

Effects of dietary fatty acids on the immune system

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UNIVERSITY OF GOTHENBURG

Gothenburg 2015

Cover illustration: Neutrophils, Luis Felipe Sánchez Heres

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ISBN 978-91-628-9644-7 (printed)

ISBN 978-91-628-9645-4 (e-print)

<http://hdl.handle.net/2077/39568>

Printed by Ineko AB, Gothenburg, Sweden 2015

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ABSTRACT

Sepsis is a deadly disease with an increasing incidence worldwide. Today, antimicrobials are the only effective pharmacological treatment. At the same time, bacteria, the pathogens behind most cases of sepsis, are becoming more and more resistant to our available antibiotics. A considerable amount of time, effort and money has been spent into finding new drug-candidates for treating sepsis. To date, none has succeeded in clinical trials. Dietary fatty acids affect the immune system. Saturated fatty acids (SFAs) increase the risk for cardiovascular diseases and promote low-grade inflammation, whereas polyunsaturated fatty acids (PUFAs) are beneficial for patients with rheumatoid arthritis and atherosclerosis, being anti-inflammatory. In this thesis, we investigated the effects of dietary fatty acids on the immune system and survival in *S. aureus*-induced sepsis in mice.

Following 8 week of either low fat diet (LFD), high fat diet (HFD) rich in SFAs (HFD-S) or HFD rich in PUFAs (HFD-P) mice were inoculated with *S. aureus* to induce sepsis or investigated for mechanistic studies. Mice fed HFD-P had a better survival in sepsis and lower bacterial load compared with mice fed HFD-S. Further, we found an increased frequency of Ly6G⁺ neutrophils and CD117⁺ hematopoietic stem cells in the bone marrow in mice fed HFD-P at uninfected state. Moreover, neutrophils from mice fed HFD-P have an improved migratory capacity. Since dietary manipulations have an effect on the whole organism, we investigated the transcriptome profile in immunologically and metabolically important organs. Remarkably, the spleen showed a major response to HFD-P, *i.e.*, down regulating both the innate and the adaptive immune system. We further investigated the mechanisms behind the increased frequency of neutrophils in mice fed HFD-P and showed an increased level of the major regulator of granulopoiesis, G-CSF, in the bone marrow. Additionally, there was an increased frequency of neutrophils in organs housing the marginated pool of

neutrophils, *i.e.* spleen, liver and bone marrow. Since HFD-P contained different types of PUFAs, both omega-3 and omega-6 PUFAs (ω -3 PUFAs and ω -6 PUFAs), additional investigations aimed to determine which type of fatty acids mediated the beneficial effects. Omega-3 PUFAs were identified as the PUFAs responsible for the positive effects on the immune system and survival in septic infection.

In conclusion, our results show that, beyond their well-recognised anti-inflammatory properties, omega-3 PUFAs have immune-modulating properties, as they influence the transcriptome profile in the spleen, increase the frequency of neutrophils in bone marrow, spleen and liver, as well as, improve neutrophil function, making this type of PUFAs a potential supplementary treatment for sepsis.

Keywords: Immune system, neutrophils, sepsis, polyunsaturated fatty acids, omega-3 fatty acids, *S. aureus*

ISBN: 978-91-628-9644-7 (printed)

SAMMANFATTNING PÅ SVENSKA

Sepsis, eller kanske mer känd som blodförgiftning, är en dödlig sjukdom som fler och fler dör av. Idag är den enda tillgängliga farmakologiskt behandlingen antibiotika. Trots adekvat behandling med antibiotika så dör mellan 40-80% av patienterna som drabbas av septisk chock. Detta, samtidigt som antibiotikaresistensen ökar lavinartat, gör att dödligheten kommer att öka ytterligare.

