	51	35	58	39	52	25	54	32	47	26	53	35
S-kol	< 4.5 mmol/L	5.9	4	3.9	3.4	4	2.5	4	3.5	4.1	3.7	5.2	3.5	5.7	4.1	4.6	2.8	4.5	2.4	3.7	3.9	5	3
S-LDL	≤ 1.8 mmol/L	4.2	2.4	2.5	2.2	2.7	1.3	2.6	2.3	2.8	2.6	3.9	2.4	3.8	3.0	3.4	1.9	3.0	1.2	2.6	2.4	3.1	1.4
S-HDL	F: ≥ 1.2 , M: ≥1.0 mmol/L	1.4	1.5	1.1	1	1.1	1.1	1.1	1	1	0.8	0.8	0.8	0.9	0.8	0.9	0.8	0.9	1.1	0.8	1.1	1.5	1.3
S-TG	< 1.7 mmol/L	1.4	0.7	1.4	1	1.2	0.71	1.3	0.9	1.4	1.4	2.5	1.5	1.9	1.75	1.5	0.7	1.4	0.52	1.4	2.1	2.1	1.3
Fasting glucose (mmol/L)	< 6.0 mmol/L	5	5.6	6.2	4.6	7.6	6.5	6	4.5	10.6	6.3	8.7	4.9	7.9	6	5.2	4.7	5.8	5	5.8	4.8	5.5	4.9
Insulin (U/L)	< 11mU/L	10.6	3.2	5.8	7.2	57.5	7.6	15.5	< 2	17.2	4.5	29.3	11.3	33.5	4.3	18.2	3.1	20.2	3.8	64.6	6.7	12.8	4.4
Insulin C-peptide (nmol/L)	0.3-1.2 nmol/L	0.8	0.4	0.2	0.9	0.3	0.8	1.4	0.2	0.5	0.7	2.3	1	1	1.2	1.8	0.5	1.6	0.4	2.2	0.81	1.1	4.8
CRP	< 5 ml	20	5	5	6	29	6	5	5	6	1	11	1	11	2	8	3	15	2	19	5	14	1

Comparison of the anthropometric and biochemical data; pre and post surgery for the obese patients

The average body weight for the obese patients was 154.6 kg at time of inclusion and 96.7 kg post surgery, this was a significant weight loss (P= < 0.005). Weight loss is accompanied with a reduction in BMI, it was a significant reduction in BMI in these patients (P= < 0.005), from an average on was 51.4 kg/m^2 pre surgery to 33.2 kg/m^2 post surgery.

There was a significant reduction (P= < 0.005) in the serum cholesterol. There were also a significant reductions in LDL cholesterol (P= < 0.005) and TG (P= 0.015). It was however no significant change in the HDL level. The HDL level is expected to increase as the lipid profile becomes more favorable.

There were experienced significant reductions in the average fasting glucose level (P= 0.006) and the insulin level (P= 0.004). However there was no significant change in the insulin C-peptide (P= 0.771).

The average CRP value was distinctly reduced, from an average of 13.0 ml pre surgery, to an average of 3.4 ml post surgery. There was a significant reduction (P= 0.001).

5.1.3 Adipose tissue biopsies received from OUHA- Intrafat pilot study

The patients included in the Intrafat pilot study were randomly selected, 2 of them at the day of surgery and the last 3 were included at the preoperative clinic. Patient identification was noted as GB (gastric bypass) and 1-5, for being the first to the fifth patient. The criterion for these patients was that they were eligible for gastric bypass surgery.

BMI > 40

BMI > 35-40 with serious co-morbidity such as:

- Cardiopulmonal condition
- Insulin treated DM Type 2
- Poorly controlled hypertension
- Arthritis
- Sleep apnea

Age 18-60 yrs

No alcohol or prescription drug abuse

No psychological disorder

Be motivated

Self understanding regarding the seriousness of situation and ability for behavioral change

Completed other diets and weight loss programs

Figure 5.1. The criteria for patient inclusion for bariatric surgery are included in the diagram above. Persons with BMI > 40 kg/m^2 or BMI 35-40 kg/m^2 with co-morbidities, as well as being 18-60 years, not having alcohol or drug abuse, no diagnosed psychological disorder, being motivated and realizing the seriousness of the situation, in addition to having completed other weight loss programs are eligible for gastric bypass surgery. From NIH conference 1991[54].

When receiving tissue from gastric bypass patients at OUHA, adipose tissue was collected from a total of 5 patients. Out of these 5, four of these patients were females (80%) and one was male (20%).

The patients included in the Intrafat pilot study had blood tests taken. Due to complications only 2 (GB3 and GB5) of the 5 included patients had a the Intrafat blood tests taken on the day of surgery, the blood test results from the other patients were obtained from a blood test taken at the preoperative clinic, approximately 2 weeks before the surgery. Weight and BMI values were noted on the day of the preoperative clinic.

The patients included donated blood tests and adipose tissue on the day of the operation.

Table 5.5. Biochemical data for the bariatric surgery patients at inclusion of the Intrafat pilot study. The blood reference values are obtained from OUHA standards.

	ID:	GB1	GB2	GB4
Measure:	Reference:			
Sex (M/F)		M	F	F
Age (yrs)		36	37	35
Weight (kg)		201	124	122
BMI at inclusion	18.5-24.9 kg/m^2	58	46	45
Fasting glucose	< 6.0 mmol/L	6.1	5.7	6.7
CRP	0-9 mg/L	6	6	5

The average BMI was 49.7 kg/m^2 and this was significantly higher than the upper recommended level of 24.9 kg/m^2 (P= 0.017) (Table 1.1).

The average fasting glucose level for the patients GB1, GB2 and GB4 was not significantly elevated when compared to the high end of the recommended value (P= 0.801). Insulin level was not obtained in this blood test. None of these 3 patients had elevated CRP level.

Table 5.6. Biochemical data for the patients who had the Intrafat blood test taken at the day of surgery. The blood reference values are obtained from OUHA standards.

	ID:	GB3	GB5
Measure:	Reference:		
Sex (M/F)		F	F
Age		48	60
Weight (kg)		125	117
BMI at inclusion (kg/m^2)	18.5-24.9 kg/m^2	44.5	38
S-kol	< 4.5 mmol/L	4	4.1
S-LDL	≤1.8 mmol/L	1.5	1.7
S-HDL	F: ≥ 1.2 , M: ≥ 1.0 mmol/L	0.78	1.48
S-TG	< 1.7 mmol/L	3.9	2.1
Fasting glucose (mmol/L)	< 6.0 mmol/L	5.9	6.7
Insulin	< 11 mU/L	85.4	10.9
Insulin C-peptide (pmol/L)	370-1470 pmol/L	4906	1387
HbA1c	4-6%	5.8	5.9
CRP	0-9 mg/L	3	4

The Intrafat blood analysis was used for patients GB3 and GB5. The S-HDL value was low for patient GB3, and the S-TG was above the recommended level. The fasting glucose levels for the 2 patients were not significantly higher than the upper recommended level (P= 0.531). However the insulin level for patient GB3 was 7 times higher than the upper recommended level, and had a C-peptide level 3.5 times higher than the upper end of the reference range. Surprisingly the same patient had a normal HbA1c. These two values were normal for patient GB5. The CRP values were within the normal range.

5.2 Method development

Pre- and post surgery fat biopsies from obese patients (subcutaneous and omental fat) and pre surgery fat biopsies from control persons (subcutaneous fat) were analyzed for infiltrating non-fat cells by flow cytometry and histology. Figure 5.2 illustrates the flow of the regime and how the isolation and the identification of the non-fat infiltrating cells in the adipose tissue were conducted.

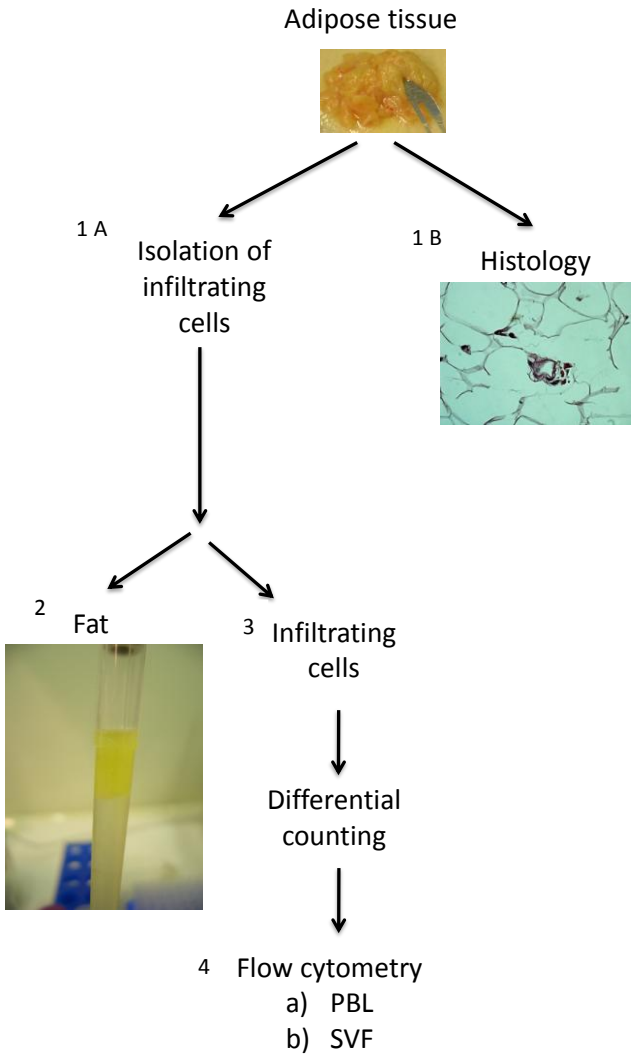


Figure 5.2. Schematic representation of the flow sheet on how fat biopsies were handled in order to be analyzed by flow cytometry and histology. For the obtained adipose tissue, one piece underwent enzymatic digestion for further isolation of the infiltrating cells, where the fat was removed and the infiltrating cells were counted and investigated using flow cytometry. A second piece of the tissue was fixated, stained with HE and sectioned for further investigation through microscope and the use of IHC for some of the tissue samples.

5.2.1 Isolation of infiltrating cells (1A in Figure 5.2)

The original method

In order to quantify and identify the infiltrating cells in WAT of obese patients, we aimed at optimizing a method in which non-fat infiltrating cells were isolated from fat tissue. These cells are found in the SVF of adipose tissue [14, 15]. The infiltrating cells are also called the SV cells. Using a gravimetric principal where cells were released from the fat tissue by enzyme digestion followed by cell separation through centrifugation, the cells could be counted in a Beckman Coulter ACT differentiation counter and quantified using flow cytometry.

We optimized a method that was originally developed at the University of Bergen (UiB). Hence, I was initially introduced to the fractionation method developed by PhD candidate Simon Dankel at HUH, [55, 56].

In short the method was as follows (step 1 to 11):

1) Fat tissue was collected in a tube containing PBS supplemented with 0.1% BSA at RT.

2) Prior to use, the fat tissue was rinsed in PBS

- To remove surface blood contaminants.

3) Next, the tissue was cut into small pieces (0.5 to 1 g) and further homogenized by further cutting.

- This was done to obtain small pieces for the digestion to be optimal.

4) The pieces were mixed with Collagenase and Liberase Blendzyme 3 (LB3) dissolved in PBS with 0.1% BSA (LB3, is designed to replace most "high quality" lots of traditional collagenase).

5) Mixing of enzyme was performed to ensure optimal digestion and the samples were incubated at 37 °C for 30 min in a humidified atmosphere, and shaken at 50 revolutions per minute (rpm) or 80 rpm orbital.

- Incubation at 37 °C with shaking was necessary in order to have optimal enzyme effect.

6) After incubation the samples were filtered into centrifuge tubes through a nylon filter using a syringe and filter holder. The samples were then left to separate, with adipocytes floating to the top.

   Optimal separation of fat (floating) and infiltrating cells (in liquid phase) was initially tested by simply letting the centrifuge tube stand on the bench (A). If this was not successful the samples would then be centrifuged for 1 min at150 x g (B). The fat cells would then be at the top and infiltrating cells in the buffer solution.

7) Independent if A or B was successful, the top phase (fat cells) was pipetted out, mixed with PBS and centrifuged at 150 x g for 1 minute before transferred to a new eppendorph tube where lysis buffer was added. The fat cells were stored separately (on ice) until the infiltrating cell sample had been analyzed.

- Samples would be stored for further fractionation if needed.

8) The filter would have unresolved matrix on it which was scraped off and added to the centrifuge tube with the SV cells. The filter was also added to this tube. The tube was centrifuged at 150 x g for 10 minutes.

9) After centrifuging, the filter and the supernatant were removed. The pellet was resuspended in lysis buffer or PBS if analyzed by flow cytometry and stored on ice.

10) If a separate matrix-fraction was desired, the matrix leftovers were transferred to a centrifuge tube, with the filter, where pure PBS was added. This was shaken carefully and centrifuged at 150 x g for 3 minutes. The matrix was transferred to an eppendorph tube and lysis buffer was added.

- This was done at 4°C (ice) and performed to homogenize the matrix tissue.

11) Infiltrating cells would be analyzed by flow cytometry.

- To analyze for the relative number of T-, B-, NK- cells as well as monocytes and macrophages.

Optimizing and establishing of the fractionation method at the University of Oslo (UiO)

At the UiO we aimed at optimizing the method in order to isolate infiltrating cells with optimal yield. We initiated the optimization by using adipose tissue from deceased patients from the Pathological Department at Rikshospitalet HF. The method was performed 14 times before the harvesting of adipose tissue from OUHA could commence. RM1- RM14 was the identification code for the fractionations completed on tissue received from the pathological department. The tissue was identified as RM1-RM14, denoting Rettsmedisinsk sample 1, 2….-14.

We did not perform flow cytometry on the tissue from the Pathological Department, as we found this irrelevant as long as this was pathological tissue. Despite this, we considered it useful to use such tissue in order to optimize the method quantitatively.

Optimization of the method was performed in a fume hood under sterile conditions, and the equipment was endotoxin- and nuclease free in order to reemploy the way the method should be executed with genuine patient materials.

The first steps (1-4) were more or less identical in the two methods. However, collagenase Liberase Blendzyme medium thermolysin (LBTM) was replaced for LB3 since LB3 was out of production. LBTM was chosen since it was considered equally good as LB3. The concentration of LBTM was initially 26 Wünsch units/vial (collagenase activity) and was diluted to a concentration of 2.6 Wünsch units / vial. The enzyme was dissolved in sterile HBSS and was aliquoted into 0.5 ml amounts in eppendorph tubes and stored at -20°C. On the day of experiment, the enzyme solution was thawed, carefully vortex and mix with a pipette 10 times.

Upon collection samples were soaked in 15 ml HBSS 5% BSA in order to rinse the tissue for excess blood and other tissue fluids. PBS as was the buffer in the original procedure was exchanged for HBSS 5% BSA, since HBSS contains a pH indicator and 5 mM glucose yielding more optimal conditions. It should be noted that protocol was optimized for one tissue sample. However, it may be preformed for two samples in parallel. In this case one of the samples will have to be stored in the HBSS/enzyme solution for some time in the incubator cabinet while the other sample is cut into pieces and homogenized. The time of incubation in HBSS/enzyme solution should be minimized.

Upon arrival with the tissue samples the incubator cabinet was set for 37 °C and the centrifuge was fast cooled to 4 °C.

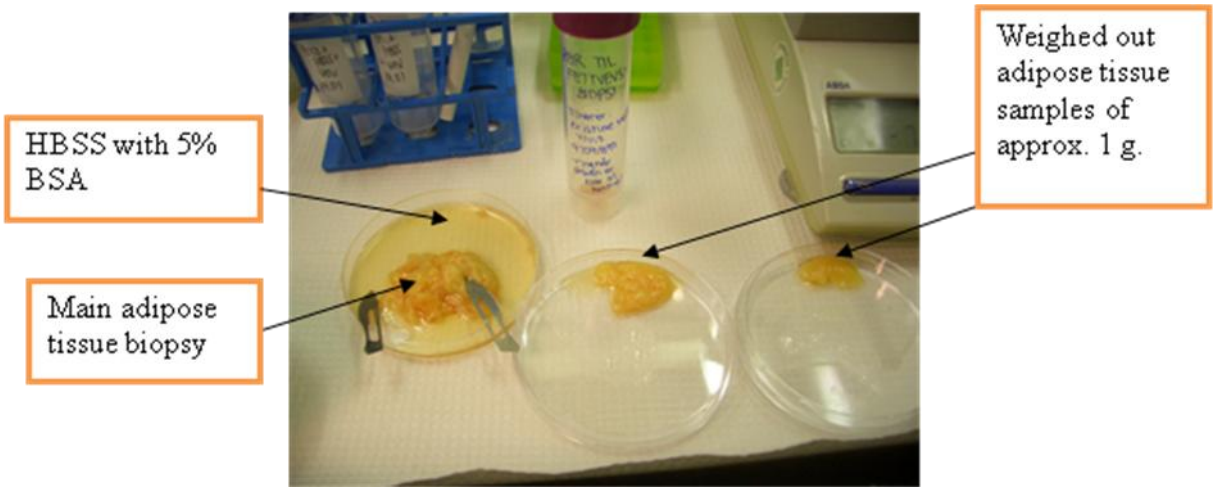


Figure 5.3. Weighing. The adipose tissue obtained was initially larger than what was needed; hence the required amount (approx. 1 gram) was weighed out on Petri dishes.

The adipose tissue biopsies received were approximately 4-5 grams, whereas required weight for the fractionation was approximately 1 gram per sample. The tissue was therefore cut to the accurate sizes, weighed and noted.

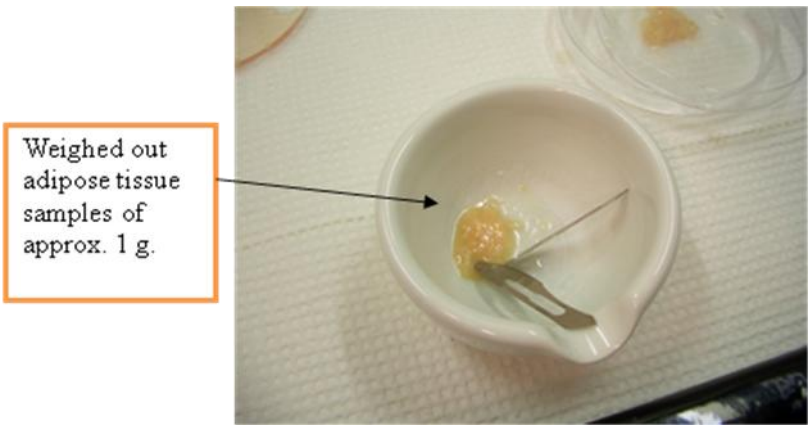


Figure 5.4. Cutting. After weighing, the adipose tissue was cut in a porcelain mortar using scalpels. The tissue was cut up until it had a purée-like consistency; this to improve the enzymatic digestion.

There was some discussion concerning using tissue samples weighing 2-3 grams, however when counting the SV cells in the SVF solution using this weight, it showed that the amount of white blood cells (WBC) was not significantly (P= 0.217) greater compared to the smaller samples (0.7-0.8 gram). The infiltrating cells are included in the WBC count.

