Effects of Dietary fatty acids on Neutrophil frequency and distribution

Master thesis in Medicine

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1. Abstract

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Introduction: Obesity is an epidemic in our time and pre-disposes to lifestyle diseases which in its turn is associated with an increased susceptibility to infections. Polyunsaturated fatty acids have been shown to decrease mortality in experimentally induced sepsis in mice. Neutrophils are the first leukocytes recruited to inflammatory sites and important for defending the body against pathogens.

Aims: In this study, the aim is to investigate the frequency of the neutrophils in the circulating pool (blood) and the frequency and distribution of neutrophils in the marginated pool which consists of bone marrow, spleen and liver.

Methods: C57BL/6J mice were randomly assigned to Low-fat diet (LFD), High-fat diet, rich in saturated fatty acids (HFD-S) or High-fat diet, rich in polyunsaturated fatty acids (HFD-P). After eight weeks of dieting the mice were sacrificed and samples were collected for analysis. Blood was examined via an automated cellcounter. Samples of spleen and bone marrow were analyzed with flow cytometry for neutrophil markers and markers for egression and retention, respectively. Spleen and liver were examined by immunohistochemistry for neutrophils.

Results: Mice fed HFD-P have increased frequency of neutrophils in both bone marrow and spleen compared to mice fed LFD and HFD-S. Neutrophils in bone marrow have decreased expression of CXCR4 (receptor for retention) in mice fed HFD-P compared to LFD, suggesting they are more easily recruited from the bone marrow. Hematologic analysis showed no increase in circulating neutrophils for mice fed HFD-P. Immunohistochemistry showed localization of neutrophils in the red pulp, predominantly in proximity of the marginal zone, but not in the white pulp. In liver the neutrophils are aligned close to the liver sinusoids.

Conclusions: This study confirms that HFD-P increases the frequency of neutrophils in bone marrow and spleen. Further, HFD-P decreases the frequency of CXCR4, suggesting the

neutrophils in those mice are more easily recruited to the circulation when needed, for example during infections.

Key words: Neutrophils, polyunsaturated fatty acids

2. Abbreviations and Frequently Used Words

CXCR2   Chemokine receptor 2, receptor for egression from bone marrow

CXCR4   Chemokine receptor 4, receptor for retention in bone marrow

HFD-P   High-fat diet rich in polyunsaturated fatty acids

HFD-S   High-fat diet rich in saturated fatty acids

LFD   Low-fat diet

NRS   Normal rabbit serum

PBS   Phosphate Buffered Saline

PUFA   Polyunsaturated fatty-acids

ROI   Region of interest

3. Background

3.1 Fatty acids and infection

Obesity is an epidemic in our time, and it is a well-known risk factor for several conditions. In addition, obesity increases the susceptibility for infections (1). The mechanism behind this relation has not been explained yet, but it has been proposed that this is due to the increased co-morbidity with diabetes mellitus in obese patients which in its turn is associated with an increased susceptibility to infections. This have been suggested to be due to amongst others damaged polymorphonuclear leukocytes, decreased levels of complement factor 4 and microangiopathy which impairs the ability to migrate to the tissues etc. (2). Although, recent studies have shown that mice fed high fat diet rich in saturated fatty acids (HFD-S) have an increased mortality rate when induced to a septic state by infection with *Stapylococcus Aureus* (*S. Aureus*) compared to mice fed a low-fat diet (LFD) (3). It has been proposed that saturated fatty acids impair the immune response (4). In line with this, studies have shown that it is not specifically the high fat diet that makes the mice less resistant to infections, but the composition of fatty-acids which alters the immune response, e.g. mice which instead were fed a high-fat diet rich in polyunsaturated fatty-acids (HFD-P) have increased survival compared with mice fed HFD-S (4).Thus, dietary fatty acid composition is important for immune function, however, the precise mechanisms behind this improved survival are still largely unknown.

3.2 The immune system

The immune system is composed of several different components including: white blood cells (granulocytes, including neutrophils, eosinophils and basophils, macrophages, T-cells and B-cells etc.), lymphoid tissues (bone marrow, thymus, spleen, lymph nodes etc.) and the circulatory system. Lymphoid tissues are defined as tissues where lymphocytes are found. Classically the lymphoid tissues are classified as either primary or secondary lymphoid tissues. Primary lymphoid tissues consist of the bone marrow, where hematopoiesis takes place in the adult human, and the thymus where T cells proliferate and mature into complete T cells. Secondary lymphoid tissues consist of mucosa-associated lymphoid tissues, skin-associated lymphoid tissues and lymph nodes. The skin- and mucosa-associated lymphoid tissues functions are to fight pathogens which have passed the first protection barriers of the

body. The lymph nodes are stations where the lymphatic fluid passes through and when doing so is examined by immune cells for pathogens. In the lymph nodes there are large numbers of resting B cells and macrophages amongst others (5). In addition to these tissues, the liver can also be thought of as a lymphoid organ due to its content of natural killer T cells (NK-cells) and Kupffer cells, a subset of macrophages (6). Furthermore, the liver has a crucial role in the acute inflammation, by altering the production of plasma proteins in the acute phase response. The liver has receptors for the cytokines IL-1, TNF and IL-6. When activated the liver amongst others decreases its production of Albumin and instead increases the production of C-reactive protein, serum amyloid A and fibrinogen. The effect of this is to fend of infections, eliminate components from damaged tissues and protect the body against inflammatory processes(7).

The blood cells consist of red blood cells (transportation of oxygen) and white blood cells (immunological active, also called leukocytes). The white blood cells can be divided according to the developmental pathway. The myeloid pathway gives rise to neutrophils, basophils, eosinophils, monocytes and macrophages. The first three are granulocytes (have intracellular granules). The lymphoid pathway gives rise to T and B lymphocytes and their effector cells.
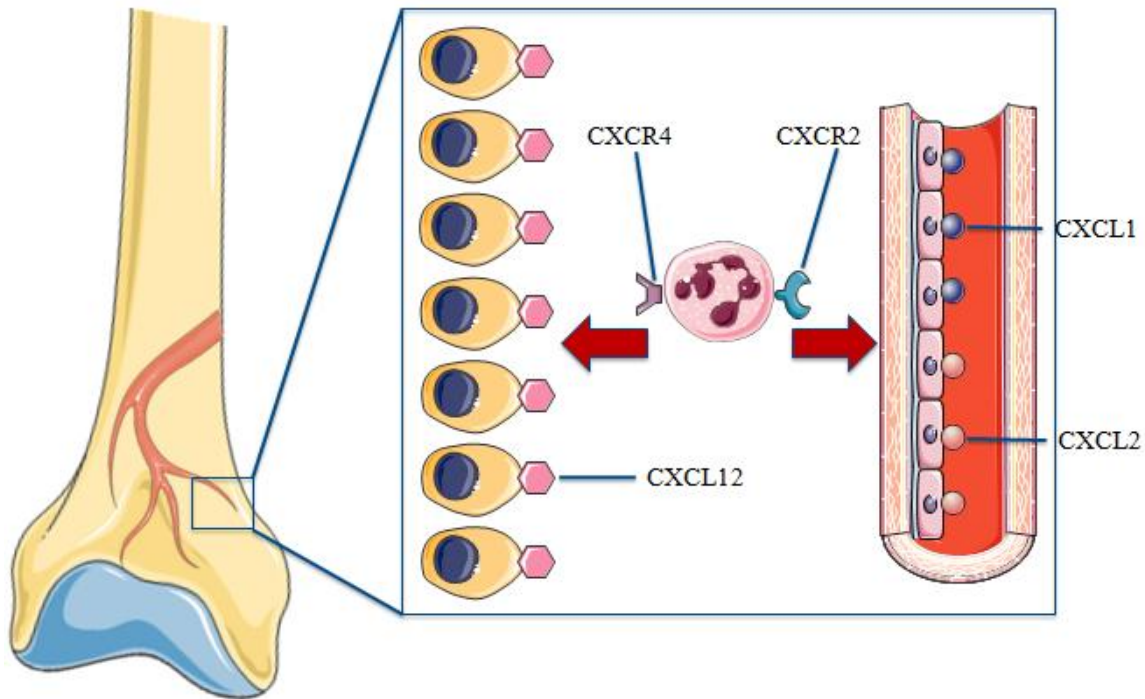
3.3 Neutrophils

The most common leukocyte in the body are neutrophils (5) and they are the first leukocyte recruited to an inflammatory site. Their role is to defend the body from a wide range of infectious pathogens. Lack of neutrophils, for example in chemotherapy-induced neutropenia, might be fatal if the patient develops febrile neutropenia which has a mortality rate of 9.5% (8). Neutrophils are continuously generated in the bone marrow and the daily production can reach up to 10×10^{10}cells/day in humans. The production is regulated by granulocyte colony stimulating factor (G-CSF) which is secreted in response to interleukin-17A (IL-17A) which is secreted by T cells. The high production rate is needed due the short circulating half time of 6-8 h for neutrophils (9). The neutrophils in the body are distributed in two populations based on where they resides: the circulating pool which are the neutrophils in the blood and

the marginated pool which are the neutrophils with prolonged transit in specific organs. The marginated pool can be found in the spleen, liver, bone marrow and the lung (9).