En av utmaningarna med att hitta nya behandlingar mot sepsis är att sepsis har en två-fasig sjukdomsbild. I början av sjukdomsförloppet löper patientens immunförsvar amok och är överstimulerat, men efter några dagar så infaller istället en förlamning av immunförsvaret. De läkemedelskandidaterna som kliniskt har testats har framförallt fokuserat på att hämma den initiala överstimuleringen utav immunförsvaret. Tyvärr har ingen kandidat kunnat visa effekt på överlevnaden och därför har inte fortsatt användning skett.

Fleromättat fett har visats ha positiva effekter i flera inflammatoriska sjukdomar, så som reumatism och åderförkalkning (åderförfattning). Mättat fett däremot har visats ha negativa effekter på åderförkalkning samt initiera en låggradig inflammation. Därför ville vi undersöka om möss som fått en högfetts diet rik på fleromättat fett överlevde sepsis bättre än möss som fått en högfettsdiet rik på mättat fett. Möss som fått högfetts diet rik på fleromättat fett överlevde till 80% medan möss som fått högfetts diet rik på mättat fett överlevde endast till 20%.

Vidare kunde vi visa att friska möss som fått högfettsdiet rik på fleromättade fetter hade högre koncentration av immuncellerna neutrofiler samt blodstamceller i benmärgen än möss som fått högfettsdiet rik på mättade fetter. Det är neutrofiler som är den viktigaste immuncellen i försvaret mot bakteriella infektioner. Neutrofiler från möss som fått högfettsdiet rik på mättat fett var även bättre på att förflytta sig till inflammationen än neutrofiler från möss som fått högfettsdiet rik på mättat fett. Vi kunde även visa att mekanismen bakom den ökade koncentrationen neutrofiler är högre nivåer av hormonet som stimulerar produktionen av neutrofiler.

En annan intressant aspekt av högfettsdieter är att de påverkar genuttrycket samt metabola parametrar i flera organ. Vi kunde visa att möss som fått högfettsdiet rik på fleromättade fetter skiljde sig kraftigt från högfettsdiet rik på mättade fetter. T.ex. levern från möss som fått högfettsdiet rik på mättade fetter

var kraftigt förfettad medan möss som fått högfettsdiet rik på fleromättade fetter hade ingen förfettnings alls.

Dessa resultat gjorde att vi var övertygade om fleromättade fetters positiva egenskaper för immunförsvaret, men vi visste inte vilken sorts fettsyra som var viktig för dessa resultat. Fleromättat fett kan delas upp i omega-3 och omega-6 fettsyror. För att undersöka vilken fettsyra det är som har de positiva effekterna på immunförsvaret, gav vi möss två nya högfetts dieter; en rik på omega-3 fettsyror och en rik på omega-6 fettsyror. Det var omega-3 fettsyrorerna som har de positiva effekterna på immunförsvaret i våra studier, både på överlevnaden i sepsis och ökningen av immunceller.

Sammanfattningsvis, fleromättade fettsyror, och då särskilt omega-3 fettsyror, har en positiv påverkan på immunförsvaret vid sepsis. Möss som fått äta omega-3 fettsyror har en bättre överlevnad vid sepsis, detta troligen beroende av att omega-3 fettsyror påverkar både produktionen av neutrofiler samt förbättrar deras funktion. På så sätt hjälper omega-3 fettsyrorerna kroppen att bekämpa bakterierna som orsakar sepsis.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