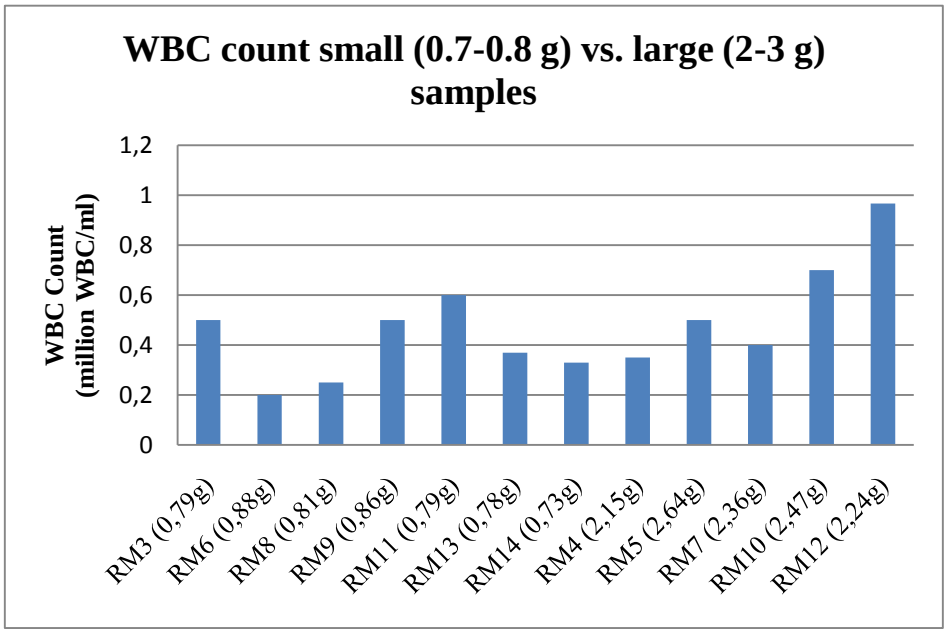


Figure 5.5. The diagram showing the weight of the samples and the detected WBCs in the SVF solution for the small (RM3, 6, 8, 9, 11, 13, 14) versus the large (RM4, 5, 7, 10, 12) samples.

The selected tissue was vigorously cut up using a scalpel and a mortar until a paste-like mash was achieved. In separate 50 ml NUNC-tubes 3.5 ml of HBSS supplemented with 0.5 mL LBTM (2.6 Wünsch units/0.5 ml) were prepared. The cut up tissue samples were added to the tube and left in the preheated incubator cabinet until digested. The samples were incubated at 37 °C for 1 hour in a humidified atmosphere, and shaken at 0.1 x g (80 rpm) orbital.

Figure 5.6. Cut up tissue incubated with HBSS/enzyme solution for enzymatic digestion. In an improvised holder for the incubation made from a Styrofoam box and tape. This created a slight angle on the tube, which increased the digestion surface and avoided solution in the cap of the tube.

After an hour, the incubation was stopped and in case of more than one parallel, one was taken out for further treatment. The other parallel sample was left to further incubate without shaking at 37 °C.

The incubation time at this step was 15-30 minutes in the initial protocol. However, we found that more time was required for optimal effect and increased the time to 1 hour.

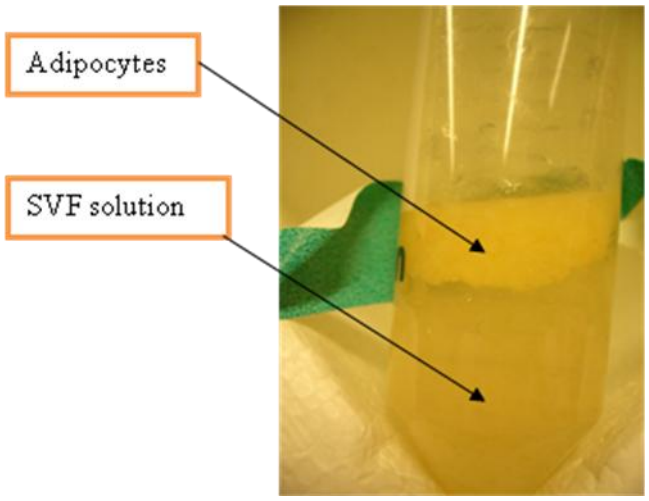


Figure 5.7. Tissue after enzyme incubation. The digested tissue had separated into two layers, the adipocytes and undigested matrix floating on top and the SVF solution being the remains of the liquid phase

One major difference between the method optimized by us and the method developed at UiB was that we were interested in the SVF fraction whereas the UiB-method was optimized for collection of the fat cell fraction. Hence after the enzyme incubation step in which the digested tissue had separated into two layers, the adipocytes that were located at the top were removed leaving the SVF solution (liquid phase) in the test tube. In order to isolate cells from the SVF fraction the solution was transferred to a 10 ml syringe and sieved through a sterile Spectra/Mesh nylon filter in order to remove any undigested matter, called the matrix. The matrix consists of connective tissue and blood vessels.

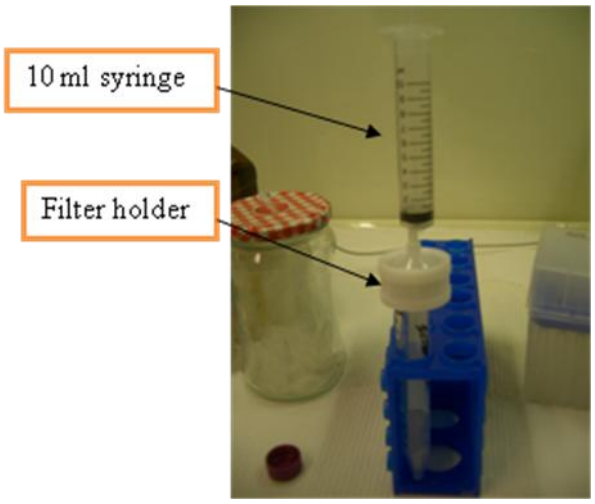


Figure 5.8. Removal of undigested matrix by filtration. A syringe was attached to the filter holder on a centrifuge tube. The tissue solution was poured into the syringe, pressed through a sterile Spectra/Mesh nylon filter.

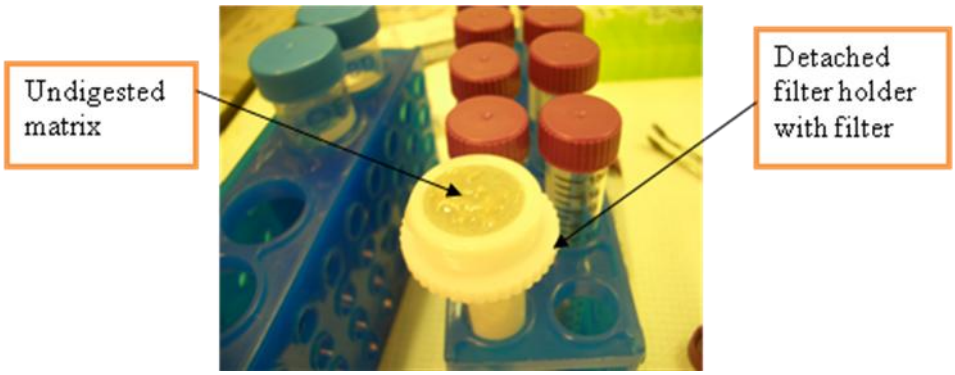


Figure 5.9. Captured undigested matrix. The Spectra/Mesh nylon filter with captured connective tissue, blood vessels and some SV cells.

After filtration, undigested matrix was left on the Spectra/Mesh nylon filter. The matrix was then treated separately to the SVF. To release valuable SV cells embedded in the matrix the filter was rinsed three times with 1 ml ice cold HBSS 5% BSA. This was done by carefully removing the syringe and opening the filter holder. The undigested matrix was manipulated to the centre of the filter using sterile scalpels. Then the filter holder was assembled, syringe put back on the filter holder before 1 ml HBSS 5% BSA was poured in the syringe and pressed through. This was repeated three times.

The remaining matrix on the filter was transferred to an eppendorph tube (labeled Ma Sup) and 1 ml of ice cold HBSS 5% BSA was added and kept on ice until use. The tube was vigorously mixed in order to release any possible SV cells left in the matrix. The SV cells in

the matrix supernatant were later counted along with the SV cells in the SVF solution. It should be noted that the matrix was not dissolved completely at any time.

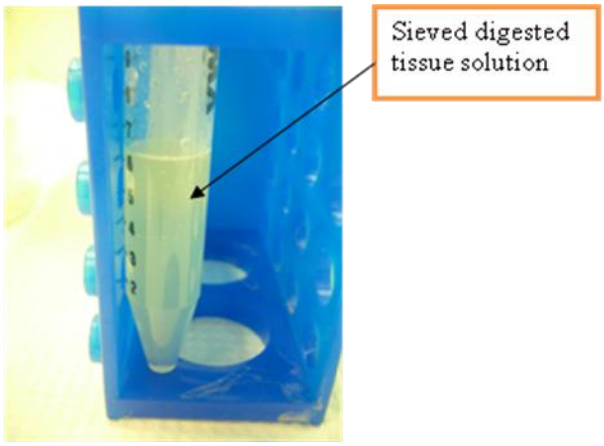


Figure 5.10. Digested, filtered and rinsed tissue. After filtration the tissue solution was rinsed with HBSS 5% BSA. The remains on the filter was washed with 1 ml HBSS 5% BSA three times in order to reduce the matrix matter left on the filter and optimal release the SV cells trapped in the matrix.

To optimize the time of separation centrifugation of samples were compared to gravimetric sedimentation (5 min at 20 °C). However, upon centrifugation (150 x g for 1 min) adipocytes were ruptured, in that the fat was smeared on the inside of the tube. In addition, the fraction had an overall oily appearance. Based on this it was decided to perform gravimetric sedimentation at this step.

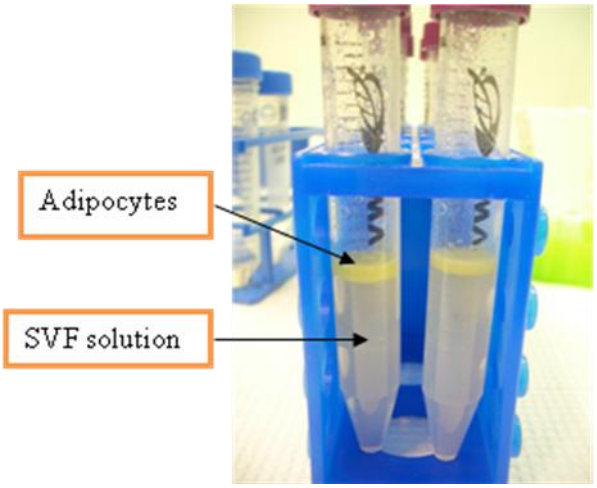


Figure 5.11. Gravimetric sedimentation of fat cells. Leaving the tubes to stand on the bench for five minutes created two clear layers, adipocytes forming the top layer.

5.2.2 Removing the fat from the digested tissue solution (2 in figure 5.2)

After separation, the adipocytes (top layer) were removed from the filtered SVF solution using a P-1000 pipette. Some SVF solution was unintentionally sucked into the pipette tip when taking out the adipocytes, therefore the pipette was let to stand in an empty centrifuge tube with tip on for 2 minutes, to let the adipocytes gravimetrically separate from the SVF solution trapped in the pipette tip.

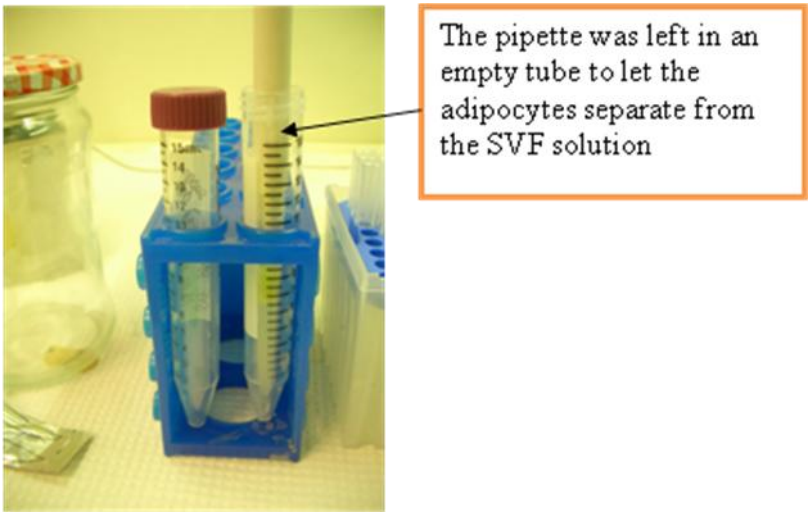


Figure 5.12. Adipocyte removal. The pipette used to remove the adipocytes (top layer) from the main SVF solution was left to stand in an empty centrifuge tube for two minutes for gravimetric separation of the adipocytes trapped in the SVF solution of the pipette tip.

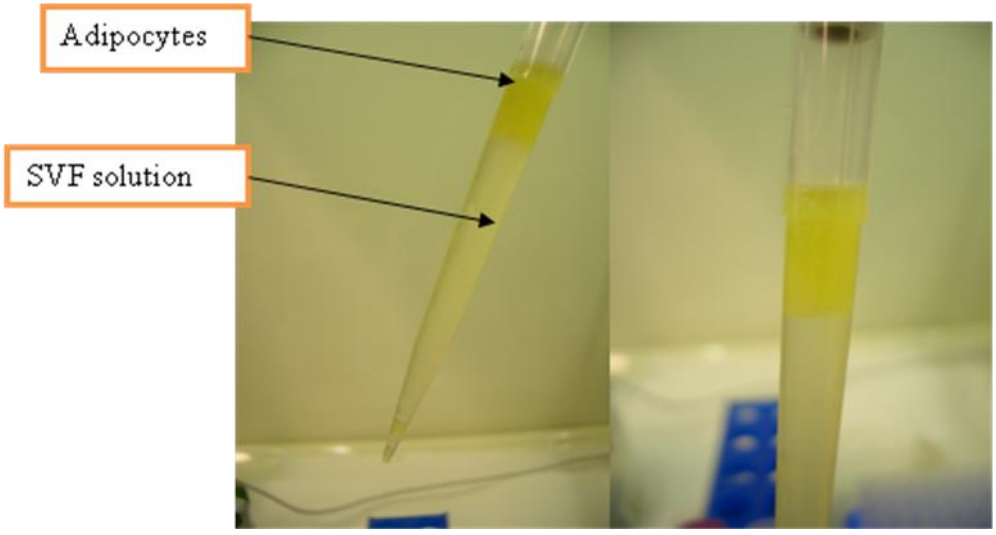


Figure 5.13. Gravimetric separation of fat cells in the pipette. After two minutes, the adipocytes had floated to the top of the trapped SVF solution in the pipette tip. The SVF solution was pressed out of the pipette back into the main centrifuge tube with the rest of the SVF solution. The adipocytes (remaining in the pipette tip) were transferred to a labeled eppendorph tube.

The SVF solution free of fat cells was pressed out of the pipette tip back into the main centrifuge tube, retaining the adipocytes in the tip. The adipocytes were transferred to a separate eppendorph tube. This step was repeated until the SVF solution was completely free of adipocytes. Once the top layer was completely removed, the adipocytes were immediately frozen at -80°C.

5.2.3 Infiltrating cells (3 in figure 5.2)

Once the fat layer had been removed, the SVF solution without the matrix fraction was aliquoted into 5-6 eppendorph tubes which were centrifuged at 585 g for 5 minutes at 4 °C. The supernatant was collected in one 15 ml centrifuge tube and was frozen directly at -80 °C and stored for future use. The cells were washed in 1 ml of HBSS 5% BSA before a second round of centrifugation. The supernatant from the second centrifugation was discarded and 0.5 ml of HBSS with 5% BSA was added to one of the eppendorph tubes. The pellet was dissolved in the HBSS and then transferred to the next tube, with the same manoeuvre performed on the remaining tubes; resulting in all pellets assembled and dissolved in the last eppendorph tube.

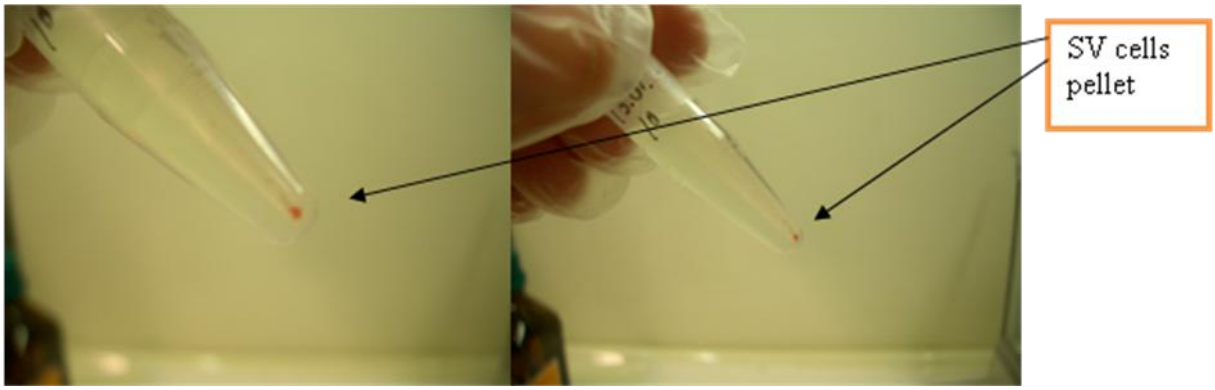


Figure 5.14. SV pellet after centrifugation.

Upon collection the infiltrating cell fraction was contaminated by RBCs. The RBCs were removed by lysis as described under material and methods. In short, the pellets were collected and centrifuged for 5 min at 585 x g and 4 °C. The supernatant was removed and the pellet dissolved in 250µl of RBC lysis buffer, at RT for two minutes. Originally the protocol suggested to use Roswell Park Memorial Institute (RPMI) medium 5% fetal bovine serum (FBS), but it was decided to continue to incubate SV cells in HBSS 5% BSA. For the tissue samples of 1.0 gram, 1 ml RBC lysis medium was used.

The cell suspension was centrifuged at 306 x g for 7 minutes. The supernatant was removed and the SV cells pellet resuspended in 0.5 ml of HBSS 5% BSA before counting. The tube containing the matrix leftovers (Ma Sup) was also prepared for counting.

The SV cells, from the SVF solution, and the SV cells released from the matrix leftovers (Ma Sup) were counted in the Beckman Coulter ACT differentiation counter where the WBCs detected were noted. The WBC count indicates the amount of infiltrating cells (SV cells). After counting all cell suspensions were frozen at -80°C.

WBC counting for the adipose tissue from the Pathology Department, Rikshospitalet HF

It should be noticed that for all of the 14 fractionation trials using pathological tissue the experiments were discontinued after counting.

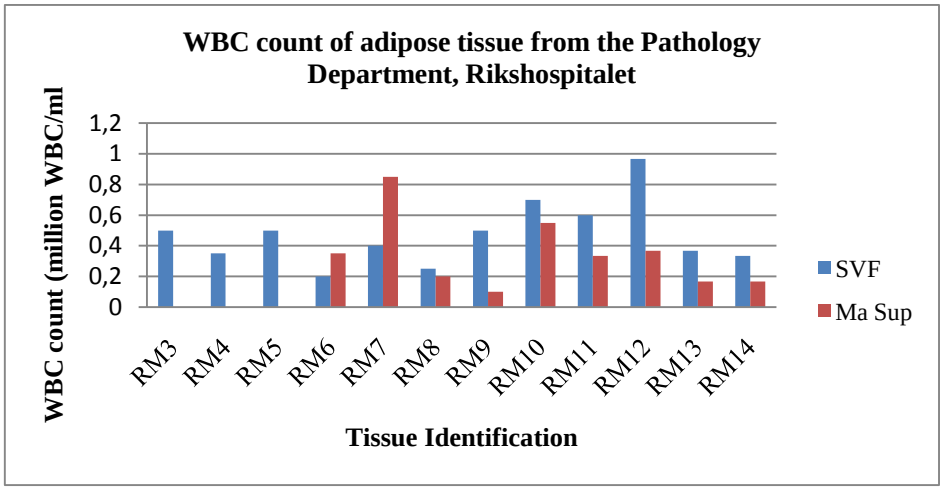


Figure 5.15. WBCs detected in the SV cells. WBCs from the SVF solution and the SV cells released from the matrix (Ma Sup). The WBC counts showed the amount of infiltrating cells in the cell suspensions.