Neutrophils can eliminate pathogens with several different procedures including phagocytose and release of antibacterial proteins or directly with antimicrobial proteases. The quantity of circulating neutrophils differs between humans and mice. In humans they constitute 50-70% of circulating leukocytes whereas only 10-25% in mice (10). The balance between production in bone marrow and clearance from the circulation are tightly regulated to maintain the neutrophil homeostasis. To keep this balance it is important to regulate the egression and retention in bone marrow. This is regulated by five important molecules: CXCR2 (receptor for egression), its ligands CXCL1 and CXCL2, CXCR4 (receptor for retention) and its ligand CXCL12 (11). CXCL1, CXCL2 and CXCL12 are constitutively expressed and released by stromal cells in bone marrow, but CXCL1 and CXCL2 are predominantly released by endothelial cells whereas CXCL12 predominantly is released by osteoblasts (12). This is functional as CXCL1 and CXCL2 will make the neutrophils travel towards the vasculature where endothelial cells are in close proximity. CXCL12 will have the opposite effect, making the neutrophils remain in the bone marrow where there are osteoblasts. During infections the cytokines IL-1 and TNF will be released to the circulation. These cytokines will bind to fibroblasts in the bone marrow which will produce G-CSF (granulocyte-colony stimulating factor) which increases the production of neutrophils (7). This function is mediated amongst others by increase of CXCL2 expression and suppression of osteoblasts.

Figure 1. Showing the neutrophil movement by chemotaxis. CXCR2 makes the neutrophils more prone to egression and CXCR4 makes the neutrophils more prone to retention in the bone marrow. Ligands for CXCR2 are CXCL1 and CXCL2, whereas CXCL12 is the ligand for CXCR4. Endothelial cells in the blood vessels running through the bone predominantly release CXCL1 and CXCL2, whereas osteoblasts release CXCL12. The chemokine-gradient causes a "tug-of-war" situation between the neutrophilreceptors for the neutrophils localization. Adapted from *Eash et al (12)* with images from *Servier Medical Art*.

CXCR4 is also expressed on senescent neutrophils which migrate back to the bone marrow for degradation. The upregulation of CXCR4 has been proposed to stimulate granulopoiesis and subsequently, maintain the balance between clearance and production of neutrophils (9).

3.4 The spleen

The spleen is an ovoid organ in the left hypochondrium of the abdomen. It's protected by the inferior thoracic cage, but nonetheless is considered the most vulnerable abdominal organ (13). It is the largest lymphoid organ in the body and is therefore important for the immune

response, predominantly so by initiating response to blood-borne antigens (14). One great difference between the spleen and other lymphoid organs is that it has no connection to the lymphatic systems and is instead connected to the systemic circulation via the splenic artery. Prenatally it functions as one of the blood-forming organs, but post-partum the bone marrow takes over this function and instead the spleen is involved in identifying, removing and destroying aged and abnormal red blood cells and platelets (13). It is an immunologic organ which amongst others is more efficient at removing non-opsonized bacteria than the liver. This is demonstrated by the increased risk for infections by encapsulated bacteria for patients who have underwent splenectomy (15). In addition it functions as storage for iron, erythrocytes and platelets.
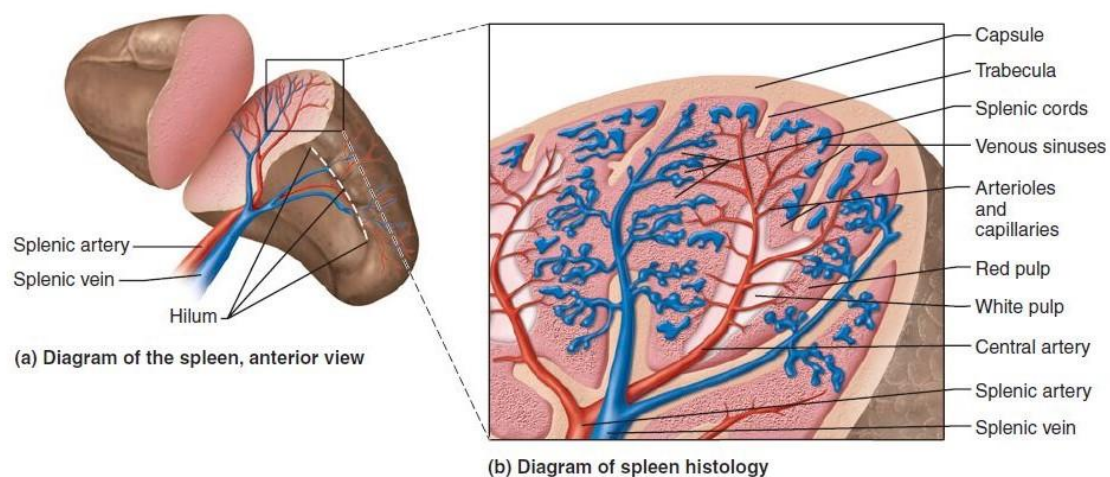
Figure 2. Showing the spleen, its blood distribution and a schematic for the spleens histology (b). Taken from *Human Anatomy* 6th edition, Marieb et al (16).

The whole blood volume filters through the spleen four times daily (5). In humans, the blood passes from the celiac trunk to the splenic artery which divides into five or more branches, called trabecular arteries, entering the splenic hilum. There does not exist any anastomoses between these branches, resulting in segmentation of the spleens vasculature (13). The trabecular arteries give rise to several central arteries which are surrounded by the white pulp wherein T cells surround the vessels. The arteries turn into arterioles and they filter blood through spaces between reticular cells to splenic cords which drains into venous sinuses. The function of this is to distinguish the normal red blood cells from the aged and abnormal which do not survive the squeeze and subsequently are lysed and secondary to that removed by phagocytic cells. A proportion of the blood circulates a series of marginal sinuses where