- I. **Svahn, S. L.***, Grahnmemo, L.*, Pálsdóttir, V., Nookaew, I., Wendt, K., Gabrielsson, B., Schéle, E., Benrick, A., Andersson, N., Nilsson, S., Johansson, M.E. and Jansson, J-O. *Dietary Polyunsaturated Fatty Acids Increase Survival and Decrease Bacterial Load during Septic Staphylococcus aureus Infection and Improve Neutrophil Function in Mice*. Infection and Immunity 2015 Feb; 83(2): 514-21.
- II. **Svahn, S.L.***, Väreemo, L.*, Gabrielsson, B., Peris, E., Nookaew, I., Grahnmemo, L., Sandberg, A-S., Wernstedt Asterholm, I., Jansson, J-O., Nielsen, J. and Johansson, M.E. *The Impact of Dietary Fat Composition on the Transcriptomes of Six Tissues Reveals Specific Regulation of Immune Related Genes*. Submitted.
- III. **Svahn, S.L.**, Ulleryd, M.A., Nookaew, I., Osla, V., Beckman, F., Nilsson, S., Karlsson, A., Jansson, J-O. and Johansson, M.E. *Dietary Polyunsaturated Fatty Acids lead to Increased G-CSF and Subsequent Neutrophil Expansion*. In manuscript.
- IV. **Svahn, S.L.**, Ulleryd, M.A., Grahnmemo, L., Ståhlman, M., Borén, J., Nilsson, S., Jansson, J-O. and Johansson, M.E. *Dietary Omega-3 Fatty Acids Increase Survival and Decrease Bacterial Load in Mice Subjected to S. aureus-induced Sepsis*. In revision.

*Authors contributed equally

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ABBREVIATIONS

AA	arachidonic acid
ALA	alpha-linolenic acid
ANOVA	analysis of variance
cDNA	complementary deoxyribonucleic acid
CLP	cecal ligation and puncture
CLS	crown like structure
DHA	docosahexaenoic acid
DXA	dual energy X-ray absorptiometry
EPA	eicosapentaenoic acid
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte macrophage-colony stimulating factor
HFD	high fat diet
HFD-P	high fat diet rich in polyunsaturated fatty acids
HFD-S	high fat diet rich in saturated fatty acids
HFD-S chol	high high fat diet rich in saturated fatty acids, added cholesterol and antioxidants
HFD- ω 3	high fat diet rich in omega-3 fatty acids
HFD- ω 6	high fat diet rich in omega-6 fatty acids
HOMAR-IR	homeostatic model assessment-insulin resistance
HP/C HF-P	high-protein-to-carbohydrate ratio high fat diet rich in polyunsaturated fatty acids
HP/C HF-S	high-protein-to-carbohydrate ratio high fat diet rich in saturated fatty acids
ICAM	intercellular adhesion molecule 1
i.p.	intraperitoneal
i.v.	intravenous
IL-10	interleukin-10
IL-17	interleukin-17
IL-1 β	interleukin-1 beta
IL-3	granulocyte-colony stimulating factor
IL-6	interleukin-6
LFD	low fat diet
LP/C HF-P	low-protein-to-carbohydrate ratio high fat diet rich in polyunsaturated fatty acids

LP/C HF-S	low-protein-to-carbohydrate ratio high fat diet rich in saturated fatty acids
LPS	lipopolysaccharides
MFI	median fluorescence intensity
mRNA	messenger ribonucleic acid
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
MUFAs	monounsaturated fatty acids
NF κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
OGTT	oral glucose tolerance test
ORO	oil red o
pHrodo particles	pHrodo™ Green <i>Staphylococcus aureus</i> BioParticles™
PPAR γ	peroxisome proliferator activated receptor gamma
PUFAs	polyunsaturated fatty acids
real-time quantitative PCR	real-time quantitative polymerase chain reaction
RvD1	resolvin D1
RvD2	resolvin D2
RvE1	resolvin E1
RvE2	resolvin E2
<i>S. aureus</i>	<i>staphylococcus aureus</i>
SFAs	saturated fatty acids
TNF α	tumor necrosis factor alfa
ω -3 PUFAs	omega-6 fatty acids
ω -6 PUFAs	omega-3 fatty acids
WAT	white adipose tissue

INTRODUCTION

In 1970, the Surgeon General of the United States of America said "time to close the book on infectious diseases, declare the war against pestilence won, and shift national resources to such chronic problems as cancer and heart disease". Now, more than 45 years later, it is more than ever abundantly clear that his statement was wrong. Infectious diseases are still a great challenge to health care in both developed and underdeveloped countries.