No data for RM1 and RM2 were obtained as the procedure failed. Furthermore, for RM3, RM4 and RM5 the Ma Sups were not counted, whereas the other fractionation results are given in the diagrams. RM7 had much greater amount WBCs in the Ma Sup than the SVF solution (0.85 million WBC/ml in the Ma Sup vs. 0.4 million WBC/ml in the SVF solution). RM12 had a lot greater amount of WBCs compared to the others in the SVF solution, 0.97 million WBC/ml. Although RM12 is one of the large samples, it was not found a significant difference between the small and the large samples (P= 0.217).

5.3 Analysis of infiltrating cells of adipose tissue from gastric bypass surgery patients, OUHA

After establishing a method for fractionation of infiltrating cells of WAT, we wanted to employ the method on genuine patient materials.

Table 5.7. The SV cell count from GB3-GB5. The SVF solution from patient GB1 (Gastric bypass 1) was isolated using the established method and found to contain 0.82 million WBC/ml whereas the Ma Sup contained 0.33 million WBC/ml. One gram of WAT from patient GB2 contained 1.65 million WBC/ml in the SVF solution and 0.53 million WBC/ml. Patient GB3 had 1.72 million WBC/ml in the SVF solution and 1.53 million WBC/ml in the Ma Sup. The SVF solution from patient GB4 contained 0.63 million WBC/ml and the Ma Sup contained 0.53 million WBC/ml, while the SVF solution of patient GB5 had 0.87 million WBC/ml and 0.47 million WBC/ml in the Ma Sup. The cell suspension volume for each SVF sample were 0.9 ml, and 0.6 ml for the Ma Sup

Tissue ID	Type	Million cells/ml	Tot cell suspension volume (ml)	Million cells in cell suspension volume (0.9 ml)
GB 3	SVF	1,8 x 106	0,9	1,62 x 106
	Ma sup	1,46 x 106	0,6	0,88 x 106
GB 4	SVF	0,87 x 106	0,9	0,78 x 106
	Ma sup	Discharded		
GB 5	SVF	0,8 x 106	0,9	0,72 x 106
	Ma sup	Discharded		

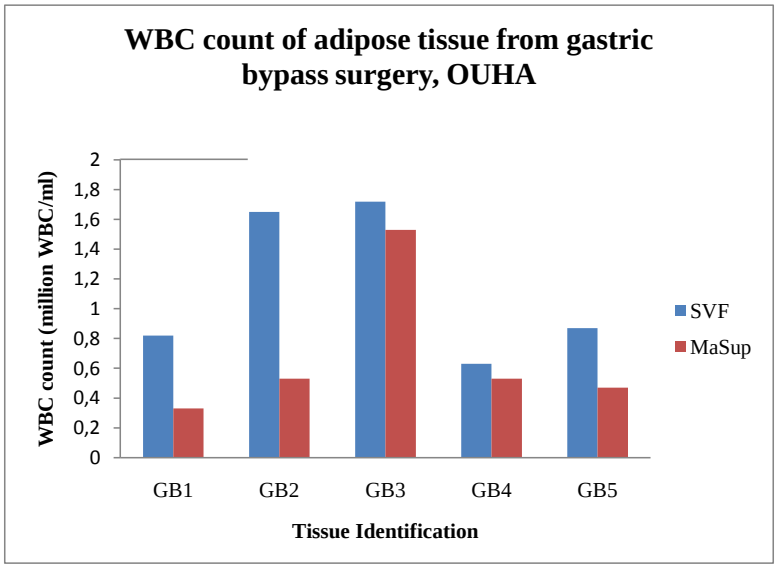


Figure 5.16. Infiltrating cells in adipose tissue of patients from OUHA. WBCs in the SVF solution and in the matrix supernatant (Ma Sup) detected in patients GB1-GB5.

Adipose tissue was received from 5 patients undergoing gastric bypass at OUHA. For patient GB1 and GB2, the experiment was terminated after cell counting; whereas flow cytometry was conducted after cell counting for patients GB3-GB5.

Finally, cell counting does not reveal the identity of the infiltrating cell. We wanted to determine the number and identity of immune cell related infiltrating cells, therefore we performed flow cytometry and IHC with antibodies to various immune cells. We also determined the form of infiltration by staining tissue samples with HE.

5.4 Identification of infiltrating cells by flow cytometry (4 in figure 5.2)

5.4.1 PBL from Ullevål University Hospital Oslo (a in figure 5.2)

In order to perform flow cytometry on isolated cells from patient material the method was optimized on PBL from healthy donors at Ullevål University Hospital Oslo. PBLs were analyzed using anti-CD4, 8, 14, 19 and 56 conjugated to FITC. For optimal result we found that 0.5-1.0 x 10^6 cells per well were optimal and 5 µl of each antibody.

5.4.2 Flow cytometry for tissue from the gastric bypass patients from OUHA (b in figure 5.2)

The SV cells for tissue GB3, GB4 and GB5 were analyzed by flow cytometry.

Flow cytometry of the SVF cells from patient GB3

For GB3, which contained the most cells the antibodies to the CD4, CD8, CD14, CD19 and CD56 markers were used. It was observed a 12% non-specific binding for the negative control (irrelevant antibody) (**Figure 5.17**).

Negative control (Mouse IgG1) for patient GB3

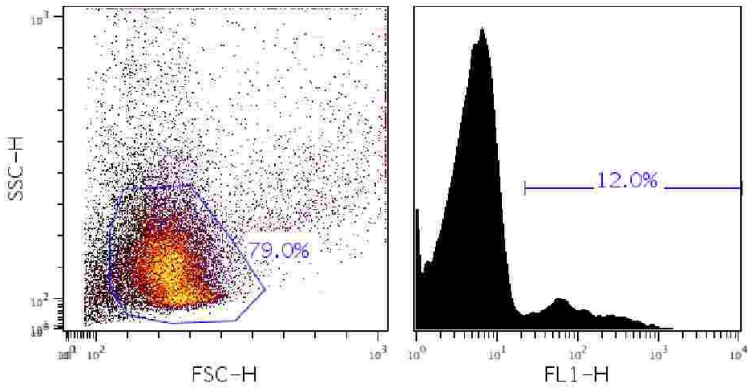


Figure 5.17. SV cells of patient GB3 stained with irrelevant antibody. The right diagram demonstrates the fluorescence at the given wavelength of the fluorochrome FITC and the corresponding cell count. It shows 12% non-specific binding in the negative control. Forward (FSC-H) and side scatter (SSC-H) denotes size and the granularity of the cells.

Moreover, 16% of the cells were positive for CD4, whereas 14.4% were positive for CD8 and, 14.8% of the cells were CD14. Furthermore, 12.4% were positive for CD19 whereas 14.6% were positive for CD56 (**Figure 5.18 A-E**).

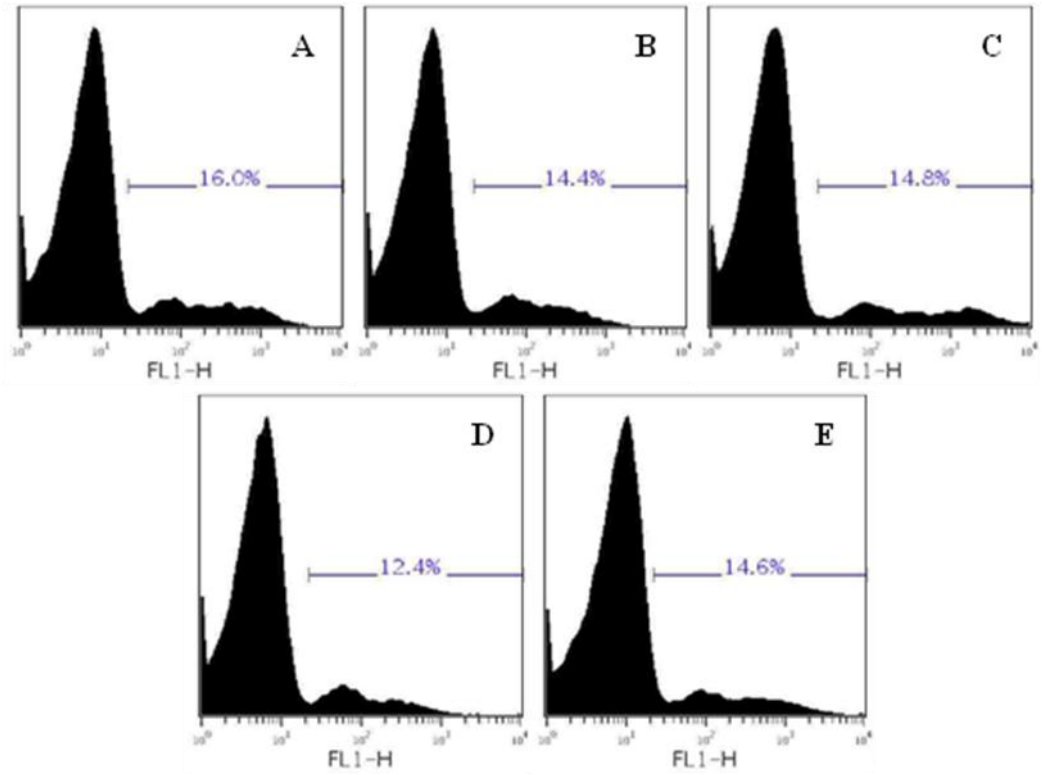


Figure 5.18. SV cells of patient GB3 stained with antibody to CD4 (A), CD8 (B) CD14 (C), CD19 (D) and CD56 (E). It is observed that 16% of the cells are CD4 positive, 14.4% are positive for CD8, 14.8% are positive for CD14, 12.4% are positive for CD19 and 14.6 % positive for CD56.

Ma Sup for patient GB3, negative control (Mouse IgG1)

As expected we monitored few cells (1.46 mill/mL) of the Ma Sup fraction of GB3. In fact we observed a great amount of non-specific binding and the peak profile was not comparable to the SV cells, although same cells were assumed to be present. No positive cells for the selection of antibodies were detected. Based on this result, flow was not conducted on the Ma Sup for the other patients (GB4 and GB5).

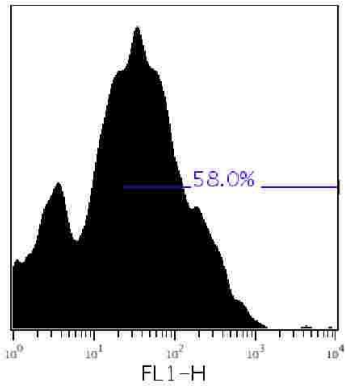
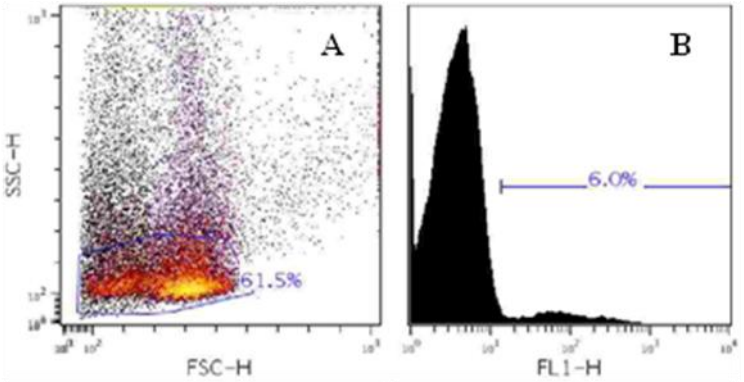


Figure 5.19. Ma Sup cells of patient GB3 stained with irrelevant antibody. Diagram showing the negative control with non-specific binding and the altered peak profile.

Flow cytometry of the SV cells from patient GB4 and GB5

As mentioned above, very few cells were isolated from patient GB4 (0.77 x 10^6 cells total) and GB5 (0.7 x 10^6 cells total). In the case of GB4, only the antibody to the CD4 marker was used, and for GB5 the antibodies to the CD4 and CD19 markers were used. For GB4 the negative control showed 6% non-specific binding, whereas the GB5 revealed 6.5% non-specific binding.

Negative control (Mouse IgG1) for patient GB4 and GB5



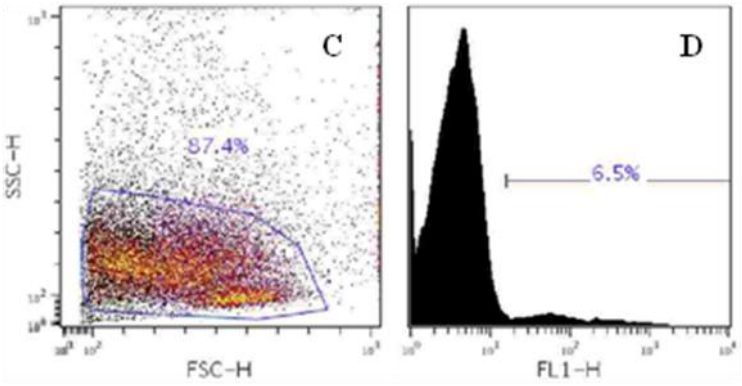


Figure 5.20. SV cells of patient GB4 and GB5 stained with irrelevant antibody. (A) Cell population according to size and granularity for patient GB4. (C) Cell population according to size and granularity for patient GB5. (B) Cell population positive for irrelevant antibody. GB4 6% non-specific binding (B) and GB5 6.5% non-specific binding (D).

The CD4 antibody used for the SV cells of patient GB4 and GB5

For patient GB4, 5.8% and for patient GB5, 7.2%, of the cells were positive for CD4 (**Figure 5.21, A and B** below).

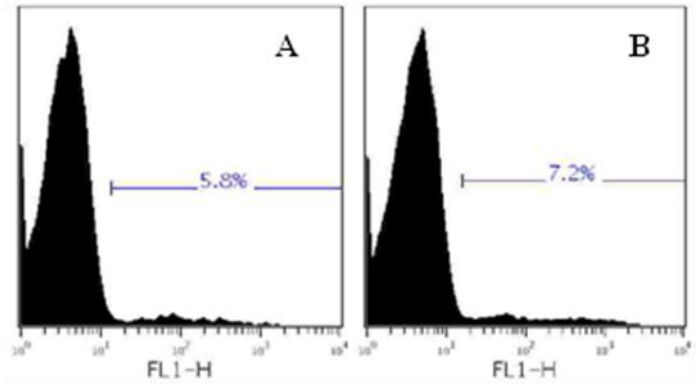


Figure 5.21. SV cells of patient GB4 (A) and GB5 (B) stained with antibody to CD4. 5.8% of the cells from patient GB4 were CD4 positive. 7.2% of the cells from patient GB5 were positive for CD4.

The CD19 antibody used for the SV cells of patient GB5

For GB5, 7.9% of the cells were positive for CD19 (**Figure 5.22**).

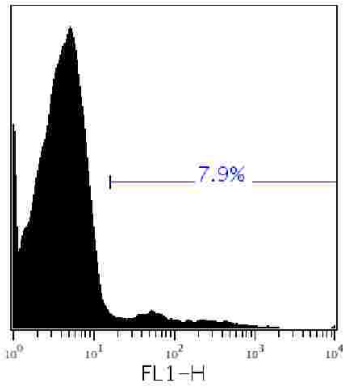


Figure 5.22. SV cells of patient GB5 stained with antibody to CD19. 7.9% of the cells from patient GB5 were positive for CD19.

5.5 Histology (1B in figure 5.2)

Fat biopsies (subcutaneous and omental fat) were received from HUH and were donated by obese patients undergoing bariatric surgery. The biopsies were investigated pre and post surgery by microscopy after HE staining and IHC. These biopsies were compared to fat biopsies (subcutaneous fat) isolated from healthy controls. Biopsies showing infiltration outside blood vessel walls and in between the fat cells were selected for IHC. The control biopsies were also prepared for IHC in order to compare. To be able to detect a possible infiltrate, the biopsies were sectioned deeper. Pathologist and physician Professor Helge Scott accompanied the observations and the decision making.

Fat biopsies of the obese patients included were a selection of the obese patients from the Dankel et al. study at UiB, as well as patients GB1- GB5 from the gastric bypass surgeries at OUHA. The biopsies from the controls were also from the Dankel et al. study.

The histological observations of the obese patients and the controls included in this part of the project are summarized in the figures below.

5.5.1 Control material

Tissue slides of subcutaneous adipose tissue biopsies from the controls 4A2, 20A2 and 27A2 were initially examined by light microscopy after HE staining. As can be observed, intact

appearing fat cells are observed (**Figure 5.23A-C**, arrows 1 and 2). No infiltrating cells, which should appear as smaller round cell of approximately size of 5-10 µm (Shido, Kobayashi et al. 1984; Krombach, Munzing et al. 1997), were observed. When performing IHC using anti-CD3, -CD20 and -CD68 we observed no positive staining for the selected antibodies concluding that there were no infiltrates of immune cells in the observed subcutaneous adipose tissue of the controls.

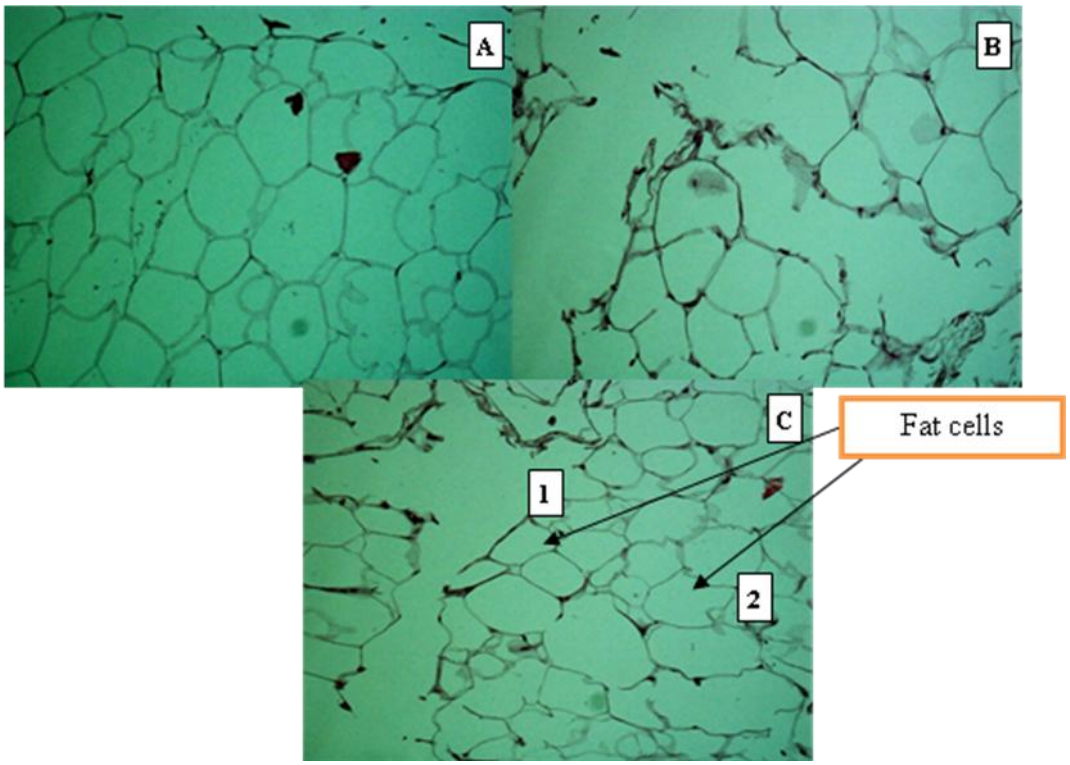


Figure 5.23. HE stained subcutaneous adipose tissue biopsies of control. Intact fat cells and none infiltrating cells are observed. Original magnification x 200.