dendritic antigen-presenting cells seeks for exogenous antigens in the blood. The third pathway for the blood in the spleen is called the closed circulation. This is a way to bypass the filtering and rapidly pass through the spleen. The blood flows from the arterioles directly to the venous sinuses and back to the systemic circulation (17).
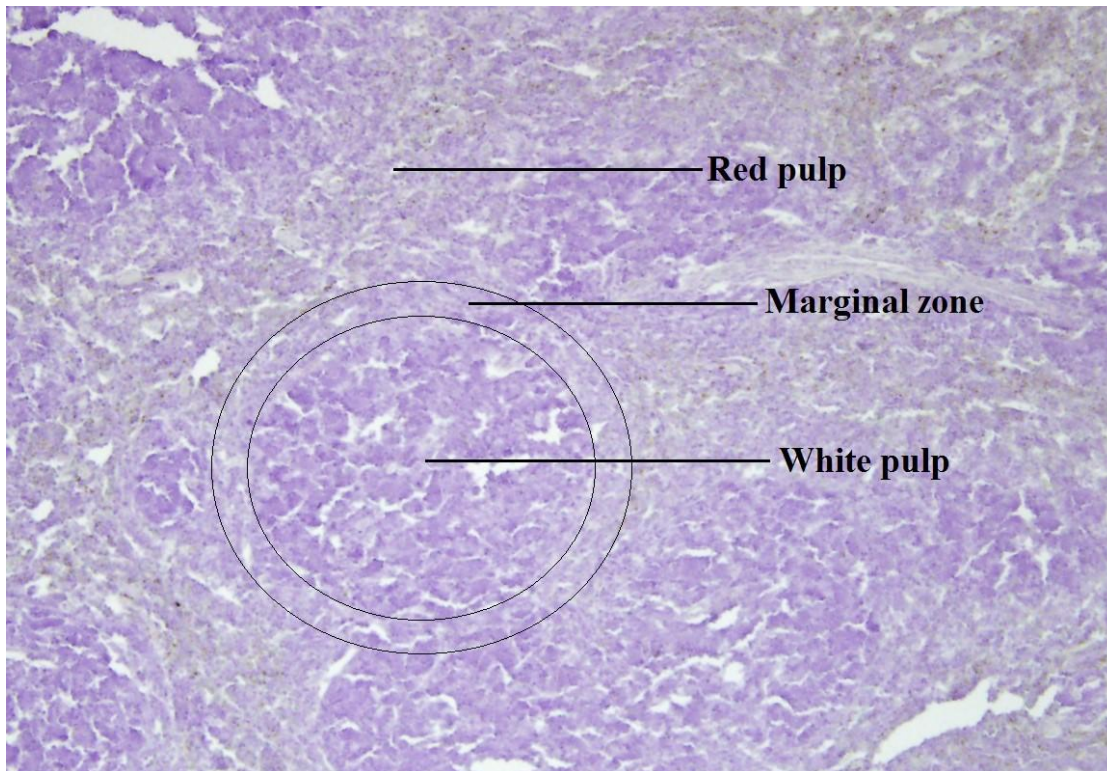
Figure 3. Showing histologic distribution of major characteristic areas in spleen of mice. *Red pulp* is the area which primarily filters the blood from particulate material. *White pulp* is a follicular area primarily consisting of lymphoid cells. Surrounding the White pulp there is an area called the *Marginal zone*, where subsets of B cells and Macrophages reside. Image is in 10 times magnification.
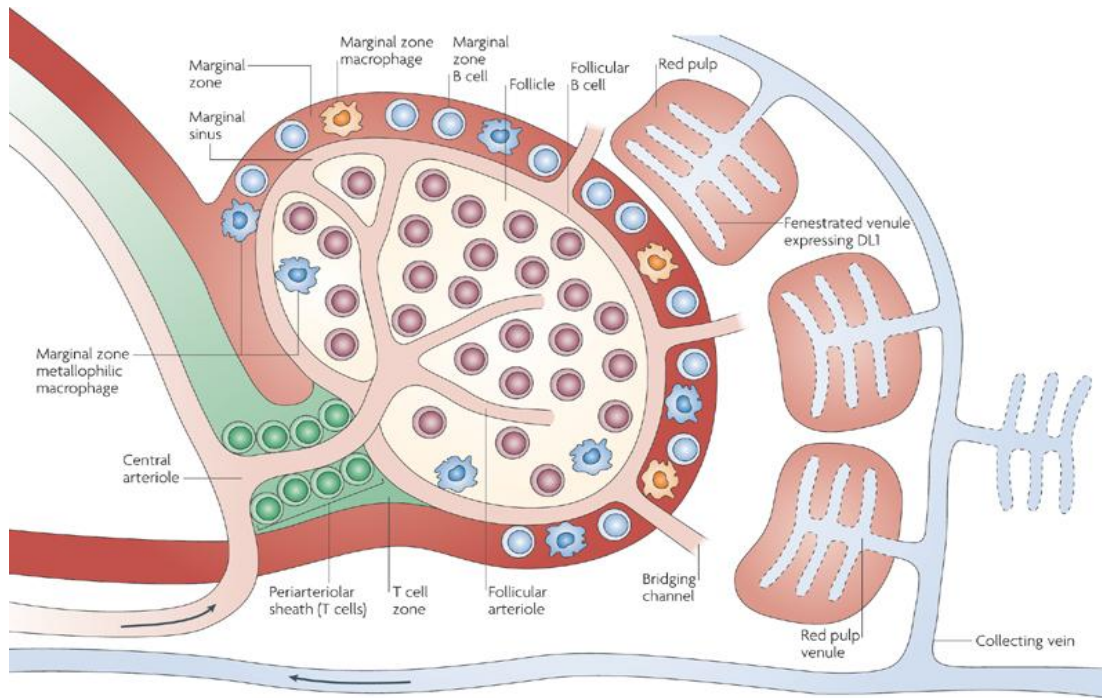
Figure 4. Showing the distribution of cells in the spleen. Taken from Pillai *et al*. (18)

The spleens immunologic function is to handle pathogens that get access to the blood, a pretty rare moment due to that most pathogens that evades the *Mucosa-associated lymphoid tissue* and *Skin-associated lymphoid tissue* and enters the tissue are collected by the lymphatic system and taken care of. Still, if the pathogen gets direct access to the blood circulation the cells of the spleen handles it (5).

4. Aim

Previous experiments have shown that uninfected mice fed HFD-P have increased number of neutrophils in the bone marrow, they also have increased recruitment of neutrophils to inflammation sites. Further, mice fed HFD-P which have undergone experimentally induced sepsis have fewer bacteria left in the kidneys compared to HFD-S (4). Neutrophils circulate in the body and can be found in organs as the liver and spleen in so called marginating pools (9). This means that they have a prolonged transit through these organs. In addition to this transit, neutrophils are also destroyed by the reticuloendothelial system, including liver, spleen and bone marrow (9). It remains unclear if the neutrophils have any functions in the spleen or if the spleen only acts as a station for degradation of the neutrophils. Therefore we want to study the localization of the neutrophils and the frequency of neutrophils to further investigate the role of the neutrophils in the spleen.

The aim of the current study was to investigate the frequency of the neutrophils in the circulating pool (blood) and the frequency and distribution in the marginated pool for mice fed low fat diet (LFD), high fat diet rich in saturated fatty acids (HFD-S) and high fat diet rich in polyunsaturated fatty acids (HFD-P).

5. Material and Methods

5.1. Experimental protocol

The protocol is similar to that described earlier by our group (4). Male six week old C57BL/6J mice were acquired from Harlan Netherlands B.V. (Horst, The Netherlands). After one week of acclimatization to the new facility the seven week old mice were randomly assigned to either low-fat diet (LFD), high-fat diet with high amount of saturated fatty acids (HFD-S) or high-fat diet with high amount of polyunsaturated fatty acids (HFD-P) for 8 weeks. Each of the groups consisted of 10 animals.

At 14 to 15 weeks of age, the mice were sacrificed with an overdose of pentobarbital (Apoteksbolaget, Sweden, 0.9 mg/g BW i.p.), as previously described (19). Blood was acquired transcardially into K-EDTA tubes for later analysis of blood cells. To avoid remaining blood from pooling in the organs they were subsequently perfused with 0.9% saline at a pressure of 100 mmHg for 10 minutes, thereafter spleen and liver were collected. Also, both femurs were collected for extraction of bone marrow cells.

5.2 Animals

To avoid bias due to hormonal alterations in female mice, only male mice were studied. The C57BL/6J mice were kept under standard conditions of light and temperature in the animal facility at the Laboratory for Experimental Biomedicine, University of Gothenburg, Gothenburg, Sweden. Water and food were available *ad libitum*. All experiments done on the animals were approved by the local animal welfare committee 2013-04-16.

5.3 Diets

The mice were randomly assigned to one of the following diets: LFD, HFD-S or HFD-P. The composition of the diets was the same as previously been described (4). They consisted of nutrients as follows: LFD (D12450B; 3.9 kcal/g, 10 kcal% fat, 20 kcal% protein, 70 kcal% carbohydrate; Research Diets, New Brunswick, NJ,USA), HFD-S (D12492; 5.2 kcal/g, 60 kcal% fat, 20 kcal% protein, 20kcal% carbohydrate; Research Diets), and HFD-P (D09020505; same composition as HFD-S of fat, protein, and carbohydrate, but 69% of the lard was replaced with menhaden oil). Menhaden oil contains a high amount of polyunsaturated fatty acids extracted from the fish Menhaden. The polyunsaturated fatty acids are predominantly omega-3 and omega-6.

5.4 Hematology and flow cytometry analysis

The blood was collected transcardially at termination. Bone marrow cells were collected from femur. The blood and the bone marrow were kept on ice until preparation.

The blood was analyzed by the VetScan HM5 (Abaxis, Union city, CA, USA) which analyzed the blood for: Total White Blood Cell Count (WBC), lymphocytes (LYM), monocytes (MON), granulocytes (GRA), Red Blood Cell count (RBC), and platelets (PLT). The instrument cannot distinguish between neutrophils, eosinophils and basophils so these parameters are grouped as granulocytes (GRA).