During my medical education, I spent a brief time in the clinic. In that time, I met these three patients: an old woman with a urinary catheter that had been kept in too long; a middle-aged man with mismanaged type-2 diabetes, with an infected wound on his foot; and finally, a middle-aged dialysis patient with a central vein-catheter. All three of these patients suffered from the infectious disease sepsis, unfortunately, not all of them survived.

Our bodies are constantly under attack from pathogens, and the immune system is our defence. With this thesis, I want to emphasize that the immune system is not disconnected from the rest of the body, but in reciprocal relation with it, and consequently, the immune system affects the body and the body affects the immune system.

Neutrophils are a type of immune cell that is of greatest importance in the defence against sepsis. Therefore, this thesis focuses considerably on neutrophils. The studies herein presented were carried out in mice. Where pertinent, I have stated the differences between human and mice.

Neutrophils

Neutrophils are the most abundant leukocyte in the human body. When the body is not fighting an infection (uninfected state) neutrophils constitute approximately 70% of the total number of leukocytes. In mice blood, however, the concentration is lower (10-25%), but they still constitute a big part of the circulating leukocytes, and have approximately the same function as in humans. During most bacterial infections, these numbers increase.

Function

Neutrophils are the first leukocyte recruited to site of inflammation (Kolaczowska and Kubes 2013). They have the capacity to defend the body from a wide range of infectious pathogens, and are truly important for the

survival of the host. How indispensable they are in the defence becomes clear when either the function or the number of circulating neutrophils is lowered. This happens, for example, in chronic granulomatous disease or chemotherapy-induced neutropenia, where patients suffering from these diseases have a strong susceptibility to infections (Andrews and Sullivan 2003; Autrel-Moignet and Lamy 2014). Nevertheless, overly excited or too numerous neutrophils are also harmful because they secrete tissue damaging agents (Rankin 2010; Day and Link 2012). Tissue damage caused by neutrophils is often observed in diseases such as acute respiratory distress syndrome and rheumatoid arthritis (Furze and Rankin 2008; Rankin 2010; Day and Link 2012), and therefore, the homeostasis of neutrophils is crucial.

Birth to death

Neutrophils are produced in the bone marrow and originate from myeloblasts (Tak, Tesselaar et al. 2013). Myeloblasts, however, do not give rise only to neutrophils, but also to basophils, eosinophils and monocytes (Summers, Rankin et al. 2010). Unlike other myeloid cells, neutrophils are terminally differentiated before they egress from the bone marrow (Wang and Arase 2014). In humans, approximately 10^{11} neutrophils are differentiated per day (Day and Link 2012). It is important to make a distinction between the differentiation of neutrophils under a non-inflammatory state and under an inflammatory state. The differentiation is driven by different factors in the two states.

Under the non-inflammatory state, the most important differentiation factor is the granulocyte-colony stimulating factor (G-CSF) (Kim, De La Luz Sierra et al. 2006; Rankin 2010; Strydom and Rankin 2013). G-CSF is thought to be produced locally in the bone marrow, but its cellular source is yet to be determined (Rankin 2010). Stromal macrophages have been suggested as a source, where G-CSF is produced after phagocytosing apoptotic neutrophils (Furze and Rankin 2008). Whether or not stromal macrophages are the only, or major, source of G-CSF production in the bone marrow remains to be further investigated. Furthermore, the mechanism behind the regulation of G-CSF is still unknown. What is known is that an increase in G-CSF concentration leads to an increased differentiation of neutrophils (Richards, Liu et al. 2003; Rankin 2010).