5.5.2 Biopsies of obese patients

Next we examined biopsies from the obese patients. Included in the figures below are the patient biopsies that displayed infiltrating cells when stained with HE. All the adipose tissue biopsies included from the obese patients were from the subcutaneous adipose tissue; this due to complete absence of infiltrates in the omental adipose tissue biopsies and due to subcutaneous adipose tissue being the only tissue obtained for the controls.

Patient 4A1

The biopsies obtained for patient 4A1 are given below in order to show the difference in the tissue before and after the surgery, magnified x 40 (**Figure 5.24 and 5.25**) and x 200 (**Figure 5.26**). For the pre surgery biopsy for patient 4A1 there was not detected any infiltration.

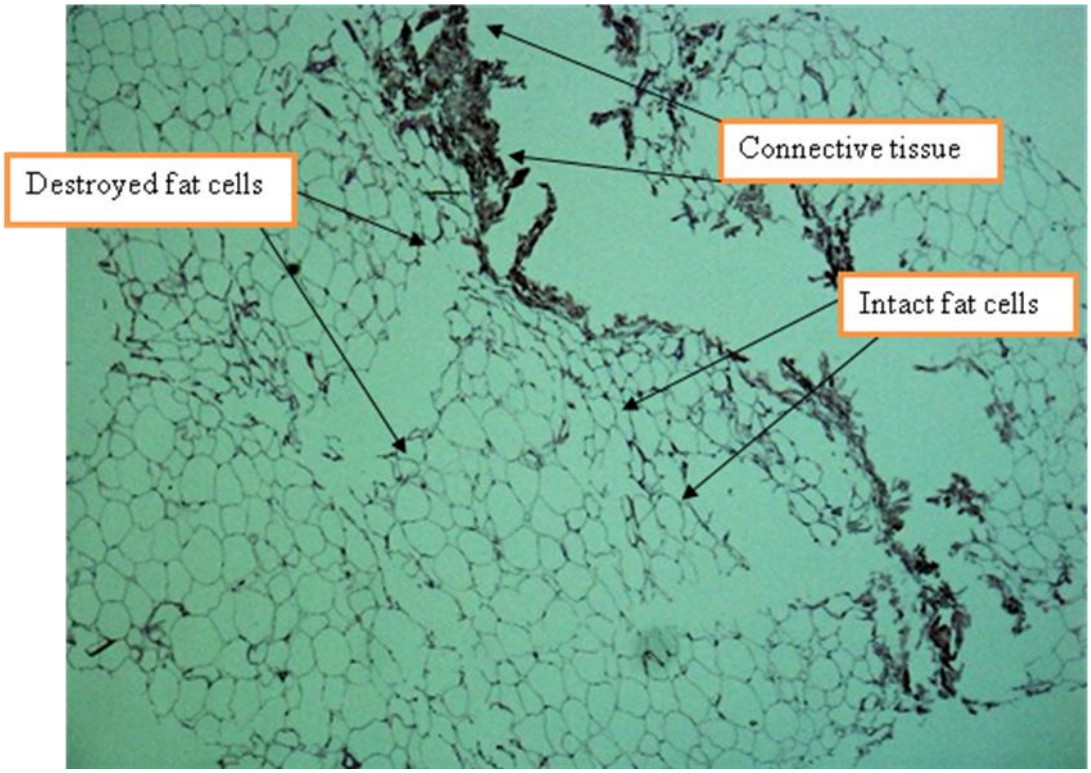


Figure 5.24. HE stained pre surgery adipose tissue of patient, 4A1. Note fat cells and connective tissues indicated by arrows, showing fat cells and connective tissue in the obese patient. Original magnification x 40.

However, when analyzing the post surgery biopsy we observed a modest infiltration of smaller cells that may represent immune cells (**Figure 5.25 and 5.26**).

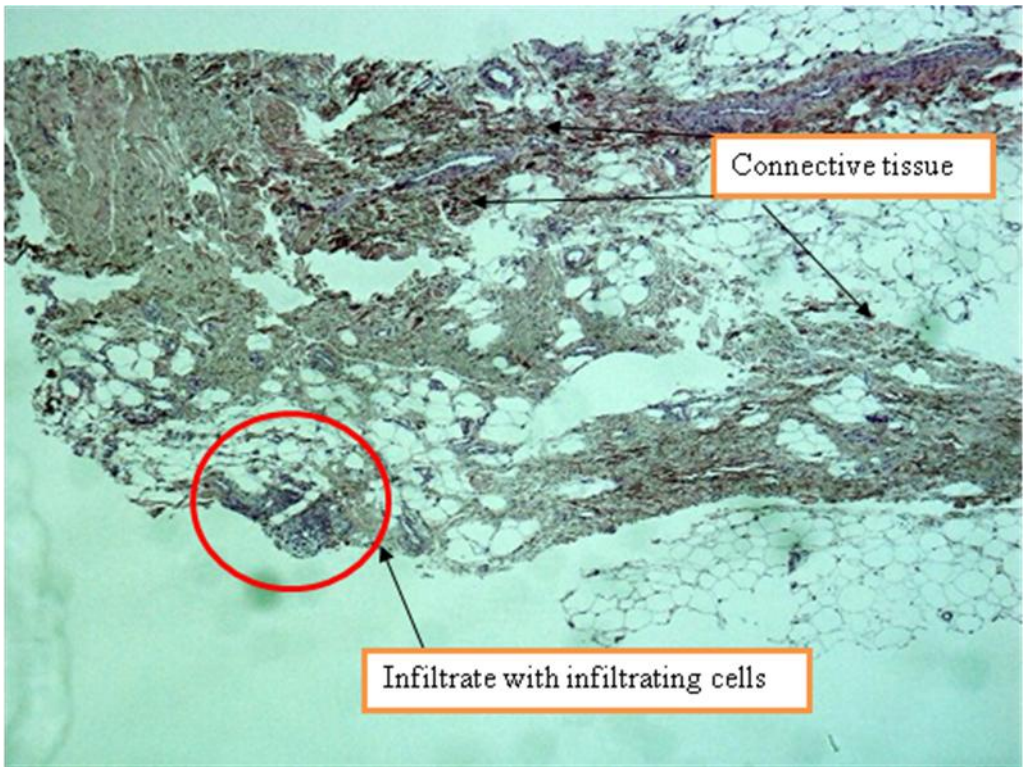


Figure 5.25. Post surgery adipose tissue biopsy stained with HE from patient, 4A1. Note infiltrating cells (red circle) and connective tissue marked by arrows. Original magnification x 40.

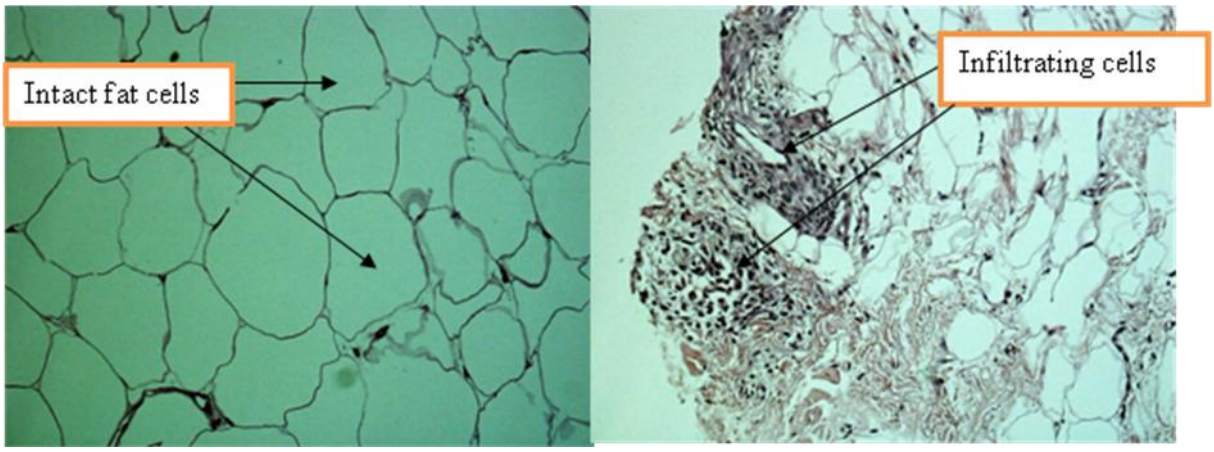


Figure 5.26. Left diagram displaying pre surgery adipose tissue biopsy from patient 4A1, stained with HE showing intact fat cells. Right diagram is post surgery showing infiltrating cells in the tissue. Original magnification x 200.

Based on this, IHC was performed for this biopsy in order to determine the identity of these cells (Fig 5.27). This demonstrated that the biopsy was positive for cells expressing the CD3 and CD68, but not for CD20 markers, respectively. Based on the antibodies used, these results suggested that there were some T cells (CD3+), and macrophages/monocytes present in the population of the infiltrating cells, however no B cells (CD20) were present.

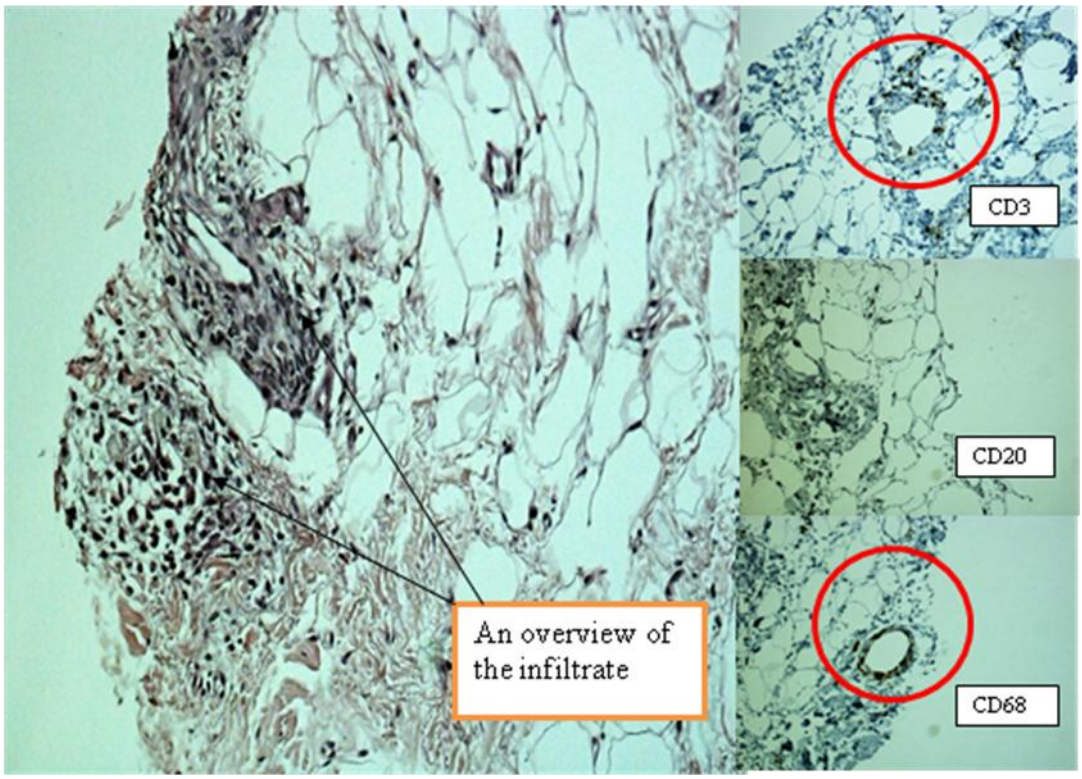


Figure 5.27. HE and IHC staining of post surgery subcutaneous adipose tissue of patient 4A1. Left panel: infiltrated post surgery adipose tissue stained with HE. Right three panels: IHC staining with anti-CD3 upper panel, anti-CD20 middle panel and anti-CD68, lower panel. Positive infiltrating cells are indicated by red circles for CD3 and CD68 whereas CD20 negative. The antibody specific infiltrating cells appears brown. Original magnification x 200.

Patient 28 A1

The biopsies obtained for patient 28A1 are given below in order to show the difference in the tissue before and after the surgery, magnified x 40 (**Figure 5.28 and 5.29**) and x 200 (**Figure 5.30**). For the pre surgery biopsy no infiltration was detected (Fig 5.28). However, infiltration was observed for the post surgery biopsy (**Figure 5.29**).

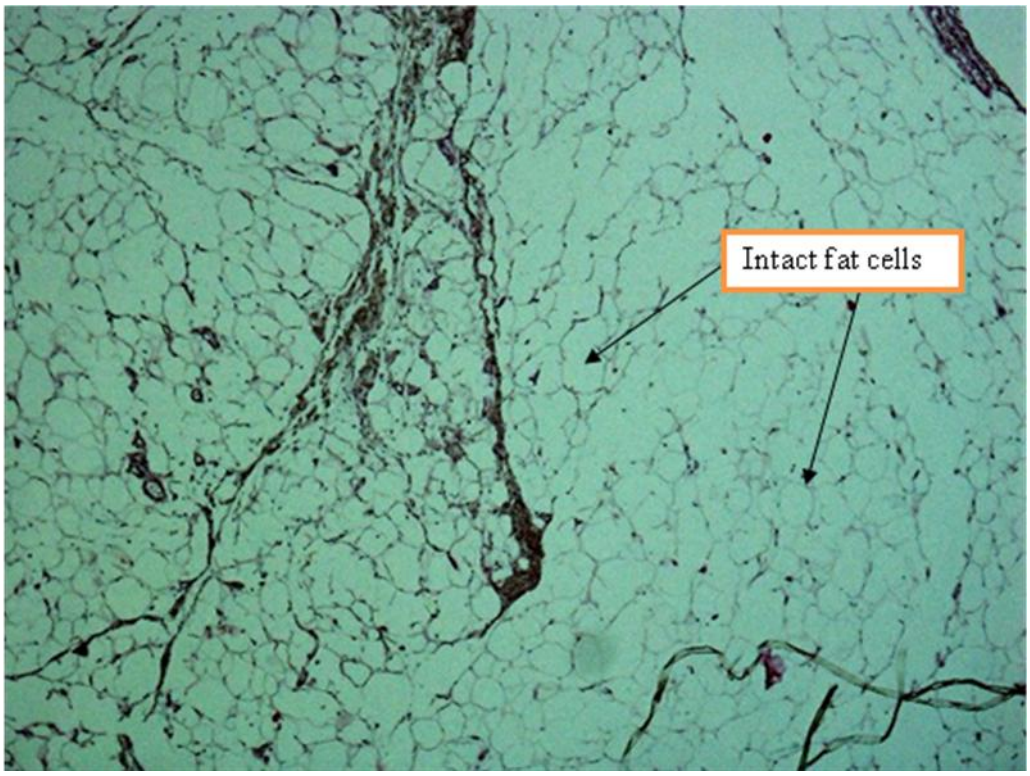


Figure 5.28. Pre surgery adipose tissue biopsy with HE stain from patient, 28A1. Note fat cells indicated by arrows, showing fat cells in the obese patient. Original magnification x 40.

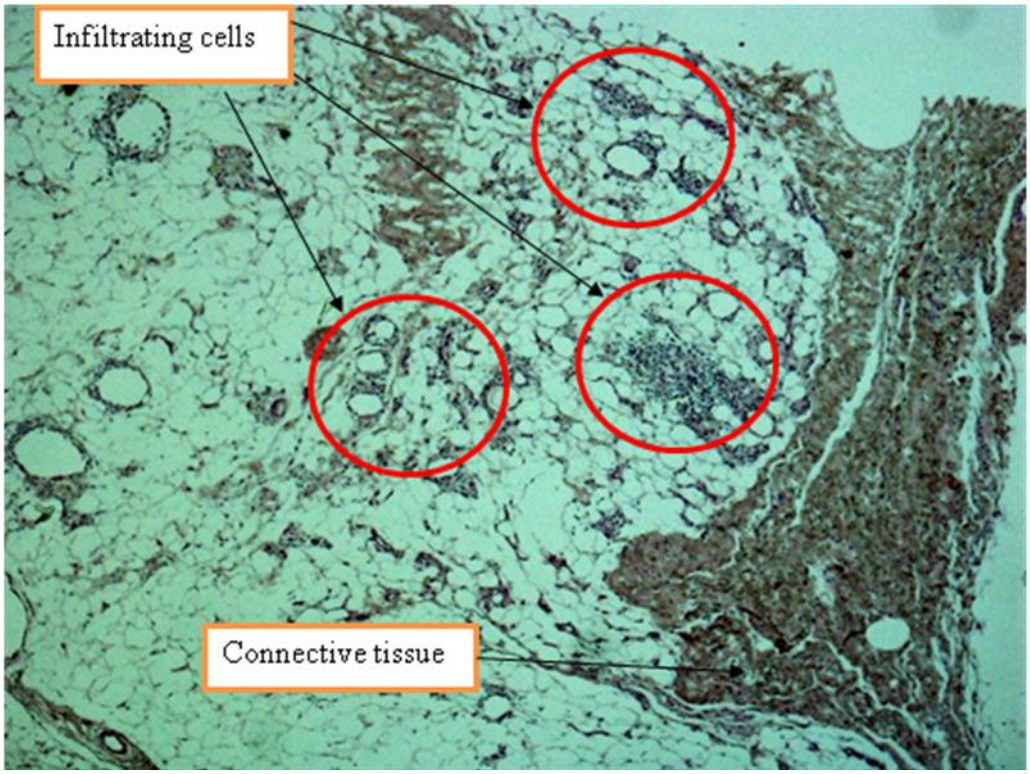


Figure 5.29. Post surgery adipose tissue biopsy stained with HE from patient, 28A1. Note infiltrating cells (red circles) and connective tissue marked by arrows. Original magnification x 40.

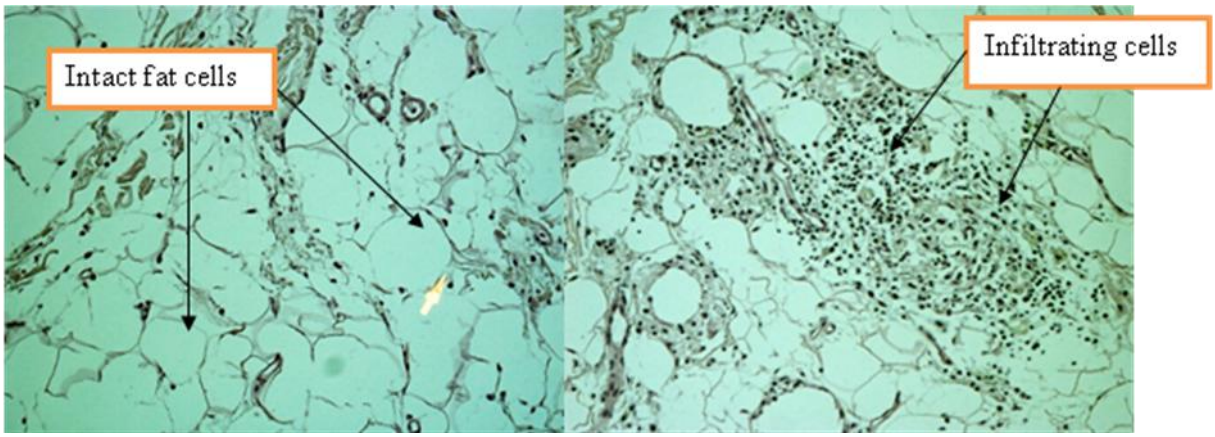


Figure 5.30. Left diagram displaying pre surgery adipose tissue biopsy from patient 28A1, stained with HE showing intact fat cells. Right diagram is post surgery showing infiltrating cells. Original magnification x 200.