To obtain cells from bone marrow one femur was first cut in both ends and then flushed with 2% PFA (Paraformaldehyde). Bone marrow samples were put on ice until further preparations. The cell suspensions were vortexed for some seconds and then the concentration of the bone marrow cells was determined with the automated cell counter (Bio-Rad, Hercules, CA, USA). From each sample $3x10^5$ cells were used for staining. Fc-block (cat no: 2.4G2, BD Bioscience, Franklin Lakes, NJ, USA) were added for 15 minutes to avoid unspecific Fc-receptor interactions. Antibodies used for labeling the cells were: Ly6G-FITC (clone 1A8, BD Bioscience, neutrophil marker), CXCR4-APC (clone 247506, R&D system, Minneapolis, MN, USA, marker for retention of neutrophils in bone marrow) and CXCR2-PE (clone 247506, R&D system, marker for egression of neutrophils in bone marrow). When different fluorochromes are used in flow cytometry there is a risk that they interfere with each other's brightness and thus alter the interpretation of the cells. To avoid the spillover brightness we used Compensation-Beads (BD Bioscience) which corrects this by "showing" the software which brightness the fluorochromes have. This is done by having samples prepared with only one fluorochrome and lets the instrument analyze all fluorochromes used one at a time. After this the software can distinguish which brightness to interpret as the different flurochromes.

Another problem with using multiple fluorochromes is that they might spread into the other channels. To avoid this we used Fluorescence Minus One Control (FMO) which is a sample where all fluorochromes except one is present, the one that does not exist in the sample is measured and compared and thus we correct for the spread. The antibodies and the samples to correct the measurement (FITC, APC, PE, FMO-FITC, FMO-APC, FMO-PE, unstained) were applied for 50 minutes and the reaction were stopped by adding PBS 1% FCS. Samples

were centrifuged for 5 minutes and the supernatant were discarded. The resuspended cells were examined by the FACSCantoA flow cytometer (BD Bioscience). Compensation was optimized by using Comp-Beads (BD Bioscience). Analysis was done with the FACSDiva software (version 6.1.; BD Bioscience). Neutrophils expressing CXCR4 were identified as $LY6G^+CXCR4^+$, similarly neutrophils expressing CXCR2 were identified as $LY6G^+CXCR2^+$.

5.5. Immunostaining

5.5.1 Preparation, fixation and sectioning

The spleens and the livers were fixated with 2% Formaldehyde for 24-48 hours. After proper fixation the organs were transferred to 30% Saccharose-solution for 24-48 hours for extraction of water from the tissue by osmosis. After the organs had sunken to the bottom of the tube they were once again moved into another Eppendorf tube filled with 30% Saccharose-solution for another 24-48 hours. The tissues were thereafter embedded in OCT Cryomount embedding medium (Histolab Products AB, Gothenburg, Sweden) and frozen with dry ice and when fully frozen stored at -80ºC freezer until sectioning.

The spleens and the livers were sectioned in a cryostat (Leica CM3050 S, Leica Biosystems, Nussloch, Germany). The spleens were cut with either 7 µm or 10 µm thickness and the livers were sectioned with 7 µm thickness. Three sections were attached to Superfrost® plus microscope slides (Thermo Fisher scientific, Waltham, MA, USA). All samples were sectioned vertically.

5.5.2 Immunostaining for neutrophils in spleen and liver.

Immunostaining were performed on spleen sections. All procedures were done at room temperature. The antibodies used were Ly6G (cat no: 551459, BD Bioscience Franklin Lakes, NJ USA) diluted 1:1000 and biotinylated rabbit anti-rat IgG (cat no: BA-4001 Vector Laboratories, Burlingame, CA, USA) diluted 1:200. As a negative control the rat IgG2a Isotype (cat no: MAB006, R&D Systems, Minneapolis, MN, USA) were used. Firstly, sections were blocked with 0.6% hydrogen peroxidase (Sigma Aldrich) diluted with methanol. Secondarily, sections were blocked with Avidin/Biotin blocking kit (Vector Laboratories) for 15+15 minutes. The last blocking step consisted of applying 10% Normal Rabbit Serum for 30 minutes, it includes various classes of immunoglobulins and serum proteins which saturate and block non-specific binding to these sites. By this the antibody later added will only bind to its true target. The Ly6G-antibody was applied for one hour and

thereafter the secondary antibody was applied for 30 minutes. For detection of the biotinylated secondary antibody, VECTASTAIN Elite ABC Kit (cat no: PK-6100, Vector Laboratories, Burlingame, CA USA) was applied for 30 minutes. Finally, binding was visualized by DAB kit (Vector Laboratories) and counterstained with Hematoxylin (HARRIS HTX, Histolab Products AB). Images were acquired by Olympus BX60F5 microscope with 10X, 20X and 40X objectives connected to an Olympus DP72 camera. The areas of the spleens were chosen in a standardized manner, i.e. area should contain one white pulp in the center of the image and white pulp should be approximately the same size. 8 spleens per group were randomly chosen for quantification. The micrographs were acquired and quantified in a blinded manner. From each individual two sections were chosen, and from each section two different areas were quantified for Ly6G$^+$ cells using cellSens Dimension analysis software (Version 1.5, Olympus Optical Company).The images quantified were in 20X magnification and manually quantified. To standardize the quantification the white pulp was excluded from quantification because those areas did not contain any neutrophils and varied in size between the images. The observer was blinded throughout the process of photographing and quantification of the sections. Numbers of Ly6G$^+$neutrophils are presented as neutrophils per area. For immunostaining of Ly6G$^+$ neutrophils in liver, 6 randomly chosen livers, two from each group were examined (n=2).

5.5.3 Histological staining and Quantification for lipids

Sections from liver were analyzed for fatty acids by staining with Oil Red O (Sigma Aldrich, Saint-Louis, MO, USA). Stock solution was prepared by dissolving 1.5 g Oil Red O in 150 ml 2-propanol (Sigma Aldrich) and mixed for approximately 1 hour. Working solution was prepared by mixing 150 ml working solution with 100 ml distilled water and subsequently stirred for 15 minutes. To remove undissolved particles the working solution was filtered. The sections used for Oil red O were 7 µm thick and they were stained with the filtered working solution for 20 minutes at room temperature. Thereafter they were rinsed in tap water, and mounted with Kaiser's glycerol gelatine (Merck Millipore, Darmstadt, Germany). Images were acquired with an Olympus BX60F5 microscope with 10X, 20X and 40X objective connected to an Olympus DP72 camera (Olympus Optical Company, Hamburg, Germany). The images of the livers were later quantified for fatty acids using cellSens Dimension analysis software (Version 1.5, Olympus Optical Company). Oil red O positive areas were automatically detected within a region of interest (ROI). Quantification was done on the 20X magnification and the ROI was chosen as the whole image. Two sections were

chosen from each individual depending on quality. From each section two different areas were chosen for quantification of lipid deposits. The areas were chosen based on the quality and a criterion was that it should include a central vein in the middle of the image.

6. Statistical Methods

All data are presented as mean and standard error of the mean (SEM). The significance level was set to 0.05. Prior to statistical analysis normality was tested using the Shapiro-Wilk test. Hematology and flow cytometry analysis were analyzed by a general line model. Since the experiments were pooled from different experimental days we initially tested for between subject effects with the nuisance factor experimental days. No effect were found, thus only differences caused by the diets were analyzed. Analysis of variance (ANOVA) followed by Tukey's post hoc test was performed on the data from quantification of Ly6G in spleen and for lipids in liver. All statistical analyses were performed using the SPSS software (IBM SPSS Statistics 20, IBM Corporation, Chicago, USA) and graphPad Prism (GraphPad Prism 5, GraphPad Software, Inc, California, USA). Differences in numbers of individuals are due to laboratory errors or lack of sample material. For the quantification of Ly6G in the spleen by immunostaining it was decided in advance that 8 individuals would be randomly chosen and quantified per group.

7. Ethics

Infectious diseases account for 25.5% of annual mortality (20). In addition, the antibiotic resistance is rising globally (21). Thereby it is crucial to find new therapies to prevent death by infectious diseases caused by bacteria that are resistant to antibiotics.

One complication to infection is Sepsis. It is when bacteria enter the bloodstream and by doing so have access to nearly all of our bodies organs. This triggers release of pro-inflammatory molecules which activate the immune system to fight the bacteria. It is a very serious state and the severe form, septic shock, has a mortality rate of 20 to 30% (22). In addition to the high mortality it has a negative effect on the quality of life. For example one third of the patients who suffered from severe sepsis had died at 6 months and 41.6% were not able to take care of themselves independently at 6 months (23). Hence, this is a very severe disease. With the alarming increase in antibiotic resistance additional treatment options is needed for patients with sepsis. In the waste majority of our experiments we do not induce sepsis in the experimental animals, as in the current study, but rather investigates the mechanisms behind the improved survival. The project was approved by the ethical committee of Gothenburg 2013-04-16 with ethical number 75-2013.