Under the inflammatory state, more players are involved in the differentiation of neutrophils. It has been shown that interleukin-17 (IL-17) increases the production of G-CSF under inflammatory state (Stark, Huo et al. 2005), but is not required to maintain homeostasis during non-inflammatory state (Ye, Rodriguez et al. 2001; Furze and Rankin 2008). Interleukin-6 (IL-6), interleukin-3

(IL-3) and granulocyte macrophage-colony stimulating factor (GM-CSF) have also been suggested to play a role in neutrophil differentiation under the inflammatory state (Christopher and Link 2007; Manz and Boettcher 2014); however, they are not essential for maintaining homeostasis during non-inflammatory state (Summers, Rankin et al. 2010). Under the non-inflammatory state, it takes 6-7 days for a neutrophil to mature from the myeloblast, while under the inflammatory state, this time can be significantly shortened (Tak, Tesselaar et al. 2013). The shorter differentiation time together with the extra differentiation factors show how important it is for the immune system to be able to increase the production of neutrophils in a time of need.

How are the neutrophils called out from the bone marrow?

Whether a neutrophil is retained within the bone marrow or not, is determined by four key players: the chemokine receptor CXCR2 and its ligand CXCL1, as well as the chemokine receptor CXCR4 and its ligand CXCL12 (Strydom and Rankin 2013). Ligation of CXCL1 to CXCR2 promotes neutrophil egression from the bone marrow, whereas ligation of CXCL12 to CXCR4 promotes neutrophil retention. G-CSF does not only regulate the production of neutrophils, it also regulates the egress of neutrophils from the bone marrow (Lieschke, Grail et al. 1994; Semerad, Liu et al. 2002). G-CSF regulates the egress by decreasing the production of CXCL12 and the expression of CXCR4 on the neutrophils (Semerad, Liu et al. 2002; Kim, De La Luz Sierra et al. 2006; De La Luz Sierra, Gasperini et al. 2007). Treatments with G-CSF have shown to increase the CXCL1 production by endothelial cells in bone marrow (Wang and Arase 2014) (Fig, 1).

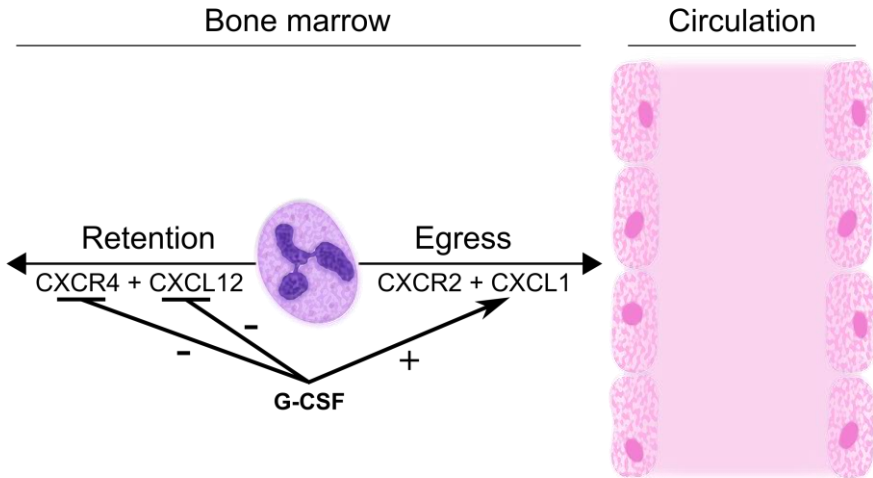


Figure 1. The chemokine receptors CXCR2 and CXCR4, together with their respective ligands CXCL1 and CXCL12, determines if the neutrophil should egress from the bone marrow or remained retained within the bone marrow. When G-CSF is present, the neutrophil down-regulates its CXCR4. A high level of G-CSF also reduces the production of CXCL12 and increases CXCL1 leading to egress of the neutrophil from the bone marrow.