Based on this finding, the biopsy was prepared for IHC to determine immune cells' identity. This identified infiltrating cells positive for only anti-CD3, whereas cells positive for anti-CD20 and anti-CD68 were not detected, suggesting that the filtrate mainly consisted of T cells.

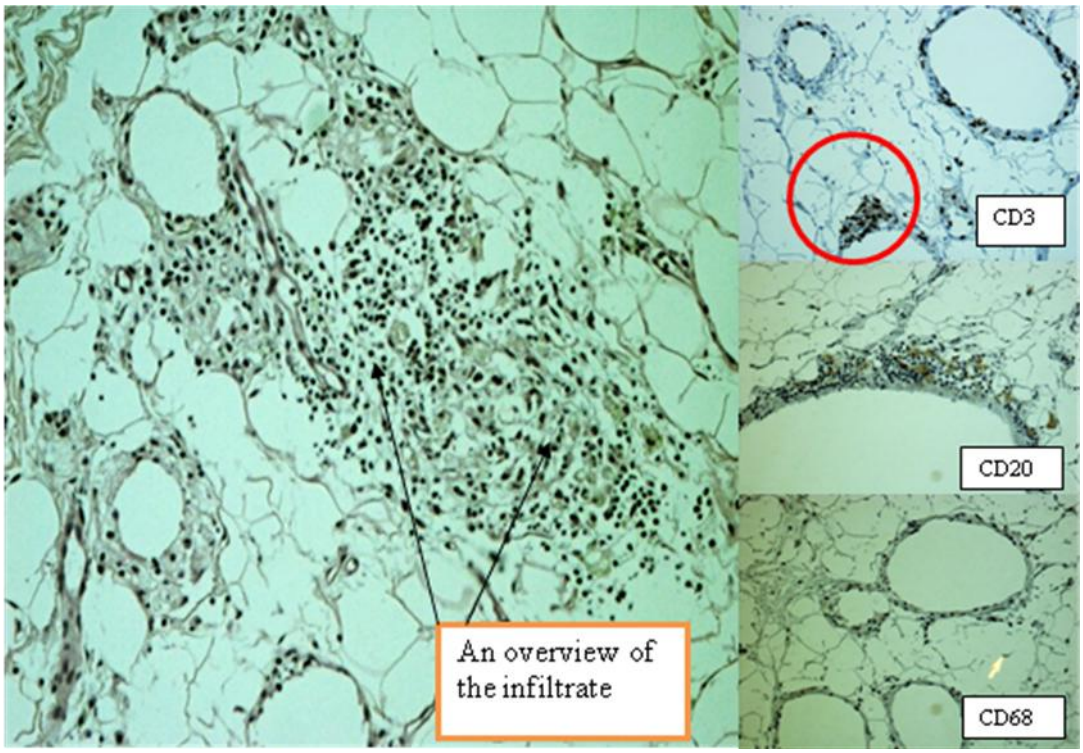


Figure 5.31. HE and IHC staining of post surgery subcutaneous adipose tissue of patient 28A1. Left diagram displaying an overview of the infiltrate in the post surgery adipose tissue biopsy stained with HE from patient 28A1. IHC diagrams to the right, showing the CD3 positive infiltrating cells (red circle) on top. None of the cells were positive for CD 20 (middle) or for CD68 (bottom). The antibody specific infiltrating cells appears brown. Original magnification x 200.

Patient 10 A1

When observing the HE stained biopsies obtained from patient 10A1 small infiltrates were detected in both pre and post surgery biopsies (**Figure 5.32**).

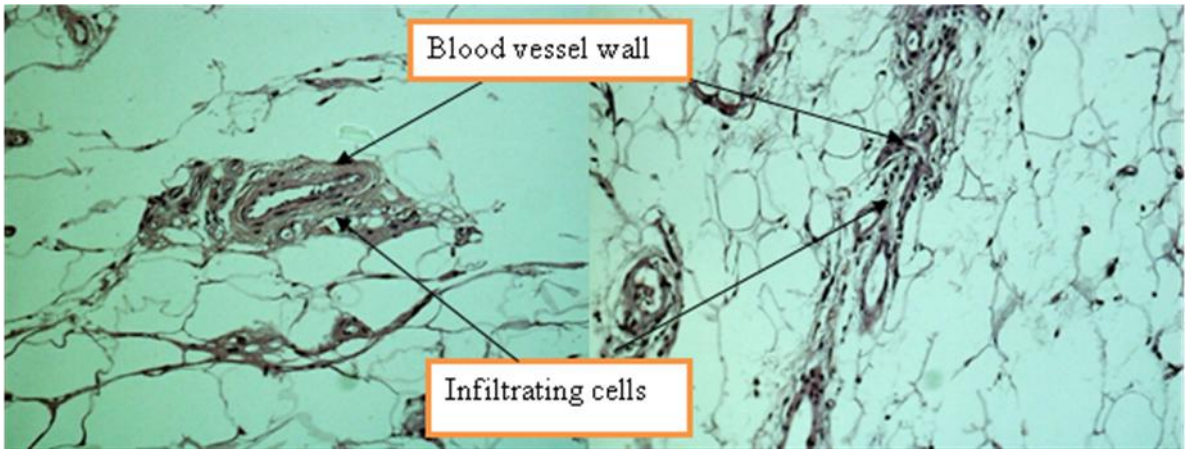


Figure 5.32. Pre- and post surgery adipose tissue biopsy from patient 10A1, stained with HE. Left panel: shows blood vessel wall and infiltrating cells, indicated by arrows. Right panel: shows blood vessel wall and infiltrating cells in the tissue indicated by arrows. Original magnification x 200.

In order to determine the cell identities by IHC, none of the cells present in the tissue were positive for the selected antibodies to the CD3, CD20 or CD68 markers (results not shown).

Patient 27 A1

When observing the HE stained biopsies obtained from patient 27A1 small amounts of infiltrating cells were detected in both pre and post surgery biopsies (**Figure 5.33**). The cells detected in the pre surgery biopsy were not considered an infiltrate, as the infiltrating cells appeared in the lumen of the blood vessel. However the cells in the post surgery biopsy were engaged in a minor infiltrate outside a blood vessel.

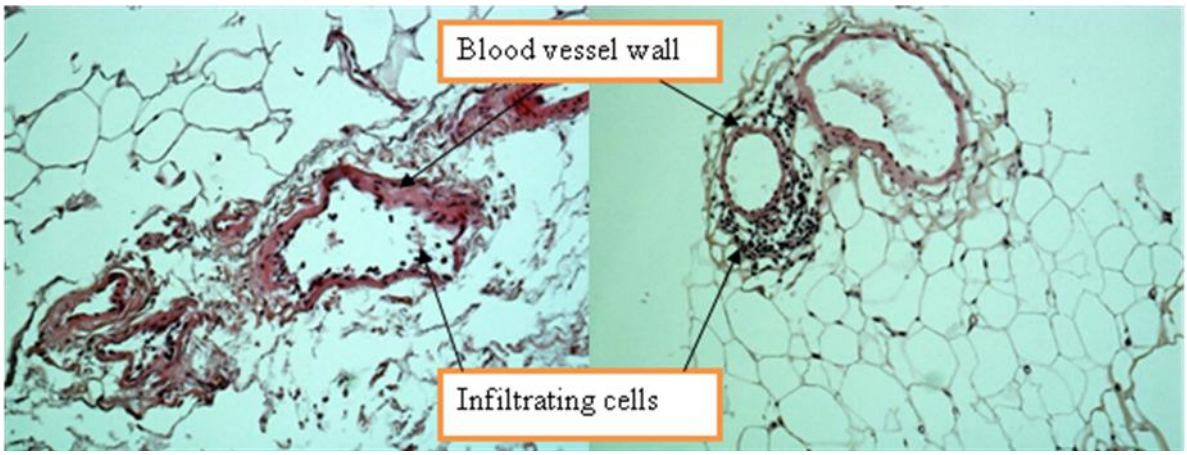


Figure 5.33. Pre- and post surgery adipose tissue biopsy from patient 27A1 stained with HE. Left diagram displaying pre surgery adipose tissue biopsy showing blood vessel wall and infiltrating cells in vessel lumen, indicated by arrows. Right diagram is post surgery showing blood vessel wall and infiltrating cells in the tissue indicated by arrows. Original magnification x 200.

When conducting IHC none of the cells present in the tissue were positive for the selected antibodies to the CD3, CD20 or CD68 markers (results not shown). Original magnification x 200.

Patient 30A1

When observing the HE stained biopsies obtained from patient 30A1 no infiltrates, only intact fat cells, were detected in the pre surgery biopsy, while a small infiltrate was observed in the post surgery biopsy. The post surgery biopsy showed destroyed fat cells (right diagram below).

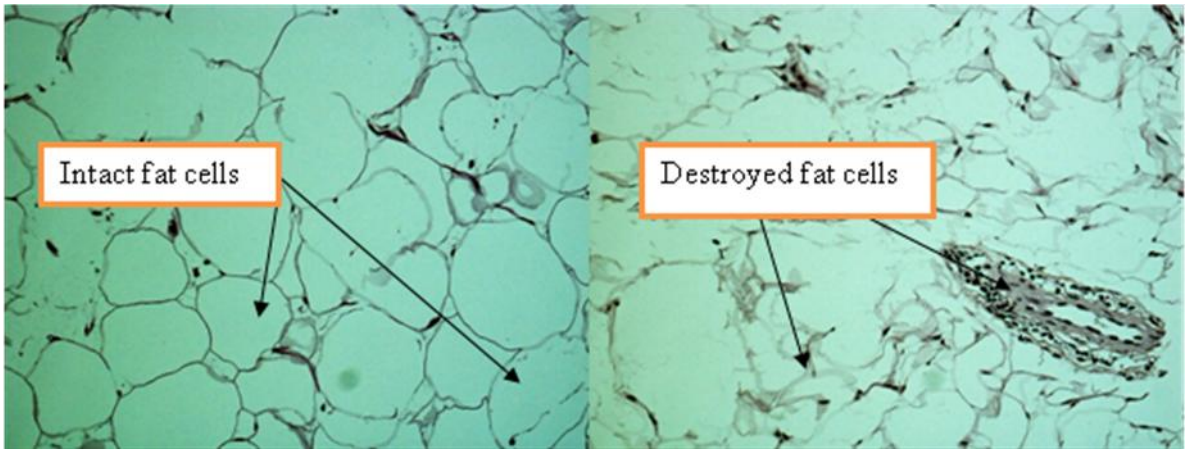


Figure 5.34. Pre and post surgery adipose tissue biopsy from patient 30A1. Left diagram displaying pre surgery adipose tissue biopsy from patient 30A1, stained with HE showing intact fat cells, indicated by arrows.

Right diagram is post surgery showing the small infiltrate, as well as destroyed fat cells, indicated by arrows. Original magnification x 200.

When conducting IHC none of the cells present in the tissue were positive for the selected antibodies to the CD3, CD20 or CD68 markers (results not shown).

Evaluation of the obese patients from HUH

Of the obese patients included in the study, 5 of the 11 patients revealed infiltrating cells and the infiltrates were only seen in the post surgery subcutaneous adipose tissue biopsies.

Based on the magnification used (x 200) it appears that the fat cells in the biopsies from the control persons in general were smaller than the fat cells from the obese patients.

5.5.3 Biopsies from obese patients from OUHA

All biopsies (omental and subcutaneous) obtained from the gastric bypass patients (GB1-GB5) were pre surgery and all were stained by HE and with the complete absence of infiltrates. The pictures below show the subcutaneous pre surgery biopsy of patient GB1 and GB2, and the omental and subcutaneous pre surgery biopsy of patient GB3, GB4 and GB5. The omental biopsy was included since the fractionation method was performed on the same tissue; these biopsies were also negative for infiltration.

IHC were performed on biopsy from patient GB1 and GB2, as expected due to the absence of infiltration, the results for all the antibodies were negative and results are not shown.

HE stained biopsy section of patient GB1 and GB2

When observing the HE stained biopsies from patient GB1 and GB2, it was only found intact fat cells and no infiltrating cells.

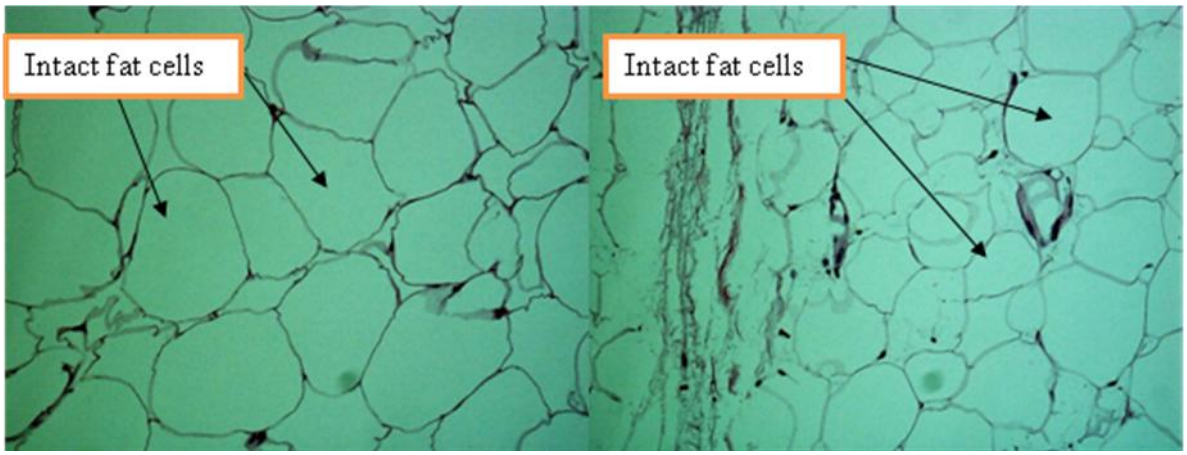


Figure 5.35. Both diagram displaying pre surgery subcutaneous adipose tissue biopsies (left: GB1, right: GB2). Biopsies stained with HE showing intact fat cells, indicated by arrows. Original magnification x 200.

HE stained biopsy section of patient GB3, GB4 and GB5

When observing the HE stained biopsies from patient GB3, GB4 and GB5, it was found intact fat cells in all patients, and a few infiltrating cells in the vessel walls (two of these shown below, indicated by arrows) in patient GB4. IHC was not performed on these tissues due to the observed absence of infiltrates.

Subcutaneous adipose tissue biopsy

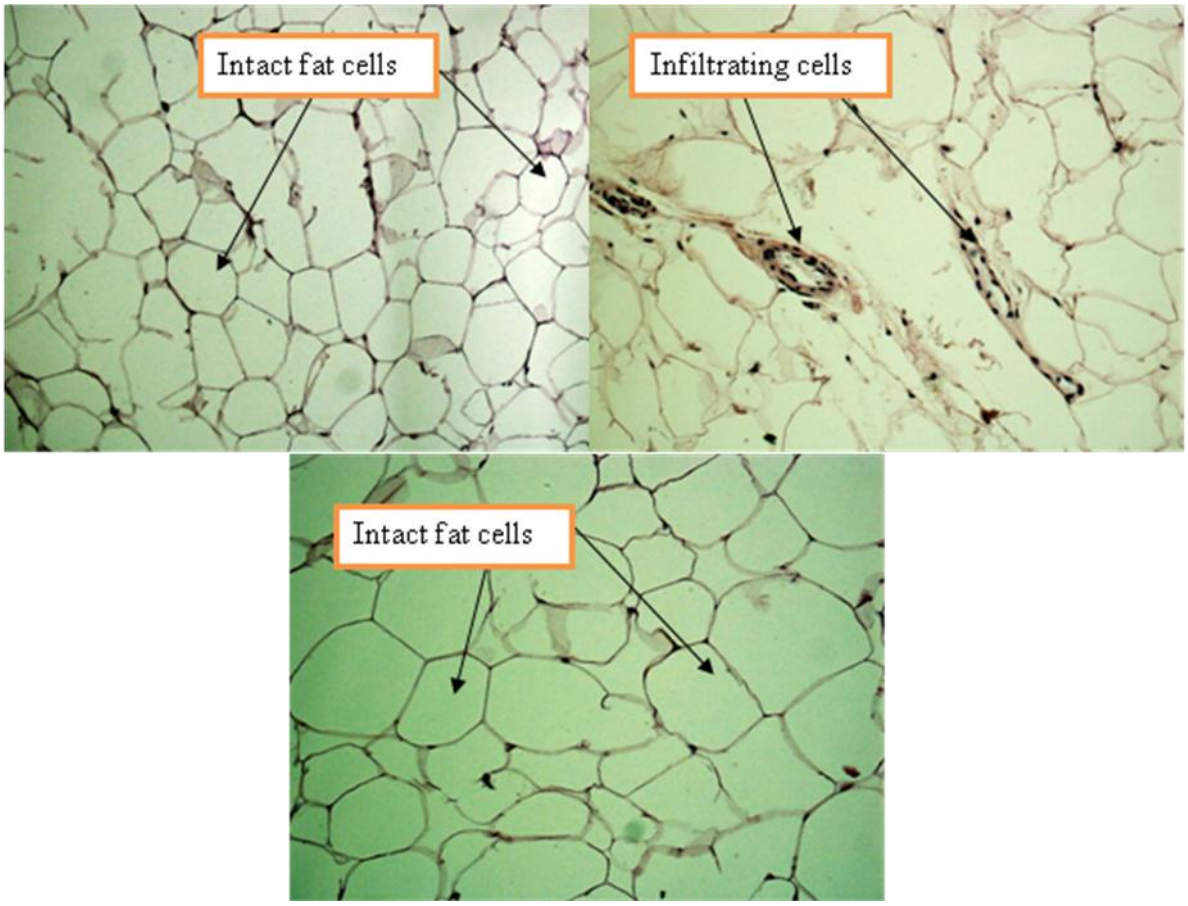


Figure 5.36. All diagrams displaying pre surgery subcutaneous adipose tissue biopsies (left: GB3, right: GB4 and bottom: GB5). Biopsies stained with HE showing intact fat cells, indicated by arrows. GB4 showing some infiltrating cells in two vessel walls. Original magnification x 200.

Omental adipose tissue biopsy

These slides were included since fractionation was performed on adipose tissue from same site.