8. Results

8.1 Dietary PUFA increases the frequency of Ly6G^{+}neutrophils in bone marrow and spleen.

Mice fed HFD-P had both an increase of Ly6G^{+}neutrophils in the bone marrow and in the spleen compared with both LFD and HFD-S (p=0.001 for all, figure 5A-B). Compared to mice fed HFD-S, mice fed HFD-P had a 28 % increased frequency of neutrophils in the bone marrow and a 222% increased frequency of neutrophils in the spleen. In addition mice fed HFD-S had a decreased amount of Ly6G^{+}neutrophils in the bone marrow compared with LFD (p=0.01, Fig.5A). The spleens of mice fed HFD-P weighed more than the spleen in mice fed HFD-S and LFD (p=0.001, Fig.5C).
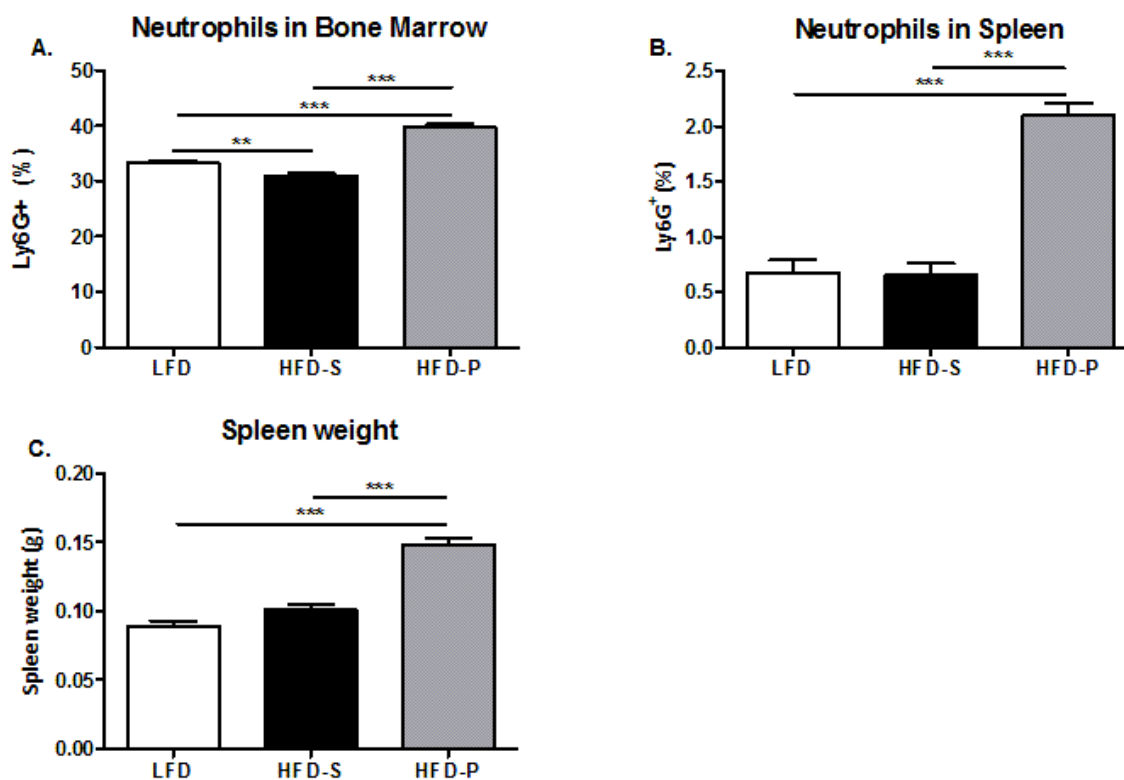

Figure 5. Eight weeks of HFD-P increases the frequency of Ly6G^{+}neutrophils in bone marrow and spleen and increases spleen weight. Flow cytometry analysis of Ly6G^{+}neutrophils in (A) bone marrow and (B) spleen. (C) Spleen weight. Data are analyzed by using general linear model (univariate). Experimental groups: Low-fat diet (LFD), high-fat diet rich in saturated fatty acids (HFD-S) or high-fat diet rich in polyunsaturated fatty acids (HFD-P). n=10/group. **p<0.01, ***p<0.001.

8.2 Dietary PUFA decreases Ly6G$^+$neutrophils retention in bone marrow.

Analysis done by flow cytometry for CXCR2 and CXCR4 show that the diets are not affecting the frequency of CXCR2$^+$Ly6G$^+$ neutrophils (Fig. 6A), suggesting that the Ly6G$^+$neutrophils are not more prone to egress from the bone marrow. Although, the Ly6G$^+$neutrophils which do express the CXCR2 receptor have a decreased expression per neutrophil after being fed HFD-P (Fig. 6B, p=0.001).

Further, mice fed HFD-P have decreased frequency of CXCR4$^+$Ly6G$^+$ neutrophils compared with mice fed LFD (Fig. 6C, p<0.05) and, in addition, Ly6G$^+$neutrophils that do express the CXCR4 have a decreased expression of the CXCR4 receptor if the mice are fed HFD-P compared with both LFD and HFD-S (Fig. 6D, p<0.05).
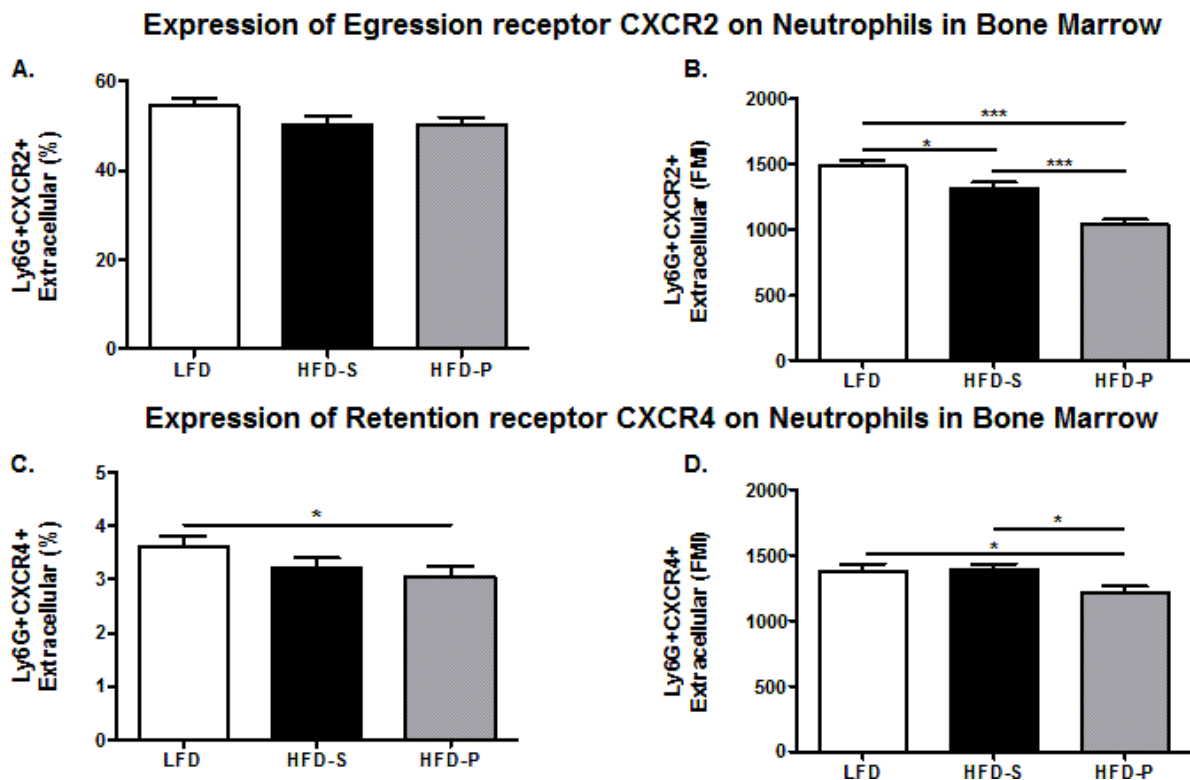
Figure 6. In bone marrow, different diets do not alter frequency of Ly6G$^+$neutrophils expressing the egress receptor CXCR2 (A), however, the Ly6G$^+$neutrophils expressing CXCR2 in mice fed HFD-P, express it to a lesser extent compared with LFD and HFD-S (decreased FMI, median fluorescence intensity) (B). Ly6G$^+$neutrophils fed HFD-P have a decreased frequency of the retention receptor CXCR4 (C), and Ly6G$^+$neutrophils expressing CXCR4 also express it to a lesser extent (D). This suggests that Ly6G$^+$neutrophils of mice fed HFD-P are less prone to stay in bone marrow. Data are analyzed by using general linear

model (univariate). Experimental groups: Low-fat diet (LFD), high-fat diet rich in saturated fatty acids (HFD-S) or high-fat diet rich in polyunsaturated fatty acids (HFD-P). For all groups in the image n=10. *p<0.05, ***p<0.001.