In humans, approximately 10^{11} neutrophils are released from the bone marrow every day (Mauer, Athens et al. 1960). Mature neutrophils in the body are divided into a circulating pool (~49%), a marginated pool (~51%) (Athens, Haab et al. 1961) and a considerably small tissue pool (Summers, Rankin et al. 2010). The neutrophils in the circulating pool are found in blood, whereas the neutrophils in the marginated pool are found in bone marrow, spleen and liver (Summers, Rankin et al. 2010). How and why neutrophils gather in the marginal pool remains to be elucidated. One can speculate that the marginal pool exists to facilitate the rapid mobilization of neutrophils in case of inflammation, or perhaps, that they are patrolling, scouting for a microbial invasion in the tissues where they are situated.

The lungs have also been proposed as a host of neutrophils in the marginated pool (Peters 1998); however, depending on the neutrophil isolation method used, their transit time in the lungs has been shown to be as little as 3.5 seconds longer than for red blood cells (Tak, Tesselaar et al. 2013). Therefore, it is still not known if neutrophils in the marginated pool also inhabit the lungs.

During inflammation, neutrophils are called out from the bone marrow by inflammatory mediators produced at site of inflammation. In humans, these mediators include: complement component 5a, CXCL8, platelet-activating factor, leukotriene B4 (Summers, Rankin et al. 2010; Phillipson and Kubes 2011; Tak, Tesselaar et al. 2013). In mice, the key mediators are CXCL1, CXCL2 and CXCL12 (Day and Link 2012). CXCL2 is the second ligand for the CXCR2 receptor. During non-inflammatory conditions, CXCL12 is involved in the retention of neutrophils in the bone marrow. However, during inflammation, the production of CXCL12 in bone marrow is down-regulated and the systemic production up-regulated, resulting in an inverted gradient that calls out the neutrophils into the circulation (Delano, Kelly-Scumpia et al. 2011).

Tissue infiltration

The neutrophil recruitment cascade consists of capturing, rolling, adhesion, crawling and transmigration (Ley, Laudanna et al. 2007; Kolaczkowska and Kubes 2013). The first step, capturing, starts with the activation of tissue residential leukocytes when encountering pathogens, leading to the release of inflammatory mediators (Kolaczkowska and Kubes 2013). Inflammatory mediators trigger the endothelium to up-regulate the expression of P-selectin and start synthesising E-selectin. The second step, rolling, occurs when these two selectins bind to circulating neutrophils, making them roll on the surface of the blood vessel (Ley, Laudanna et al. 2007). The third step, adhesion, is dependent on the endothelial cell surface molecule intercellular adhesion molecule 1 (ICAM). Even though neutrophils are by now adhered to the endothelium, they might transmigrate into the tissue in another location. This is the fourth step, crawling, and it might occur because neutrophils prefer to transmigrate at cell-cell junctions between the endothelial cells. Crawling is also dependent on ICAM (Kolaczkowska and Kubes 2013). The last step, transmigration, is dependent on several integrins and adhesion protein (Kolaczkowska and Kubes 2013). The neutrophil can either pass paracellularly or transcellularly to arrive to site of inflammation.

Clearing and halftime

As stated previously, too many neutrophils or overly activated neutrophils are hazardous for the body. Therefore, it is of great importance that the degradation and clearing of old neutrophils is carried out in an efficient and controlled manner to prevent any leak of tissue damaging agents when they succumb due to necrosis (Korns, Frasch et al. 2011). The clearance of neutrophils, in the same way as the production, is regulated in two ways depending on the inflammatory state of the body. Under the non-inflammatory state, the neutrophils are cleared

from circulation by macrophages in the organs of the marginal pool: bone marrow, spleen and liver (Saverymuttu, Peters et al. 1985; Furze and Rankin 2008; Rankin 2010). It has been postulated that the clearance of neutrophils in bone marrow and spleen is controlled by their migration to these organs, whereas the liver clearance is constant, regardless of the inflammation status (Furze and Rankin 2008). Up-regulation of CXCR4 precedes neutrophils homing back to the bone marrow, and once they are back, they do not re-enter the circulation, as they become apoptotic and cleared by macrophages (Strydom and Rankin 2013).