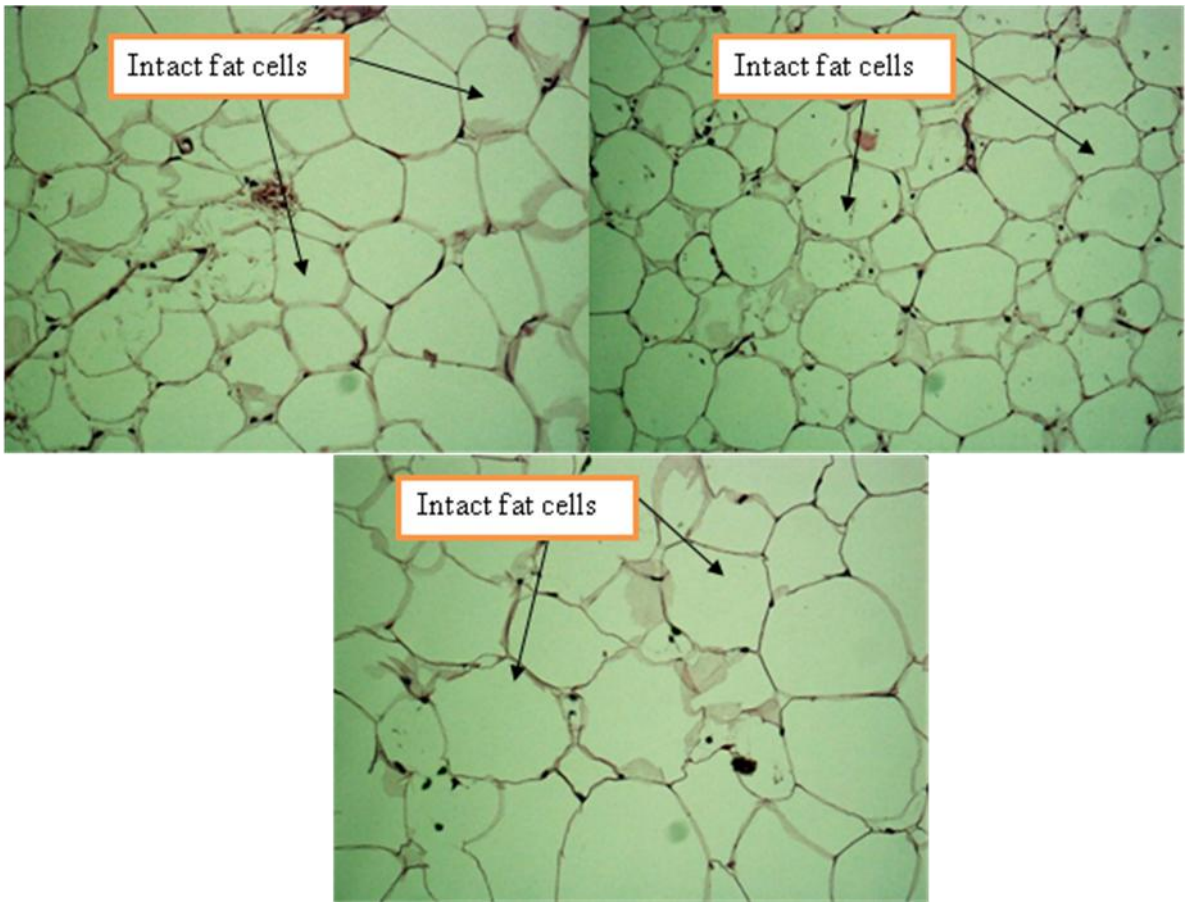


Figure 5.37. All diagrams displaying pre surgery omental adipose tissue biopsies (left: GB3, right: GB4 and bottom: GB5). Biopsies stained with HE showing intact fat cells, indicated by arrows. Original magnification x 200.

Evaluation of the obese patients from OUHA

Adipose tissue biopsies pre surgery was obtained, both subcutaneous and omental. Neither of the biopsies showed any infiltrates or accumulation of infiltrating cells.

6 Discussion

6.1 Patient inclusion of obese patients from HUH and OUHA

6.1.1 Numbers for female/male ratio for bariatric surgery

For the adipose tissue biopsy part of the project, biopsies from 11 obese patients were received from HUH. These patients were eligible for biliopancreatic diversion with duodenal switch surgery. Out of these 11 obese patients, 77.7% were female, and 22.3% of them were male.

When including obese patients at OUHA for the Intrafat pilot study, adipose tissue from 5 patients was collected. Out of these five, four of these patients were females (80%) and one was male (20%).

Relating these percentages to the literature in regards to the abundance of females undergoing bariatric surgery compared to men, it seems that the patient selection from HUH and OUHA based on sex, comply with several findings. Evidence shows that the majority of patients undergoing bariatric surgery are women, contributing to about 80% of all surgeries [39, 57-59].

6.1.2 Blood samples obtained from the obese patients and controls at inclusion

Blood samples from all the patients included at HUH obese (N=11) and the controls (N=3), were obtained.

Unfortunately similar comprehensive blood tests (Intrafat blood analysis) were only obtained from 2 out of the 5 included patients at OUHA. This was due to time restrictions for the staff taking the blood tests for the other 3 patients. The regular blood test was obtained for the 3 remaining patients and results were therefore incomplete in terms of metabolic parameters such as lipids, glucose and insulin values.

Interestingly for some of the patients, glucose levels were normal and the insulin levels were higher, this may indicate insulin resistance. For the obese patients included at OUHA, the blood glucose levels were similar to the obese patients at HUH, as they were considered normal, however one of the two patients who had the specific Intrafat blood analysis done showed dramatically increased insulin level, 85.4 mU/L (ref: <11 mU/L), being same observation seen in the obese patients from HUH. Furthermore, value that was increased for the same patient was Insulin C-peptide, this was 4906 pmol/L (ref: 370-1470 pmol/L). C-peptide is a better indication of pancreatic insulin secretion than the level of insulin itself, reflecting that the β-cells are producing insulin [60]. Despite that these are only speculations based on the fact that many of the obese patients may have been on glucose lowering drugs, e.g. metformin [61]. However, as clinical information was restricted we can only speculate on this.

For the lipid profile, the level of the total cholesterol was elevated for 5 of the 11 obese patients included at HUH, and for 2 of the 3 control persons. For all the obese patients the LDL cholesterol was greatly elevated, a feature that is observed in overweight and obese people, and a strong contributor to the development of atherosclerosis [62, 63].

The lipid values for the patients included at OUHA showed normal values, except from elevated TG values, this also being a contributor to atherosclerosis as explained above.

The CRP values for the obese patients from HUH were significantly higher than for the controls (P= 0.004). It is recognized that overweight and obesity contribute to a low grade inflammation, which develops as fat mass increases [13, 64, 65]. Other pro-inflammatory factors in addition to CRP are also found to be elevated in obesity; these include TNF- α and IL-6 [24, 25, 64, 66]. The CRP values for the patients from OUHA were within the normal range.

Based on the knowledge about elevated CRP values in terms of inflammation and that studies have found CRP and other inflammatory factors to be elevated in obese subjects, the motivation for developing a method in which could investigate infiltrates of immune cells and the detection of other inflammatory markers in obese patients was established.

6.2 Discussion of methods

The consensus from recent research has shown that there is an increase in immune cells that correlates with weight gain and that obese people have a plasma and serum profile similar to those suffering from chronic diseases (Bruun et al. (2005), Lionetti et al. (2008), Curat et al. (2004), Weisberg et al. (2003)). These infiltrating cells, monocytes, macrophages, T- and B-cells have been found in the adipose tissue; in blood vessel walls, lumen of blood vessels and in between the adipocytes. Based on these findings, a fractionation method was established in order to isolate infiltrating cells in adipose tissue biopsies from obese patients and identifying these cells by flow cytometry. Adipose tissue was also investigated by the use of histology methods, HE staining and immunohistochemistry.

6.2.1 Development of the fractionation protocol

In order to determine the identity of the various cell infiltrates of WAT, we addressed this by fractionation. In order to do so we made changes for the optimization of a gravimetric fractionation protocol initially developed by others [55, 56].

Receiving adipose tissue biopsies

When the adipose tissue biopsies were immersed HBSS 5% BSA, the medium was recommended to be at RT, or close to body temperature. Cold medium create extra stress for the body temperate adipose tissue once immersed.

Medium

Phosphate PBS was initially the medium of choice, but was changed to HBSS. HBSS contains glucose, which imitates a more physiological environment and will reduce any additional cellular stress, as well as containing a pH indicator.

In addition to HBSS, M199 and FBS as also suggested as washing media, however this idea was rejected as HBSS was considered sufficient for washing and for incubation based on previous experiments on immune cells isolated from human blood [67].

BSA

The BSA had to be protease-, lipid- and immunoglobulin free in order to maintain optimal conditions of the adipose tissue and to prevent any further post operative triggering of inflammatory cells. Primarily, the BSA concentration for PBS was 0.1%. However based on previous experiments in the lab on immune cells this was modified to 5% in HBSS (Skålhegg personal conversation). The BSA product chosen only contained small amounts of fatty acids (0.01%) and endotoxins (0.3 Eu/mg) and was free of proteases and IgG (none detected).

Enzyme

Initially, it was suggested to dilute the collagenase enzyme in 6 ml PBS 0.1% BSA. The enzyme was added to pure HBSS, as it was experienced that the BSA interfered with the enzyme activity (Dankel personal conversation).

The enzyme used initially was collagenase LB3, an enzyme blend that contained thermolysin. This was later changed to an enzyme mixture that contained dispase, collagenase Liberase 1, which was thought to be more favorable for the tissue. We decided to use LBTM Research Grade (previously called Collagenase LB3), as it turned out that the Collagen Liberase 1 was out of production. The new LBTM contains the same amount of thermolysin as LB3.

Sample weight of the adipose tissue biopsy

The weight of the adipose tissue biopsy was larger than the weight required for the fractionation protocol; therefore while optimizing the protocol, the additional adipose tissue was stored in the refrigerator overnight to repeat the protocol for practice purposes. The weight of tissue used for the fractionation protocol was decided to be approximately 1.0 gram per sample. Parallels were prepared for each adipose tissue biopsy obtained.

The initial weight of the samples was between 0.7-0.8 grams. The sample weight was for some time adjusted to be between 2-3 grams by Simon Dankel as they achieved great cell counts with this weight. This increased tissue weight resulted in a greater amount of HBSS used, with15 ml of HBSS being mixed with 0.5 ml of the enzyme.

This became one of the reasons as to why the final amount of tissue used ended up being 1 gram. Using large amounts of tissue not only required larger amount of buffer, but was also

more time demanding. The final weight of adipose tissue to use for the final protocol was decided to be as close to 1 gram as possible.

Incubation equipment

In addition to the change in tissue weight and amount of HBSS, so was the incubation equipment and time. It was suggested to use a roller-mixer, which due to the amplified mixing movements created greater conditions for the enzyme and the chance of the complete digestion of the matrix fraction. It was experienced that the tissue did not reach a complete digestion when using the roller in RT. Hence, after the roller incubation, the tubes were transferred to the usual incubator cabinet which was set for 37 °C. This enhanced the digestion; however it lengthened the incubation time up to 2 hours. A further suggestion was to put the roller mixer in a incubator cabinet set for 37 °C, this could not be accommodated in Oslo, therefore the idea with the roller mixer was abandoned and the initial incubator cabinet was used again. From this experience it seemed that the enzyme had a better effect when incubated at 37 °C and it seemed that an incubation time of one hour was sufficient for the tissue to be digested, as long as the tissue was well cut up.

Centrifugation

The strength of the centrifugation when centrifuging the SVF solution was conferred. In the initial protocol it was suggested to, in the washing steps, centrifuge at a force of 1500 x g for 10 minutes. The speed was used for blood analysis and was reduced as it was concluded that this force was to powerful for the SV cells and lead to disrupted cells (Dankel personal conversation). Hence, it was concluded that 585 x g for 5 minutes was the most appropriate force and time, and this was kept as speed all through the fractionation protocol.

Matrix

The literature mentions the remains of connective tissue, the matrix, post the collagenase digestion and how this fraction contains amounts of SV cells [26, 68]. It was initially suggested to incubate the undigested matrix together with the filter in the sieved SVF solution once the fat had been removed, and further treat the matrix and the SVF as one. Because the matrix did not completely dissolve, the matrix was not further treated other than mixed with

the HBSS 5% BSA in a separate tube to release the SV cells embedded in the tissue, the supernatant was then counted for WBCs.

6.2.2 The isolation and identification of non-fat cells from adipose tissue

Cell lysis

The original amount of ACT buffer being 1 ml in the initial protocol was considered too much based on the low number of cells. The 200 µl amount was used when the adipose tissue sample was 0.7-0.8 grams and increased according to the tissue weight. When the samples were between 2-3 grams the amount of lysis buffer was about 580 µl. When the weight of the adipose tissue was finalized to be 1.0 gram, it resulted in the amount of cell lysis buffer to be 250 µl.

As for the HBSS 5% BSA volume, it was changed to 800 µl when the adipose tissue sample was 0.7-0.8 grams. When testing with the larger tissue sample, this amount was increased in order to maintain the same conditions between the buffer and the medium. The eppendorph tubes used could fit 1.5 ml, therefore the SVF solution was further divided into two eppendorph tubes in order to accommodate for the required quantity. The decision of having the HBSS 5% BSA amount to be 800 µl was quite random, but seemed to work in relation to the amount of the lysis buffer. The eppendorph tube could fit this amount and one tube was used for the progression of the protocol.

Cell counting

It is known that the total WBC count, as well as the count of TNF-α, IL-6 and hsCRP, in blood tests are indicators of the metabolic syndrome and other inflammatory conditions in obese adults [69, 70]. In this thesis the WBCs were counted in the SVF of the adipose tissue obtained, the normal proportion of the WBCs in the SVF of adipose tissue is unknown. However the proportions of the identified immune cells can reveal information about the condition of the patient.

When optimizing the fractionation method, the adipose tissue obtained from the Pathology Department at Rikshospitalet HF was from deceased patients. The prerequisites given were to

minimize the time between death and autopsy, tissue showing no sign of autolysis and that the adipose tissue was taken from the mesentery, the membrane that attaches the intestines to the abdominal wall. This location is close to where the adipose tissue was collected from the gastric bypass patients. Suggestions of variables that might have affected the adipose tissue upon collection were time delay between death and autopsy, temperature variations, if the deceased had a fever at time of death, if the deceased was recently operated in the abdomen and general cause of death.

The WBC count obtained from the adipose tissue biopsies from the Pathology Department could have been affected by many variables, such as the time of death of the patients, unknown clinical information about the patients, and storing time of the adipose tissue. With the main issue being that the adipose tissue was from deceased patients, the WBC count was not compared to the WBC count from the adipose tissue obtained from the obese patients at OUHA. Since not comparable, the adipose tissue biopsies received from the Pathology Department were rather recognized as indispensable material during the optimization of the fractionation protocol before the adipose tissue from the obese gastric bypass patients from OUHA were obtained.

Flow cytometry

The selected antibodies were against the CD4, CD8, CD14, CD19 and CD56 markers and were all conjugated to the fluorochrome FITC. These were introduced when optimizing the flow cytometry protocol on PBL. Flow cytometry was not performed on the tissue from the Pathology Department at Rikshospitalet HF, but on the SV cells from patient GB3, GB4 and GB5. The antibodies against these markers were chosen due to the broad range of cell identification they provide in a heterogenic cell suspension such as PBL and SV cells.

It was recommended to have between 0.5- 1.0 million WBCs per well, and preferably 12 wells were going to be used (parallels of the 6 antibodies), if sufficient amount of cells. When optimizing the flow cytometry method using PBL there average cell concentration was 7.5 million cells/ml, which was sufficient amount of cells as the total cell suspension volume was 5 ml. This volume made it possible to up concentrate the cells. However, when applying the flow cytometry method on the SV cells from patients GB3, GB4 and GB5, the average cell concentration was 1.2 million cells/ml, and the total cell suspension volume was 0.9 ml in which made it, due to the low cell count, impossible to up concentrate so that sufficient cell

number for the 12 wells was obtained. In result, the cells from patient GB3 were up concentrated as allowed by the volume in order to supply 6 wells (negative control, CD4, CD8, CD14, CD19 and CD56) with cells, although aware that cell volume was insufficient. This was mainly done for practice purposes. For patient GB4, the WBC count only allowed 2 antibodies to be tested (negative control and the antibody for CD4). The CD4 was selected based on suggestions from the literature that T-cells precedes the infiltration of macrophages and initiate macrophage recruitment in mice [71] and that CD4+ cells possibly diminish in high-fat diet fed mice [16]. CD4 also showed more positive cells when conducting flow on the PBL, compared to CD8. For patient GB5, the cell count allowed for negative control, CD4 and CD19 to be tested, basically to compare to the results from patient GB3 and GB4.

When performing flow cytometry on the Ma Sup, the results indicated that there were no infiltrating cells of immunological nature, seeing as the literature suggests that SV cells are embedded in the matrix, it might of interest to develop a method in which is suited for this cell suspension [26]. The Ma Sup for patient GB4 and GB5 did not undergo flow cytometry based on the experiences from patient GB3.

The flow software Cell Quest was taught during optimization and the settings saved from the PBL trials were applied for the SV cells from the adipose tissue. The settings were slightly changed; similar peaks were obtained between the two tissue types, but the scatter plot displayed a dissimilar pattern, indicating the cell population of the SV cells is different to the PBL. Due to limited cell number and the total cell suspension, titration of the antibodies were not conducted, therefore the most suited antibody concentration for the SV cells from adipose tissue was unknown.

Table 6.1. Identified lymphocytes and macrophages in the SV cells from the obese patients from OUHA.

	GB3	GB4	GB5
CD4	16 %	5.8 %	7.2 %
CD8	14.1 %		
CD14	14.8 %		
CD19	12.4 %		7.9 %
CD56	14.6 %		

A complete range of the selected antibodies were applied to the SV cells of patient GB3, whereas the low SV cell count resulted in incomplete antibody identification for patient GB4 and GB5.

Suggested immune cell distribution in WBCs from blood: Granulocytes: 40-60%, Lymphocytes: 20-40% and Macrophages: 2-8% [72]. If lymphocytes are elevated it indicates chronic inflammation ([73] and Helge Scott personal conversation). The total percentage of lymphocytes from patient GB3 from the SV cells was 57%.

Cell counting and flow cytometry for GB1-GB5

The obese patients from OUHA had an average BMI of 49.7 kg/m^2 (classified morbidly obese). Based on the evidence that as fat mass increases so does the adipokines and the chemokines in the blood stream, and hence the inflammatory factors, such as macrophages and leukocytes, it was assumed that the WBC count would be elevated in the SVF solution from these patients [20, 23, 27, 74]. The average number of WBCs obtained from the cell counts was 1.2 million cells / ml, however, the WBC count results could only be compared to other obese patients and to lean healthy controls in order to elucidate whether the obese patients' WBC count was elevated.

The optimization of flow cytometry was conducted on PBL from apparently healthy donors before flow cytometry was performed on SVF solution from adipose tissue collected from obese patients. The detected WBCs in the SVF solution were from adipose tissue, where the distribution of the various cells was unknown. It is therefore inappropriate for the WBCs from the SVF solution to be compared to the normal distribution range of the various cell types found in blood. The result from the flow cytometry for patient GB3 showed that 57% of the cells in the SVF solution were lymphocytes. This percentage could only be used to as a reference when flow cytometry was performed on SVF solution from other obese patients, as well as from healthy persons. Based on the established evidence from the literature that obesity contributes to a systemic low grade chronic inflammation, where the immune cells are increased, it can only be speculated if the obtained amount of lymphocytes from patient GB3 was increased compared to lean subjects.

The results from the cell counting and flow cytometry were not the main drive behind the experiment, but rather optimization and establishment of the methods for the continuation of the project.