8.3 No increased amount of granulocytes in blood for mice fed HFD-P.

Although the spleen contains more Ly6G$^+$neutrophils, hematology analysis of blood by an automated cell counter does not show any differences in granulocytes between the diets. A significant increase of monocytes where found for mice fed HFD-S compared to LFD ($p<0.01$). Monocytes were also increased in mice fed HFD-P compared to LFD ($p<0.05$). Platelets were significantly decreased for HFD-S compared to both LFD ($p<0.01$) and HFD-P ($p<0.05$) (Table 1).

Table 1.Hematologic analysis of blood constituents

|	LFD	HFD-S	HFD-P
WBC	2.06±0.38	3.1±0.36	3.01±0.34
LYM	1.56±0.29	2.36±0.27	2.19±0.26
MON	0.06±0.02	0.13±0.02*	0.12±0.02#
GRA	0.45±0.12	0.63±0.11	0.72±0.1
RBC	10.06±0.13	9.91±0.12	9.97±0.12
PLT	544.79±16.25	484.5±15.2*	531.71±15.22¤

WBC =white blood cells, LYM= Lymphocytes, MON=Monocytes GRA=Granulocytes, RBC=red blood cells, PLT=platelets. Experimental groups: Low-fat diet (LFD) n=8, high-fat diet rich in saturated fatty acids (HFD-S) n=9 or high-fat diet rich in polyunsaturated fatty acids (HFD-P) n=10.Standard error of the mean (SEM). All mean and SEM data are expressed in 10^9 except for RBC which are expressed in 10^{12}. Data are analyzed by using general linear model (univariate). The comparisons between the groups (vs.) show the p-values for each. *=LFD vs. HFD-S $p<0.01$, #=LFD vs. HFD-P $p<0.05$, ¤=HFD-S vs. HFD-P $p<0.05$.

8.4 Ly6G$^+$neutrophils are localized in the red pulp of spleen

Ly6G$^+$neutrophils are found in different frequency in the red pulp of the spleen. There is a tendency to be an increased frequency of Ly6G$^+$neutrophils around the white pulp in the close proximity of the marginal zone. There were no neutrophils in the white pulp. The micrographs of the immunostained spleens were manually quantified in a blinded manner for Ly6G$^+$neutrophils. This quantification did not find any difference of the diets for Ly6G$^+$neutrophils.
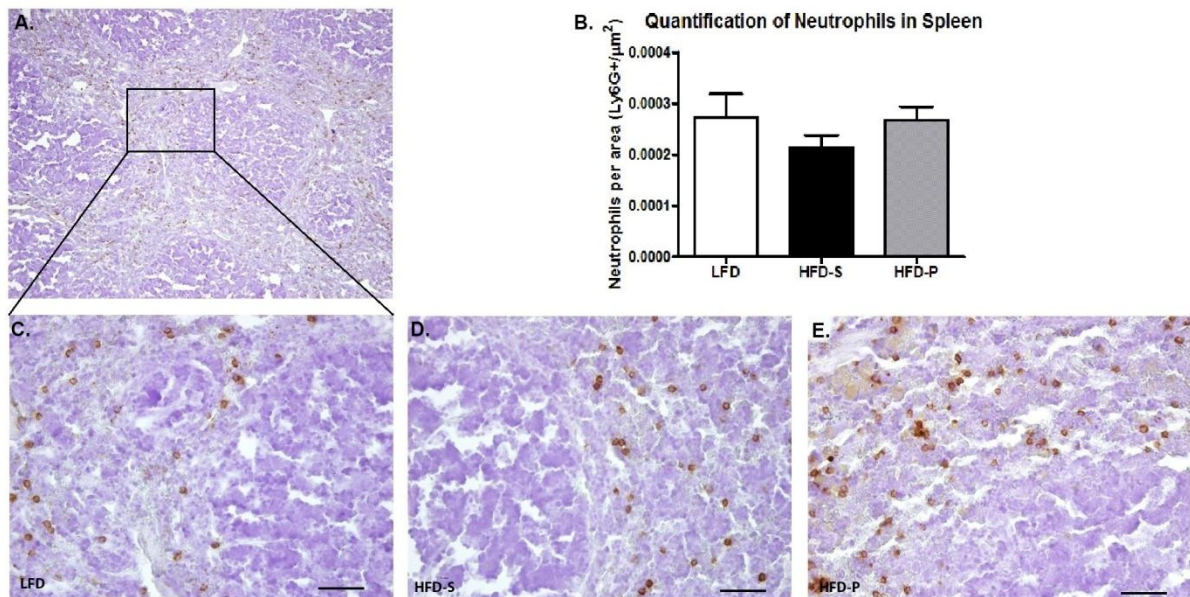

Figure 7. Representative micrographs of immunostained spleen in mice. (A) Overview micrograph (10X) for the selected areas in C-E. (B) Neutrophil quantification based on immunostained sections. From each individual two sections were chosen in a standardized manner and from each section two images were quantified in 20X magnification. Ly6G Immunostaining at 40X in mice fed LFD (C), HFD-S (D) and HFD-P (E). Ly6G$^+$neutrophils are stained brown with DAB. Neutrophils are located in the marginal zone and the red pulp but not in the white pulp of the spleen. Experimental groups: Low-fat diet (LFD), high-fat diet rich in saturated fatty acids (HFD-S) or high-fat diet rich in polyunsaturated fatty acids (HFD-P). *n*=8 randomly chosen individuals for each group. Scalebar represents 50 µm.

8.5 Ly6G$^+$neutrophils are localized in proximity of liver sinusoids

The liver is an immunological organ with its content of NK-cells, Kupffer cells and its vital part in the acute phase response. As in spleen and bone marrow, neutrophils are destroyed

there. Subsequently, immunostaining were performed for Ly6G in liver to examine if neutrophils are present, and in that case, where? There are Ly6G$^+$neutrophils present in liver and they are found in close proximity of the liver sinusoids. Although no statistical analysis was conducted for Ly6G in liver, there seems to be decreased frequency of Ly6G$^+$neutrophils in liver compared to spleen. In addition there is a tendency that HFD-P has increased frequency of Ly6G$^+$neutrophils compared to HFD-S and LFD (Fig. 8).
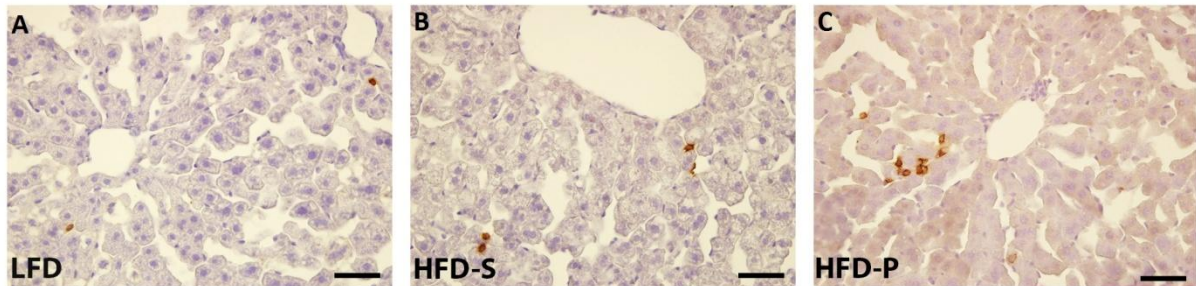

Figure 8. Micrographs of spleens immunostained for Ly6G$^+$neutrophils in liver. Ly6G$^+$neutrophils are stained brown by DAB. No quantification was conducted for Ly6G$^+$neutrophils in liver. (A-C). Micrographs in 40X magnification. Ly6G$^+$neutrophils are aligned in close proximity of the liver sinusoid, especially shown in (C). Experimental groups: Low-fat diet (LFD), high-fat diet rich in saturated fatty acids (HFD-S) or high-fat diet rich in polyunsaturated fatty acids (HFD-P). Scalebar represents 50 µm.