6.3 Biopsy slides from the patients from HUH

6.3.1 HE stained biopsies from the obese patients

Upon the microscopic observations, it was experienced that only a few of the adipose tissue biopsies had infiltrates of immune cells, in which all of these were exclusively from the post surgery subcutaneous adipose tissue biopsies. In fact, none of the slides with infiltrating cells were from the pre- surgery subcutaneous or omental biopsies or post surgery omental adipose tissue biopsies. Due to the total size of the adipose tissue of the obese patients, infiltrations might have been unintentionally excluded due to the location of which the biopsy was taken from. The number of infiltrates per patient is unknown and therefore the negative adipose tissue biopsies might have infiltrates on other locations of the tissue. However, the adipose tissue biopsies were obtained from the approximately same place on each of the patients. The negative results might also be due to the immune infiltrates being further inside the biopsy. A serial of sections of all the biopsies were obtained in order to possibly detect infiltrating cells deeper in the biopsy, though this did not affect the results.

Metabolic disorders, such as T2DM, dyslipidemia and HT, improves or resolves as a result of the dramatic weight loss experienced post bariatric surgery [38, 75-77]. This is thought to occur due to the reduction in fat mass, hence the decreased secretion of inflammatory factors such as TNF-α, IL-6, MCP-1, other cytokines and attracted monocytes and macrophages [32, 74, 78, 79]. Reduction in macrophage number found in morbidly obese has also been reported post surgically induced weight loss [27]. Based on these results, it was not expected to find infiltrating cells in the post surgery biopsies, but rather in the pre surgery biopsies.

Findings from the literature suggest that the visceral adipose tissue is more metabolically active than the subcutaneous in terms of the secretion of inflammatory factors (TNF-α, IL-1β, IL-6) by adipose tissue macrophages (ATM) and other cells in the SVF of the adipocytes [19, 20]. Others have shown that there is a greater infiltration rate of monocytes in visceral tissue than in subcutaneous due to the increased release of MCP-1 from this fat depot which is important for the macrophage activation in adipose tissue [18, 75]. Adding on to these indications, Cancello et al. found twice as many macrophages in omental/visceral fat depot as in the subcutaneous fat depot from 55 obese subjects [64]. The biopsies studied in this project that showed infiltrating cells were solely from the subcutaneous adipose tissue, conflicting

with the results mentioned above. Little can be concluded from these observations, however based on this patient material, we suggest that there is some low degree of immune activity post surgery in the subcutaneous adipose tissue.

A study, Cancello et al. showed images of adipocytes and macrophages pre bariatric surgery and post surgery in morbidly obese subjects [27]. The images of macrophages surrounding adipocytes in a CLS pre surgery were thought to be a typical artifact of low chronic inflammation, seeing as no such formations were observed after the surgery and weight loss. They as well as numerous of other studies have showed images of crown-like formation of macrophages surrounding adipocytes before weight loss surgery. These CLSs are thought to be macrophages surrounding adipocytes with features of necrosis, implying that the macrophages exhibit some phagocytic properties. When adipocytes reach a critical size, it supposedly triggers apoptosis, hence the macrophage infiltration. These CLSs have been reported in adipose tissue from both mice and humans [33, 80-82]. These CLSs are referred to as a general structure of macrophage infiltration observed in the adipose tissue pre surgery. What was surprising in this project was that no such structures or similar ones were detected when observing the biopsy sections through the microscope. Not even in the biopsies displaying infiltrates. The total absence of these macrophage formations seen in our sample might imply that these structures are not a significant sign of inflammation, but rather showing one of the functions of the macrophages infiltrating the adipose tissue. By investigating the diagrams of the studies that have detected these formations, the CLS are not abundant in the adipose tissue, but rather scattered around in the tissue.

The post surgery subcutaneous biopsy for patient 28A1 showed the most infiltrating cells. It was different to the other infiltrates observed as there were large fat vacuoles (oil cysts), infiltrating cells and an increase in connective tissue. These observations suggested that the part of the adipose tissue in this patient was necrotic; where the fat vacuoles had engulfed the fat from the destroyed adipocytes, while the empty adipocytes created connective tissue. It was suggested that macrophages were involved in this process (Helge Scott personal conversation). This observation indicated that the adipose tissue is active post surgery; however this could have been brought by an external factor, such as a tight belt, a firm grip or a punch etc.

As mentioned previously, no infiltrations were found in the biopsies from the control patients. The adipose tissue of lean and normal weight people have been estimated to contain about

10% macrophages, whereas obese humans are assumed to have 50% of macrophages in adipocytes [34]. Other biochemical data obtained from the controls were normal; hence the expectation of the complete absence of infiltrating cells in the adipose tissue.

The obese patients which donated the adipose tissue biopsies had a significant weight loss (P= <0.005), as well as significant reduction in the CRP value (P= <0.001) post surgery, indicating, based on findings from the literature, that inflammation is reduced. It was, however, only observed infiltrating cells in the post surgery subcutaneous biopsies. This proposes the idea that other immune factors are involved in the course of the inflammatory process which has not been investigated in this thesis.

6.3.2 Immunohistochemistry of biopsies from the patients

When conducting IHC on the cells present in the infiltrates of the biopsy sections, the aim was to categorize the cells by using the antibodies for T- and B- cells, and for macrophages, CD3, CD20 and CD68 respectively. The antibodies selected were chosen as these immune cells have been detected in morbidly obese subjects in previous studies [22, 27, 83]. Due to the small amounts of cells present in the infiltrates detected, it was appropriate to not use an extensive range of antibodies in order to identify the infiltrating cells. The positive biopsies were sectioned onto super frost glass slides and prepared for IHC. The biopsies of the control patients were also prepared.

When observing the stained sections, it was experienced that the infiltrations were shallow. Some of the infiltrates did not penetrate the 10 consecutive sections prepared for IHC making it impossible to investigate the three antibodies in the same infiltrate, seeing as section 1-3 was for one antibody and so on. Based on the microscopic investigation of the slides, it seemed that more T- and B- cells were present in the infiltrates than CD68 presenting macrophages. Theories from recent research have questioned the possibility that there might be a T- and B-cell stimulation prior to the macrophage infiltration in adipose tissue [16, 31], something that might explain the apparent majority of the T- and B- lymphocytes. However, due to the overall small amount of infiltrating cells, the perceptible abundance of T- and B-cells was not necessarily a correct observation, and a result can only be drawn from this if more biopsies would have been positive for infiltration. In addition to this, the findings were conflicting with the other literature stating a reduction in inflammation post surgery [27, 84].

Although some of the HE stained biopsies were showing infiltrating cells, the markers used in the IHC (antibodies for CD3, CD20 and CD68) turned out not recognizing any of the cells present in the infiltrates for patient 10A1, 27A1 and 30A1, hence IHC did not aid identification of the infiltrating cells. Due to these outcomes, the results were not shown.

It should be noted that there was no difference between the blood test of the patients in which showed an infiltrate after IHC was performed (Patients 4A1 and 28A1) compared to the other patients who did not show any infiltrating cells, the CRP was significantly reduced, as well as insulin and blood glucose level post surgery. What it is that initiates the infiltrations, or at least increase the infiltrating cells when the biochemical parameters obtained from blood tests are close to normalized one year after the surgery can be a range of variables.

Given that the minority of the infiltrating cells were identified, it can be questioned what kind of cells are present in the infiltrates. Granulocytes were not investigated but can be suggested as present cells; however these cells are usually present early in inflammations and are the first cells to be at the site of damage [85, 86]. Based on this it has been discussed whether the possible presence of granulocytes are there due to a recent exterior impact, ex. wearing a tight belt or tight clothes, experienced a recent minor incident creating a punch, or a firm grip in the area where the biopsies are collected from post surgery (Helge Scott personal conversation).

There were no infiltrating cells for the control patients, they appeared as the pre surgery adipose tissue biopsies; only smaller fat cell size was noted as difference.

6.4 Biopsy slides from the patients from OUHA

The biopsies were from pre surgery, and as experienced with the biopsies from HUH, the pre surgery biopsies were free of infiltrating cells. Although several sections were prepared no infiltrates were observed and no staining was positive when performing IHC. These findings support the other findings in the biopsies from HUH, indicating that there seem to be a slight up regulation of unidentified immune cells post surgery, rather than pre surgery, in which creates an inflammatory looking tissue. It will be interesting to observe biopsies from these patients post surgery (one year after surgery), in order to compare if the same is experienced for these patients as for the patients and the post surgery biopsies from HUH.

7 Conclusion

Blood tests collected from the obese patients at HUH showed a significant decrease in CRP values post surgery, results that propose a reduction of inflammatory conditions after significant weight loss.

A method for the fractionation of adipose tissue in order to isolate and to further identify immune cells was optimized in this present study. Enzymatic digestion enabled the separation of fat cells and the SVF, where the cells of interest are embedded. A fraction of these immune cells were identified by performing flow cytometry, with antibodies for T- and B- cells, macrophages and NK-cells. These cells are believed to be involved in the low grade chronic inflammation observed in obese subjects.

From HUH, adipose tissue biopsies were obtained from 11 bariatric surgery patients before and after surgery and 3 control patients. Microscopic observations of HE stained sections, pre surgery adipose biopsies from morbidly obese patients revealed no infiltrates of immune cells. These results were further supported by pre surgery adipose tissue biopsies from 5 patients at OUHA where no infiltrates were found. No infiltrates were found in the control biopsies.

Post surgery subcutaneous adipose tissue biopsies revealed infiltrates of immune cells, with 5 of 11 patient biopsies being positive.

Analysis by IHC concluded that 2 of 5 biopsies were infiltrated with immune cells as judged by the antibodies used.

Modest conclusions can be drawn from these observations, as only 2 of the 11 patients displayed infiltrating cells in which was identified by IHC. However based on the findings that only post surgery biopsies showed infiltrates, although CRP significantly reduced, might suggest an underlying mechanisms in which low grade inflammation is present post surgery.

8 Reference list

1. Cassell, J.A., Social anthropology and nutrition: a different look at obesity in America. J Am Diet Assoc, 1995. **95**(4): p. 424-7.

2. Renzaho, A.M., Fat, rich and beautiful: changing socio-cultural paradigms associated with obesity risk, nutritional status and refugee children from sub-Saharan Africa. Health Place, 2004. **10**(1): p. 105-13.

3. Bluher, M., Adipose tissue dysfunction in obesity. Exp Clin Endocrinol Diabetes, 2009. **117**(6): p. 241-50.

4. Bornstein, S.R., et al., Is the worldwide epidemic of obesity a communicable feature of globalization? Exp Clin Endocrinol Diabetes, 2008. **116 Suppl 1**: p. S30-2.

5. Yach, D., D. Stuckler, and K.D. Brownell, Epidemiologic and economic consequences of the global epidemics of obesity and diabetes. Nat Med, 2006. **12**(1): p. 62-6.

6. Hossain, P., B. Kawar, and M. El Nahas, Obesity and diabetes in the developing world--a growing challenge. N Engl J Med, 2007. **356**(3): p. 213-5.

7. Ogden, C.L., et al., Prevalence of overweight and obesity in the United States, 1999-2004. JAMA, 2006. **295**(13): p. 1549-55.

8. Catenacci, V.A., J.O. Hill, and H.R. Wyatt, The obesity epidemic. Clin Chest Med, 2009. **30**(3): p. 415-44, vii.

9. Sheipe, M., Breaking through obesity with gastric bypass surgery. Nurse Pract, 2006. **31**(10): p. 12-4, 17, 18, 21; quiz 22-3.

10. WHO, Obesity: preventing and managing the global epidemic. Report of a WHO consultation. World Health Organ Tech Rep Ser, 2000. **894**: p. i-xii, 1-253.

11. Gomes, F., et al., [Obesity and coronary artery disease: role of vascular inflammation]. Arq Bras Cardiol, 2010. **94**(2): p. 255-61, 273-9, 260-6.

12. Schaffler, A. and J. Scholmerich, Innate immunity and adipose tissue biology. Trends Immunol, 2010.

13. Trayhurn, P. and I.S. Wood, Signalling role of adipose tissue: adipokines and inflammation in obesity. Biochem Soc Trans, 2005. **33**(Pt 5): p. 1078-81.

14. Astori, G., et al., "In vitro" and multicolor phenotypic characterization of cell subpopulations identified in fresh human adipose tissue stromal vascular fraction and in the derived mesenchymal stem cells. J Transl Med, 2007. **5**: p. 55.

15. Fain, J.N., Release of interleukins and other inflammatory cytokines by human adipose tissue is enhanced in obesity and primarily due to the nonfat cells. Vitam Horm, 2006. **74**: p. 443-77.

16. Nishimura, S., et al., CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. Nat Med, 2009. **15**(8): p. 914-20.

17. Suganami, T. and Y. Ogawa, Adipose tissue macrophages: their role in adipose tissue remodeling. J Leukoc Biol, 2010.

18. Hajer, G.R., T.W. van Haeften, and F.L. Visseren, Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. Eur Heart J, 2008. **29**(24): p. 2959-71.

19. Bays, H.E., et al., Pathogenic potential of adipose tissue and metabolic consequences of adipocyte hypertrophy and increased visceral adiposity. Expert Rev Cardiovasc Ther, 2008. **6**(3): p. 343-68.

20. Cancello, R. and K. Clement, Is obesity an inflammatory illness? Role of low-grade inflammation and macrophage infiltration in human white adipose tissue. BJOG, 2006. **113**(10): p. 1141-7.

21. Curat, C.A., et al., From blood monocytes to adipose tissue-resident macrophages: induction of diapedesis by human mature adipocytes. Diabetes, 2004. **53**(5): p. 1285-92.

22. Wu, H., et al., T-cell accumulation and regulated on activation, normal T cell expressed and secreted upregulation in adipose tissue in obesity. Circulation, 2007. **115**(8): p. 1029-38.

23. Stehno-Bittel, L., Intricacies of fat. Phys Ther, 2008. **88**(11): p. 1265-78.

24. Fain, J.N., S.W. Bahouth, and A.K. Madan, TNFalpha release by the nonfat cells of human adipose tissue. Int J Obes Relat Metab Disord, 2004. **28**(4): p. 616-22.

25. Bastard, J.P., et al., Elevated levels of interleukin 6 are reduced in serum and subcutaneous adipose tissue of obese women after weight loss. J Clin Endocrinol Metab, 2000. **85**(9): p. 3338-42.

26. Fain, J.N., et al., Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. Endocrinology, 2004. **145**(5): p. 2273-82.

27. Cancello, R., et al., Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. Diabetes, 2005. **54**(8): p. 2277-86.

28. Xu, H., et al., Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest, 2003. **112**(12): p. 1821-30.

29. Fuentes, L., T. Roszer, and M. Ricote, Inflammatory mediators and insulin resistance in obesity: role of nuclear receptor signaling in macrophages. Mediators Inflamm, 2010. **2010**: p. 219583.

30. Karalis, K.P., et al., Mechanisms of obesity and related pathology: linking immune responses to metabolic stress. FEBS J, 2009. **276**(20): p. 5747-54.

31. Kintscher, U., et al., T-lymphocyte infiltration in visceral adipose tissue: a primary event in adipose tissue inflammation and the development of obesity-mediated insulin resistance. Arterioscler Thromb Vasc Biol, 2008. **28**(7): p. 1304-10.

32. Lionetti, L., et al., From chronic overnutrition to insulin resistance: the role of fat-storing capacity and inflammation. Nutr Metab Cardiovasc Dis, 2009. **19**(2): p. 146-52.

33. Wentworth, J.M., et al., Pro-inflammatory CD11c+CD206+ adipose tissue macrophages are associated with insulin resistance in human obesity. Diabetes, 2010.

34. Weisberg, S.P., et al., Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest, 2003. **112**(12): p. 1796-808.

35. Pedersen, J.O., et al., [Lifestyle intervention in the treatment of severe obesity]. Ugeskr Laeger, 2006. **168**(2): p. 167-72.

36. Bult, M.J., T. van Dalen, and A.F. Muller, Surgical treatment of obesity. Eur J Endocrinol, 2008. **158**(2): p. 135-45.

37. Monteforte, M.J. and C.M. Turkelson, Bariatric surgery for morbid obesity. Obes Surg, 2000. **10**(5): p. 391-401.

38. Aasheim, E.T., et al., [Surgical treatment of morbid obesity]. Tidsskr Nor Laegeforen, 2007. **127**(1): p. 38-42.

39. Buchwald, H., et al., Bariatric surgery: a systematic review and meta-analysis. JAMA, 2004. **292**(14): p. 1724-37.

40. Sjostrom, L., et al., Lifestyle, diabetes, and cardiovascular risk factors 10 years after bariatric surgery. N Engl J Med, 2004. **351**(26): p. 2683-93.

41. Carlos do Rego Furtado, L., Procedure and outcomes of Roux-en-Y gastric bypass. Br J Nurs, 2010. **19**(5): p. 307-13.

42. Bays, H.E., et al., Adiposopathy and bariatric surgery: is 'sick fat' a surgical disease? Int J Clin Pract, 2009. **63**(9): p. 1285-300.

43. Herpertz, S., et al., Does obesity surgery improve psychosocial functioning? A systematic review. Int J Obes Relat Metab Disord, 2003. **27**(11): p. 1300-14.

44. Karlsson, J., L. Sjostrom, and M. Sullivan, Swedish obese subjects (SOS)--an intervention study of obesity. Two-year follow-up of health-related quality of life (HRQL) and eating behavior after gastric surgery for severe obesity. Int J Obes Relat Metab Disord, 1998. **22**(2): p. 113-26.

45. Goldbacher, E.M. and K.A. Matthews, Are psychological characteristics related to risk of the metabolic syndrome? A review of the literature. Ann Behav Med, 2007. **34**(3): p. 240-52.

46. Kinder, L.S., et al., Depression and the metabolic syndrome in young adults: findings from the Third National Health and Nutrition Examination Survey. Psychosom Med, 2004. **66**(3): p. 316-22.

47. Kinzl, J.F., Morbid obesity: Significance of psychological treatment after bariatric surgery. Eat Weight Disord, 2010.

48. van Hout, G. and G. van Heck, Bariatric psychology, psychological aspects of weight loss surgery. Obes Facts, 2009. **2**(1): p. 10-5.

49. Dankel, S., et al., Switch from stress response to homeobox transcription factors in adipose tissue after profound fat loss. 2009.

50. Biocompare. DAB Immunohistochemistry Substrate from GenWay Biotech, Inc. 2010 [cited 2010 08.06].

51. Boenisch, T., et al. Immunochemical Staining Methods. 2001 [cited 2010 23.05].

52. SchoolofPublicHealth, Dietitian's Pocket Book. 2006: Curtin University of Technology.

53. FürstLaboratory, Fürst Handbook. 2008.

54. NationalInstituteofHealth, NIH conference. Gastrointestinal surgery for severe obesity. Consensus Development Conference Panel. Ann Intern Med, 1991. **115**(12): p. 956-61.

55. Dankel, S., Project description: Induction of Early-response Genes in Adipose Tissue During Stress Exposure and Adipose Tissue 2009.

56. Yang, K., Adipose Tissue Protocols, in Methods in Molecular Biology. 2008, The University of Western Ontario: Ontario.

57. Flum, D.R. and E.P. Dellinger, Impact of gastric bypass operation on survival: a population-based analysis. J Am Coll Surg, 2004. **199**(4): p. 543-51.

58. Smith, F.J., et al., Incidence of bariatric surgery and postoperative outcomes: a population-based analysis in Western Australia. Med J Aust, 2008. **189**(4): p. 198-202.

59. Lee, W.J., et al., Clinical significance of central obesity in laparoscopic bariatric surgery. Obes Surg, 2003. **13**(6): p. 921-5.

60. Van Cauter, E., et al., Estimation of insulin secretion rates from C-peptide levels. Comparison of individual and standard kinetic parameters for C-peptide clearance. Diabetes, 1992. **41**(3): p. 368-77.