8.6 Mice fed HFD-S have increased accumulation of lipids in liver

It is known that fatty acid composition in diets can influence lipid metabolism in the liver, consequently I examined the livers for content of fatty acids. The livers were stained with Oil Red-O (ORO) which stains lipids red and thereafter quantified for amount of lipids in the liver. Despite the fat content in the diets being the same for both HFD-S and HFD-P, only mice fed with HFD-S developed extensive lipid accumulation in the liver compared to HFD-P and LFD (Fig. 9).
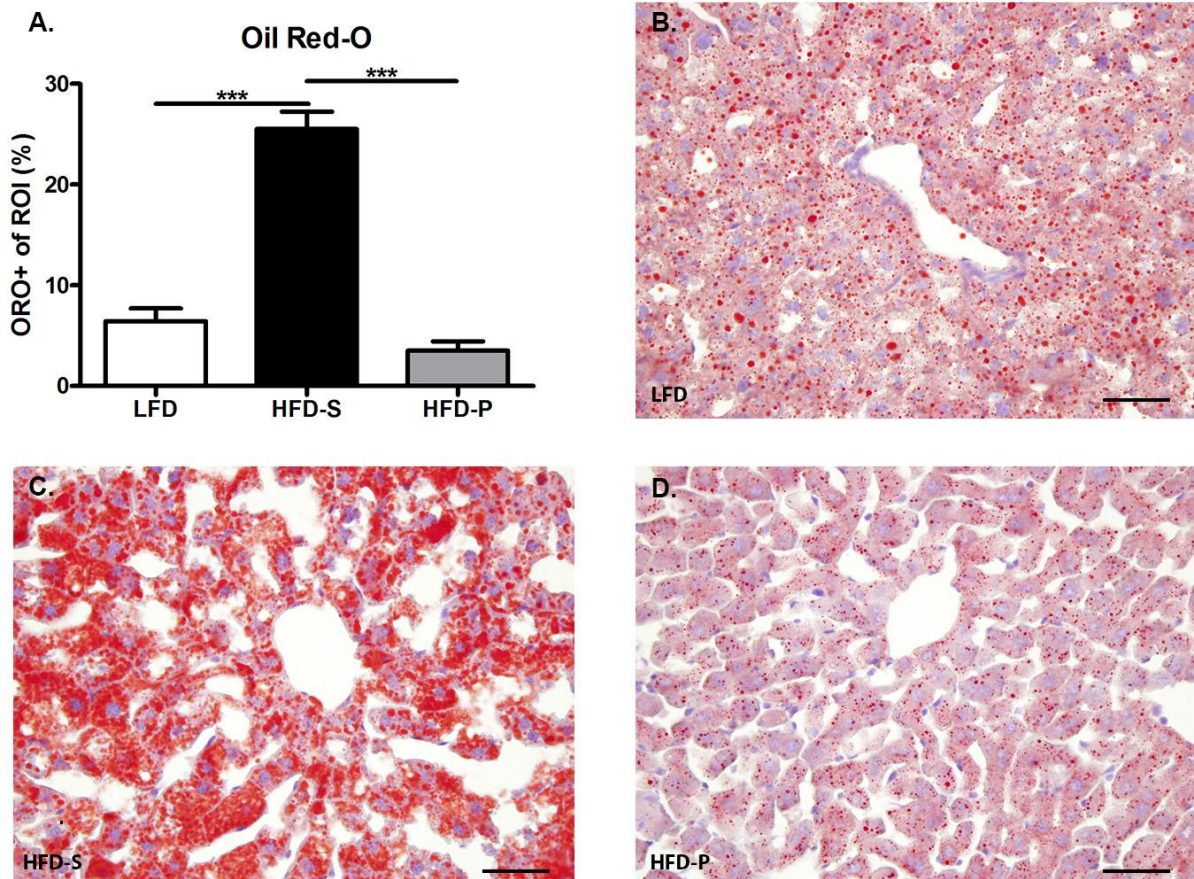
Figure 9. Mice fed HFD-S accumulate more lipids in the liver. Representative micrographs of lipid staining (Oil Red O) in liver. (A) Demonstrating the increased amount of lipid accumulation in the livers for mice fed HFD-S compared to both LFD and HFD-P. (B-E) Representative micrographs of livers demonstrating the differences in lipid accumulation for LFD (B), HFD-S (C) and HFD-P (D). Lipids are accumulated ubiquitously in the hepatocytes in the liver. ***p<0.001. For all groups *n*=6 randomly chosen individuals. From each individual two sections were chosen in a standardized manner and from each section two images were quantified in 20 times magnification. Data were analyzed by using a one-way ANOVA. Experimental groups: Low-fat diet (LFD), high-fat diet rich in saturated fatty acids (HFD-S) or high-fat diet rich in polyunsaturated fatty acids (HFD-P). ***p<0.001.Scalebar represents 50 µm.

9. Discussion

The main finding in the current study is that mice fed with HFD-P have increased frequency of neutrophils in bone marrow and in the spleen. In spleen Ly6G$^+$neutrophils are only found in the red pulp, and most abundant in the close proximity of the marginal zone. In liver Ly6G$^+$neutrophils are found in lesser extent than in the spleen. There is a tendency that Ly6G$^+$neutrophils are localized alongside liver sinusoids.

9.1 The effect of HFD-P on neutrophil frequency in bone marrow

In line with previous data from Svahn et al (4) mice fed HFD-P had increased frequency of neutrophils in bone marrow. An increased frequency of white blood cells (*leukocytosis*) is a common feature of an inflammatory reaction, particularly bacterial infections (24). However, previous data from our group have shown that the pro-inflammatory cytokines IL1b, IL6, IL17, IFN-γ, MCP-1 and TNF-α), as well as the anti-inflammatory cytokine IL10 are not increased in the mice fed HFD-P (25). Hence, it is not likely that the increased frequency is a sign of an ongoing inflammation. A previous study showed that mice fed HFD-P had an increased survival compared with mice fed HFD-S in a *S. Aureus* induced sepsis model (4). Rather, the increase in neutrophil frequency suggests that mice fed PUFA have an increased ability to early neutralize pathogens and therefore defend against infections. This hypothesis builds on that large numbers of neutrophils are required to terminate the pathogens, and thus we assume that if mice fed PUFA have increased frequency of neutrophils in the bone marrow, their ability to early fight the pathogens might be improved. In addition, it appears that the type of polyunsaturated fatty acids is also important. A recent study showed that mice fed High fat diet rich in omega-3 fatty acids had increased survival after undergoing experimental sepsis compared to both mice fed HFD-S and high fat diet rich in omega-6 fatty acids (25). The PUFA in our study were both omega-3 and omega-6.

To further characterize the neutrophil population I analyzed the neutrophils in bone marrow for chemokine-receptors important for egression (CXCR2) and retention (CXCR4), to see if the neutrophils in HFD-P were more easily departed from the bone marrow or were retained in the bone marrow. As described above, the egress of neutrophils from the bone marrow into the circulation is regulated by CXCR2 (egression) and its ligands CXCL1 and CXCL2. The retention of neutrophils to the bone marrow is regulated by CXCR4 and its ligand CXCL12.

Feeding the mice HFD-P caused a decrease in the expression of the retention receptor CXCR4 suggesting that the neutrophils are more prone to migrating out from the bone marrow. However, only approximately 4 % of the neutrophils express this receptor, which raises the question whether this difference is biologically important. Although, we found that the neutrophils which did express the receptors in mice fed HFD-P actually did it to a lesser extent for both the retention receptor CXCR2 and the egress receptor CXCR4. What this decreased amount of receptors per neutrophil does biologically are yet to be explained. One hypothesis could be that the mice fed PUFA have an increased turnover of the neutrophils and therefore have less time to produce as many receptors per neutrophil as the mice from the other diets which have a decreased turnover. But this might be far-fetched so further studies are needed when both receptors and ligands are analyzed to fully understand this.

When we both have an increased frequency of neutrophils in the bone marrow and decreased expression for CXCR4 one might assume that this would increase the neutrophils in the blood as well. However, there were no differences in the amount of granulocytes circulating in the blood. This is in line with our findings that the mice fed HFD-P are not inflammatory which could be harmful considering the increased risk of amongst others malignant disease (26).

9.2 The frequency of neutrophils in spleen are increased

The blood enters the spleen via the splenic artery, exits the circulation in the marginal sinus and then circulates back to the venous circulation. On its way it passes the marginal zone to the white and red pulp. In the marginal zone there are B-cells residing which search the blood for blood-borne antigens (27). Neutrophils have been found in spleen but little have been known about the localization and function of neutrophils in the spleen. Shi et al. showed that endotoxemia (by injection of lipopolysaccharide) lead to large accumulation of neutrophils in spleen, liver and lungs (28). In the same study they showed that neutrophils underwent apoptosis in spleen, liver and lungs but to a very small extent in lungs and spleen compared with the liver.

I found that HFD-P increased the frequency of neutrophils in spleen compared to mice fed LFD or HFD-S when analyzed by flow cytometry. This raises a question, what are the neutrophil functions in non-inflammatory mice as our mice are? Puga et al. found a special phenotype of neutrophils which like the B-cells resided in the peri-marginal zone of the spleen (29). They showed that in a non-inflammatory state neutrophils stimulated B-cells to

produce more immunoglobulins. They also showed a suppression of T-cells by the neutrophils. However they found that during inflammation the neutrophils became disorganized and instead infiltrated lymphoid follicles in the spleen to function as an antigen presenting cell for T-cells.

I was able to show that there were an accumulation of neutrophils in close proximity of the marginal zone, this confirms the previous study by Puga et al. and in addition, I did not find any neutrophils in the white pulp. My result support the view that the neutrophils in addition to their function in eliminating pathogens as a part of the innate immune system are also important in the adaptive immune system. The potential to earlier activate the adaptive immune system and also potentiate its effect might explain the increased survival in the study by Svahn et al.(4) and this can be accomplished by the increased amount of neutrophils in the spleen of mice fed HFD-P.

I tried to reproduce the findings of flow cytometry with immunohistochemistry and quantification of micrographs, but was not able to show the increased frequency of neutrophils in spleen for mice fed HFD-P. This is probably due to selection bias. The spleens of the mice fed HFD-P had increased size of the white pulps and those areas were excluded from the quantification. I tried to standardize the micrographs as much as possible by choosing areas with approximately the same sized white pulps in the middle. This led to fewer possible areas to choose from in mice fed HFD-P and also a proportionally decreased area evaluated respectively than for the other diets. Subsequently we showed that there were no difference in amount of neutrophils per area, but if we instead would have quantified the whole spleen instead of only a little area, there would most likely have been a significant increase for neutrophils in spleen also with this analysis. Another strategy could be to use *Stereology*, which is a method where random areas of a two-dimensional section is chosen and easily counted. By this larger areas would be covered and the selection would be unbiased.

The HFD-P fed mice had increased spleen weight. This was in accordance to the increased amount of neutrophils in spleen, but it is highly unlikely that this was the sole explanation for the spleens increased weight. It is more likely that the increased weight is due to proliferation of splenic cells, however this remains to be further investigated. Another possible reason

behind the increased spleen weight is increased accumulation of fatty acids, however, this was not the case in the current study (data not shown).

9.3 The effect of HFD-P in liver

The liver is also a lymphoid organ which plays a role in detecting antigen from the circulation by Kupffer cells (a subset of macrophages) and innate lymphocytes (6). Overwhelming activation of neutrophils in the liver can induce liver damage (30), therefore we conducted immunohistochemistry analysis for the neutrophil marker Ly6G. By visual analysis there were no apparent difference in the amount of neutrophils in the liver and therefore no quantification was performed. The livers of HFD-S were bigger than the livers of the other two diets and thus we conducted a stain for lipids with Oil Red-O on the livers and thereafter a quantification which showed significant increase of lipid accumulation in the liver for mice fed with HFD-S compared to LFD and HFD-P. This is in line with previous findings where PUFA can act as inhibitors of hepatic lipogenesis (31) which may explain that although the mice in the HFD-groups get the same amount of fat content in their diets, only mice fed HFD-S developed the lipid accumulation. Ferramosca et al. provides a possible explanation that this may be caused by the decreased effect on mitochondrial citrate carriers which PUFA exerts, but saturated fatty acids does not exert. This would implicate that mice fed HFD-P are less prone to transport citrate outside mitochondria resulting in less cytosolic fatty acid biosynthesis (32). If this is also the case here remains to be determined.

10. Conclusions and Implications

In conclusion this study shows that intake of dietary polyunsaturated fatty acids can increase neutrophils in bone marrow and spleen. This can in its turn at least partly explain the decreased mortality in mice fed polyunsaturated fatty acids which were found in earlier studies. Furthermore it shows that high intake of saturated fatty acids increases lipid accumulation and therefore should be avoided by people in risk for developing Non alcoholic fatty liver disease.

After verification in human, this knowledge may be used in general dietary guidelines to stress the importance of increasing the amount of polyunsaturated fatty-acids in the diet instead of the saturated acids. In medicine this can be tested on subjects prone to develop neutropenia, for example patients undergoing chemotherapy. One other possible group that may benefit from this is patients planned for elective surgery, the time prior to surgery they might be instructed to increase their dietary intake of polyunsaturated fatty acids to possibly develop less post-operative infections.

11. Future directions

It remains unclear whether the decreased mortality rate of mice fed PUFA are due to the increased neutrophil frequency or other effects as well. This should be investigated. In addition further analyses should be conducted on the spleens for mice fed PUFA to decide what the increased weight of the spleens is caused by. Analysis with stereology should be conducted on the spleens for the neutrophil marker Ly6G to get an unbiased quantification, and probably confirm the Flow cytometry data for mice fed HFD-P.

Analysis of both the ligands and the chemokine receptors for neutrophils should be conducted to determine if the decreased frequency of CXCR4 for mice fed HFD-P compared to mice fed LFD is biologically important.

The findings that there were widespread liver steathosis in HFD-S but not in HFD-P and LFD should also be investigated. Does this influence the immunological functions of the mice? Analysis of the acute-phase proteins could be a possible strategy to determine this.

12. Populärvetenskaplig sammanfattning på svenska

Vi lever i en tid då fetmaepidemin sprider sig som en löpeld över världen med dess följdsjukdomar i släptåg. Det är känt att fetma ökar benägenhet att drabbas av infektioner. Detta kan bland annat förklaras av ökad samsjuklighet i diabetes, men även molekylärbiologiska förändringar i kroppen. Dock verkar fettet i sig inte vara boven i dramat, utan typen av fettintag är det avgörande. Tidigare studier har fastslagit att möss som får en diet med hög andel fleromättat fett istället för dieter med hög andel mättat fett överlever experimentellt orsakad blodförgiftning i högre grad. Detta var grunden för mitt arbete, att förklara varför det är så.

En av de vita blodkropparna kallas för neutrofiler och det är dessa som är först på plats vid infektion eller inflammation. De har en avgörande roll i oskadliggörandet av bland annat bakterier och därmed valde jag att fokusera på dem.

Möss fick under åtta veckor en av de ovanstående dieterna eller låg-fetts-diet och därefter tog jag till vara på provmaterial från dem och analyserade med s.k. flödescytometri. Det är en teknik där en maskin används vilken kan räkna antal celler från ett prov för bedömning av bl.a. cellslag och receptorer. Denna använde jag för att räkna antal neutrofiler i benmärg och mjälte samt räkna hur många av neutrofilerna som uttryckte receptorer för att skickas ut från benmärgen eller stanna kvar i den.

Jag tillämpade också en annan teknik s.k. immunhistokemi, då utnyttjar man antikroppar för att åskådliggöra specifika celltyper på vävnadssnitt. Det använde jag för att bedöma var neutrofilerna befann sig i mjälte och lever. Jag använde också färgning med Oil Red O. Då används inte antikroppar utan molekyler som fäster till olika biokemiska molekyler, i detta fall till fett. Denna teknik använde jag i lever för att se om mössen drabbades av leverförfettning.

Mina undersökningar visade att dieter med hög andel fleromättat fett ökar antalet neutrofiler i benmärg och mjälte, vilket åtminstone delvis kan förklara den ökade överlevnaden hos möss som utsätts för experimentell blodförgiftning. Dessutom har denna grupp minskat antal av receptorn som signalerar för att stanna kvar i benmärgen, indikerande att de lättare kan rekryteras från benmärgen till blodbanan och sjuka vävnader, då mindre håller dem kvar i

benmärgen. I levern fann vi kraftig leverförfettning hos mössen som fått mättat fett, men normal fettansamling hos de som fått fleromättat fett eller lågfettsdiet. Dessa resultat behöver verifieras hos människa, därefter kan de användas till kostrekommendationer för patienter i riskgrupper för att utveckla brist på vita blodkroppar, exempelvis inför cellgiftsbehandling. Dessutom kan de användas i generella kostriktlinjer för att minska leverförfettningen genom att ersätta det mättade fettet med fleromättat fett.

13. Acknowledgement

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