61. Becker, M.L., et al., Genetic variation in the multidrug and toxin extrusion 1 transporter protein influences the glucose-lowering effect of metformin in patients with diabetes: a preliminary study. Diabetes, 2009. **58**(3): p. 745-9.

62. Siri, P.W. and R.M. Krauss, Influence of dietary carbohydrate and fat on LDL and HDL particle distributions. Curr Atheroscler Rep, 2005. **7**(6): p. 455-9.

63. Siri-Tarino, P.W., et al., Saturated fat, carbohydrate, and cardiovascular disease. Am J Clin Nutr, 2010. **91**(3): p. 502-9.

64. Cancello, R., et al., Increased infiltration of macrophages in omental adipose tissue is associated with marked hepatic lesions in morbid human obesity. Diabetes, 2006. **55**(6): p. 1554-61.

65. Trayhurn, P. and I.S. Wood, Adipokines: inflammation and the pleiotropic role of white adipose tissue. Br J Nutr, 2004. **92**(3): p. 347-55.

66. Esposito, K., et al., Effect of weight loss and lifestyle changes on vascular inflammatory markers in obese women: a randomized trial. JAMA, 2003. **289**(14): p. 1799-804.

67. Fried, S.K. and N. Moustaid-Moussa, Culture of adipose tissue and isolated adipocytes. Methods Mol Biol, 2001. **155**: p. 197-212.

68. Fain, J.N., et al., Comparison of PGE2, prostacyclin and leptin release by human adipocytes versus explants of adipose tissue in primary culture. Prostaglandins Leukot Essent Fatty Acids, 2002. **67**(6): p. 467-73.

69. Lee, Y.J., et al., Metabolic syndrome and its association with white blood cell count in children and adolescents in Korea: the 2005 Korean National Health and Nutrition Examination Survey. Nutr Metab Cardiovasc Dis, 2010. **20**(3): p. 165-72.

70. Shim, W.S., et al., The association of total and differential white blood cell count with metabolic syndrome in type 2 diabetic patients. Diabetes Res Clin Pract, 2006. **73**(3): p. 284-91.

71. Bouloumie, A., L. Casteilla, and M. Lafontan, Adipose tissue lymphocytes and macrophages in obesity and insulin resistance: makers or markers, and which comes first? Arterioscler Thromb Vasc Biol, 2008. **28**(7): p. 1211-3.

72. Bagby, G., Leukopenia and leukocytosis. 23 ed. 2007: Saunders Elsevier.

73. Ferrero-Miliani, L., et al., Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1beta generation. Clin Exp Immunol, 2007. **147**(2): p. 227-35.

74. Bruun, J.M., et al., Diet and exercise reduce low-grade inflammation and macrophage infiltration in adipose tissue but not in skeletal muscle in severely obese subjects. Am J Physiol Endocrinol Metab, 2006. **290**(5): p. E961-7.

75. Compher, C. and K.O. Badellino, Obesity and inflammation: lessons from bariatric surgery. JPEN J Parenter Enteral Nutr, 2008. **32**(6): p. 645-7.

76. Frezza, E.E., C. Wei, and M.S. Wachtel, Is surgery the next answer to treat obesity-related hypertension? J Clin Hypertens (Greenwich), 2009. **11**(5): p. 284-8.

77. Salameh, J.R., Bariatric surgery: past and present. Am J Med Sci, 2006. **331**(4): p. 194-200.

78. Clement, K., et al., Weight loss regulates inflammation-related genes in white adipose tissue of obese subjects. FASEB J, 2004. **18**(14): p. 1657-69.

79. Forsythe, L.K., J.M. Wallace, and M.B. Livingstone, Obesity and inflammation: the effects of weight loss. Nutr Res Rev, 2008. **21**(2): p. 117-33.

80. Shaul, M.E., et al., Dynamic, M2-like remodeling phenotypes of CD11c+ adipose tissue macrophages during high-fat diet--induced obesity in mice. Diabetes, 2010. **59**(5): p. 1171-81.

81. van Eijk, M., et al., Reducing glycosphingolipid content in adipose tissue of obese mice restores insulin sensitivity, adipogenesis and reduces inflammation. PLoS One, 2009. **4**(3): p. e4723.

82. West, M., Dead adipocytes and metabolic dysfunction: recent progress. Curr Opin Endocrinol Diabetes Obes, 2009. **16**(2): p. 178-82.

83. Cottam, D.R., et al., Dysfunctional immune-privilege in morbid obesity: implications and effect of gastric bypass surgery. Obes Surg, 2003. **13**(1): p. 49-57.

84. Belza, A., et al., Effect of diet-induced energy deficit and body fat reduction on high-sensitive CRP and other inflammatory markers in obese subjects. Int J Obes (Lond), 2009. **33**(4): p. 456-64.

85. Palmblad, J., The role of granulocytes in inflammation. Scand J Rheumatol, 1984. **13**(2): p. 163-72.

86. Yang, D., et al., Alarmins link neutrophils and dendritic cells. Trends Immunol, 2009. **30**(11): p. 531-7.

9 Appendix

Appendix 1 Intrafat blood analysis requisition

Akershus Universitetssykehus HF
Sentrallaboratoriet Sinsen tlf. 22 89 44 42
Ernæringslaboratoriet Sinsen tlf. 22 89 49 66
Klinisk kjemisk avd. og blodbank Ski tlf. 64 85 65 31

605764

001

Analyseprofil: **Intrafat**

Rekvirent ID:

Rekvirent: **JONKRI**
Jon Kristinsson 2158 655

HPR-nr.

Kopi av svar sendes til (legesenter/kontor, lege)

INTRAFAT
Hb,MCV,Rø,Trc,Hv,Diff,Ca
Kreat,Urat,Bil,Kol, TG, HDL, LDL, Alb
Na,K,CRP,ASAT,ALAT,ALP,GT,Glu, HbA1C, HbA1C
Ferri,HormS,Screen,xgl-EDTA, xgl-se-gel
Glass du trenger: 3x4 ml EDTA,2x6 ml EDTA
3x5 ml se-gelrør,1x4ml Li-hep-gel

☐ Ø-hjelpsvar ☐ Mistanke om blodsmitte

Pasient
Ekstern-ID:
Fødselsdato Personnr. Kvinne Mann
Pasientnavn
Adresse
Postnr. Poststed

Betales av:
☐ Trygdekontor
☐ Institusjon
☐ Bedriftshelsetj.

Prøvetaker:
Signatur

Prøvetakingsdato:
D D M M Å Å
Kl.slett: T T M M

Hematologi, HbA1C, Genetiske analyser [1]

☐ HB	☐ Reticulocytter	☐ Kuldeag.
☐ Hct	☐ Eosinofile	☐
☐ MCV	☐ Maskindiff	☐ HbA1C
☐ Røde	☐ Utstryk	☐
☐ Hvite	☐ MCH	☐ FaktorV Leiden
☐ Trombocytter	☐ MCHC	☐ ProtrombinG20210A

Brett her

Hemostase [2]

☐ INR	☐ D-dimer	☐ Protein S-akt.
☐ Cephotest	☐ Antitrombin	☐ Heparin/Lavmol
☐ Fibrinogen	☐ Protein C	☐ APC resistens

Klinisk kjemi, Vitaminer, Proteiner, Medikament, Tumormarkører, Hormoner [3]

☐ Na	☐ Kreatinin	☐ Elektroforese
☐ K	☐ Urinstoff	☐ Haptoglobin
☐ Kalsium	☐ Urinsyre	☐ IgG
☐ Ion. kalsium [4]	☐ Jern	☐ IgA
☐ Fosfat	☐ Ferritin	☐ IgM
☐ Magnesium	☐ Transferrin	☐
☐ CRP	☐ Transf. met.	☐
☐ Glukose	☐ Kolesterol	☐ Digitoksin
☐ Tot. prot.	☐ HDL-kol.	☐ Paracetamol
☐ Albumin	☐ LDL-kol.	☐ Etanol
☐ ASAT	☐ Triglycerid	☐
☐ ALAT	☐	☐ PSA-tot.
☐ Gamma GT	☐	☐ PSA-fritt [6]
☐ Alk.fosfatase	☐ Osmolalitet	☐ CEA
☐ Bilirubin	☐ Monospot	☐
☐ Pan.amylase	☐	☐ TSH
☐ LD	☐ Vit. B12	☐ FT4
☐ CK	☐ Folinsyre	☐
☐ CKMB	☐	☐
☐ TnT	☐	☐

Ernæringsprøver
Se rettledning for prøvetaking og forsendelse på baksiden.

☐ Thiamin (B1) [1]	☐ Vitamin C [8]
☐ Vitamin B6 [8]	☐ S-Karnitin [8]
☐ Vitamin A [8]	☐ U-Karnitin [5]
☐ Vitamin E [8]	☐

Belastninger/Pusteprøver/Timeavtale
Sinsen tlf. 22 89 48 81 Bygg 8 / Ski tlf. 64 85 65 31

☐ Glukose belastning	☐ 13 C-xylose
☐ Laktose absorbsjon	☐ 13 C-MTG
☐ Glukose absorbsjon	☐
☐ 13 C-Urea	☐

Analyser i URIN [5]

☐ Urinstix	☐ Kreatinin	☐ D-Xylose
☐ Na	☐ Kreat.clearance	☐ Heller
☐ K	☐ Urinsyre	☐ Glukose
☐ Kalsium	☐ Citrat	☐ Pan. amylase
☐ Magnesium	☐ Albumin-Kreat-ratio	☐
☐ Fosfat	☐ Totprot-Kreat-ratio	☐
☐ Totalprotein	☐ Mikroalbumin	☐
☐ Karbamid	☐ Elektroforese	☐

Samleurin:
Fra: _____
Til: _____
Diurese: _____ ml

Avføring:
☐ Elastase
☐ Blod

Andre undersøkelser:
☐ Homocystein [1,7]
☐ Venesectio
☐

Forklaring:
1: Rør m/EDTA, lilla
2: Rør m/Na-citrat, blå
3: Rør m/gel, gul
4: Ikke ta av korken
5: Urin
6: Avpip. og fryses innen 3t
7: På isvann. Avpip. innen 1t
8: Rør u/tilsetn.

For laboratoriet:
☐ EDTA ☐ Urin
☐ Citrat ☐ Avføring
☐ Gelrør ☐ Annet
☐ Serum
Sign.

Wittusen & Jensen®

Aker universitetssykehus HF
Hormonlaboratoriet

FOR LABORATORIET

Pasient

Ref. nr. (for elektroniske svar)

Fødselsdato | Personnr. (må oppgis) | Kvinne ☐ | Mann ☐

Navn

Adresse

Postnr. | Betales av (må oppgis)

Kommunenr. | ☐ Inst. ☐ Trygdekontor

Relevante opplysninger

Hormonlab rutineanalyser (skal registreres)
1 x 5 ml gelglass til insulin, C-peptid, 25-OH-D

Rekvirent (fullstendige adresseopplysninger må oppgis)

Jon Kristinsson
Ekspedisjonen, Kirurgisk klinikk
Oslo universitetssykehus, Aker
Trondheimsveien 235, 0514 Oslo

Legens hpr.nr 2158655

Rekvirentkode Intrafat

Forskningsseksjonen Hormonlab (skal til forskning)
2 x 5 ml SST-glass → ring 94744
2 x 6 ml EDTA
1 x 25 ml Urinprøve Intrafat-prosjekt

Dato og kl.slett for prøvetaking Sign

Fastende ☒

Siste menstruasjon første dag

Gravid ☐

Supplerende analyser ønskes Ja ☐ Nei ☐

Informasjon til prøvetaker* - se baksiden **Utredningsforslag - se baksiden**

BLOD

Tyreoidea

Intrafat

- ☐ TSH
- ☐ FT_4, Fritt tyroksin
- ☐ T_4, Tyroksin
- ☐ FT_3, Fritt trijodtyronin
- ☐ T_3, Trijodtyronin
- ☐ TBG, Tyroksinbindende globulin
- ☐ Anti-TPO
- ☐ Anti-Tg
- ☐ TRAS, TSH-reseptor antistoff
- ☐ rT_3, revers trijodtyronin

Graviditet

- ☐ hCG

Gonader

- ☐ FSH
- ☐ LH
- ☐ Prolaktin
- ☐ Østradiol
- ☐ Progesteron
- ☐ Testosteron
- ☐ DHT, Dihyd
- ☐ Androst
- ☐ SHB
- ☐

Binyrer

- ☐ Kortisol kl 07-09
- ☐ Kortisol kl 18-21
- ☐ Kortisol kl.....
- ☐ ACTH* kl 07-09
- ☐ ACTH* kl 18-21
- ☐ ACTH* kl.....
- ☐ Kortisol i spytt* kl 07
- ☐ Kortisol i spytt* k
- ☐ Kortisol i spyt
- ☐ CBG, Kor
- ☐ 17-OH
- ☐ 17
- ☐

Ann

☐ sf.

ml

Samletidsrom:

- ☐ Fritt kortisol*
- ☐ Aldosteron*
- ☐ Jod*
- ☐ DPYD, Deoksipyridinum tverrbind.
- ☐ NTx, N-term. telopeptid type 1 kollagen
- ☐ Anabol-androgene steroider*, 50 ml
- ☐ Medikamentanalyse*(**ønske understrekes**) Beta-blokkere, diuretika, narkotika, stimulantia, 50 ml

Katekolaminer

- ☐ Adrenalin*
- ☐ Noradrenalin*
- ☐ VMA, Vanilinmandelsyre*

stende

eprotein 1

- ☐ 25-
- ☐ 1,25-(
- ☐ DBP, V
- ☐ Osteokals
- ☐ ICTP, C-term
- ☐ Alk.fosfatase (

agen

sulin stimulert

- ☐ C-peptid stimulert
- ☐ Proinsulin stimulert
- ☐ Anti-GAD
- ☐ Anti-IA2
- ☐ Anti-insulin

Aker universitetssykehus HF
Hormonlaboratoriet
Adresse: Trondheimsveien 235 0514 OSLO
Telefon: 22 89 47 08
Telefax: 22 15 87 96
Internett: www.hormonlaboratoriet.no

SE BAKSIDEN FOR PRAKTISKE OPPLYSNINGER HELSE SØR-ØST

Aker universitetssykehus HF
Hormonlaboratoriet

FOR LABORATORIET

Pasient

Ref. nr. (for elektroniske svar)

Fødselsdato | Personnr. (må oppgis) | Kvinne ☐ | Mann ☐

Navn

Adresse

Postnr. | Betales av (må oppgis)

Kommunenr. | ☐ Inst. ☐ Trygdekontor

Relevante opplysninger

Hormonlab rutineanalyser (skal registreres)
1 x 5 ml gelglass til insulin, C-peptid, 25-OH-D

Kommentar ønskes ved patologiske prøvesvar ☐

Rekvirent (fullstendige adresseopplysninger må oppgis)

Legens hpr.nr 2158655

Jon Kristinsson
Ekspedisjonen, Kirurgisk klinikk
Oslo universietssykehus, Aker
Trondheimsveien 235, 0514 Oslo

Rekvirentkode Intrafat

Forskningsseksjonen Hormonlab **(skal til forskning)**
2 x 5 ml SST-glass → ring 94744
2 x 6 ml EDTA
1 x 25 ml Urinprøve Intrafat-prosjekt

Siste menstruasjon første dag | Gravid ☐

Dato og kl.slett for prøvetaking Sign

Fastende ☒

Supplerende analyser ønskes Ja ☐ Nei ☐

Informasjon til prøvetaker* - se baksiden **Utredningsforslag** - se baksiden

BLOD

Intrafat

Tyreoidea
- ☐ TSH
- ☐ FT_4, Fritt tyroksin
- ☐ T_4, Tyroksin
- ☐ FT_3, Fritt trijodtyronin
- ☐ T_3, Trijodtyronin
- ☐ TBG, Tyroksinbindende globulin
- ☐ Anti-TPO
- ☐ Anti-Tg
- ☐ TRAS, TSH-reseptor antistoff
- ☐ rT_3, revers trijodtyronin

Graviditet
- ☐ hCG

Gonader
- ☐ FSH
- ☐ LH
- ☐ Prolaktin
- ☐ Østradiol
- ☐ Progesteron
- ☐ Testosteron
- ☐ DHT, Dihydrotestosteron 2 ml
- ☐ Androstendion 1 ml
- ☐ SHBG, Seksualhormonbindende globulin
- ☐ FTI, Fri testosteron-indeks 1 ml
  (FTI = testosteron x 10/SHBG)
- ☐ Anti-ovaricantistoffer
- ☐ Inhibin A
- ☐ Inhibin B

Kalsium og beinvev
- ☐ PTH*
- ☐ Ionisert kalsium*
- ☒ 25-OH-Vit. D
- ☐ 1,25-$(OH)_2$-Vit. D 1,5 ml
- ☐ DBP, Vit. D-bindende protein
- ☐ Osteokalsin*
- ☐ ICTP, C-term. telopeptid type 1 kollagen
- ☐ Alk.fosfatase (beinspesifikk)

Binyrer
- ☐ Kortisol kl 07-09
- ☐ Kortisol kl 18-21
- ☐ Kortisol kl.....
- ☐ ACTH* kl 07-09
- ☐ ACTH* kl 18-21
- ☐ ACTH* kl.....
- ☐ Kortisol i spytt* kl 07-09
- ☐ Kortisol i spytt* kl 21-23
- ☐ Kortisol i spytt* kl.....
- ☐ CBG, Kortikosteroidbindende globulin
- ☐ 17-OH-Progesteron
- ☐ 17-OH-Pregnenolon
- ☐ DHEA-Sulfat
- ☐ DHEA, Dehydroepiandrosteron
- ☐ 11-Deoksikortisol
- ☐ DOC, Deoksikortikosteron 4,25 ml
- ☐ Aldosteron 1 ml
- ☐ Reninaktivitet* 2 ml
- ☐ Metanefrin og Normetanefrin* 3 ml
- ☐ Anti-21-hydroksylase

Vekst og metabolisme
- ☐ Veksthormon
- ☐ IGF-1*
- ☐ IGF-bindeprotein 3

Diabetes
- ☒ Insulin fastende
- ☒ C-peptid fastende
- ☐ Proinsulin fastende
- ☐ IGF-bindeprotein 1

- ☐ Insulin stimulert
- ☐ C-peptid stimulert
- ☐ Proinsulin stimulert
- ☐ Anti-GAD
- ☐ Anti-IA2
- ☐ Anti-insulin

Annet
- ☐ % CDT, Karbohydrat deficient transf.
- ☐ EPO, Erytropoietin
- ☐ Leptin
- ☐ LTFR, Løslig transferrinreseptor
- ☐ ADH, Antidiuretisk hormon* 2,25 ml
- ☐ Glukagon*
- ☐ PTHrP, PTH-relatert peptid* 1 ml
- ☐ HFE, "Hemokromatose-mutasjon"* 3 ml
- ☐ Laktoseintoleranse gentest* 3 ml

Andre analyser - kun etter avtale
- ☐ Andre genetiske analyser* 5 ml
- ☐
- ☐

URIN

Diurese ml

Samletidsrom:

- ☐ Fritt kortisol*
- ☐ Aldosteron*
- ☐ Jod*
- ☐ DPYD, Deoksipyridinum tverrbind.
- ☐ NTx, N-term. telopeptid type 1 kollagen
- ☐ Anabol-androgene steroider*, 50 ml
- ☐ Medikamentanalyse*(**ønske understrekes**)
  Beta-blokkere, diuretika, narkotika, stimulantia, 50 ml

Katekolaminer
- ☐ Adrenalin*
- ☐ Noradrenalin*
- ☐ VMA, Vanilinmandelsyre*

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