Under the inflammatory state, degradation and clearing of apoptotic neutrophils promotes the resolution of the inflammation. At site of inflammation, neutrophils are, as they were under the non-inflammatory state, also cleared by macrophages (Rankin 2010; Wang and Arase 2014). Interestingly, phagocytosis of apoptotic neutrophils by macrophages induce a switch in the macrophages from an inflammatory M1 phenotype to a more anti-inflammatory M2 phenotype that promotes resolution rather than inflammation (Korns, Frasch et al. 2011; Dalli and Serhan 2012; Wang and Arase 2014). Furthermore, apoptotic neutrophils can dampen the recruitment of new neutrophils by producing lactoferrin and annexin A1 (Wang and Arase 2014). However, during inflammatory state, macrophages can also delay apoptosis of neutrophils by releasing the vital signals; interleukin-1beta (Il-1 β), G-CSF and GM-CSF (Wang and Arase 2014).

The lifetime of circulating neutrophils has previously been considered very short, approximately 8 hours in humans and 1.5 hours in mice (Kolaczowska and Kubes 2013). It is important to notice that these numbers are based on experiments that were conducted on neutrophils that were labelled *ex vivo* and then transplanted back *in vivo* to where the time measurements took place. In a more recent study, where neutrophils were labelled *in vivo*, the measured lifetime of neutrophils in circulation was longer: 5.4 days in humans and 12 hours in mice (Pillay, den Braber et al. 2010). An important aspect of this study is that its method was criticized because the bone marrow neutrophils may have also been labelled, leading to an overestimation of the lifetime of the circulating neutrophils (Li, Turner et al. 2011; Tofts, Chevassut et al. 2011). Overall, the exact lifetime of circulating neutrophils is still a topic for debate; however, regardless of the exact time-span, the lifetime for neutrophils is short compared with other leukocytes, such as macrophages and B-cells.

Neutrophils interact with the adaptive immune system

For a long time, neutrophils have been viewed as blunt killers that only get to the site of inflammation to kill pathogens and then die (Jaillon, Galdiero et al. 2013). Nowadays, this view is changing as it has been shown that neutrophils also interact with the adaptive immune system. For example, neutrophils promote naive T-cells to transition into inflammatory T helper type 1 cells, and interact with B-cells in the spleen (Puga, Cols et al. 2012). Also, neutrophils bind to B cell-derived immunoglobulin G and A that have been attached to microbes to facilitate opsonisation (Pasquier, Launay et al. 2005; Tsuboi, Asano et al. 2008). This is a new and interesting aspect of the neutrophils life that needs to be further investigated.

Sepsis

Many people regard sepsis as a disease that used to kill us, but is nowadays treatable. This is, however, not fully true. Sepsis is the most common cause of death in patients admitted to the intensive care unit (Hotchkiss, Monneret et al. 2013) and patients that survives sepsis often sustain morbidity and organ dysfunction (Guirgis, Khadpe et al. 2014).

Sepsis definition

Sepsis is a very broad disease. Patients may suffer from sepsis to different degrees of severity, depending on the underlying diseases.

How to define sepsis has been a topic of considerable discussion, both for facilitating research as well as for improving its diagnosis (Bone, Balk et al. 1992; Dellinger, Levy et al. 2008; Dellinger, Levy et al. 2013). Today, sepsis is defined as the presence, suspected or proven, of infection together with a systemic inflammatory response syndrome (Dellinger, Levy et al. 2013; Gille-Johnson, Hansson et al. 2013). A patient is said to have a systemic inflammatory response when he or she is presenting two or more of the following symptoms: temperature $>38^{\circ}\text{C}$ or $<36^{\circ}\text{C}$, heart rate $>90/\text{min}$, respiratory rate $>20/\text{min}$, and white blood cell count $>12 \times 10^9/\text{l}$ or $<4 \times 10^9/\text{l}$ (Gille-Johnson, Hansson et al. 2013).

If sepsis is not diagnosed or controlled, it can progress into severe sepsis. Severe sepsis is defined as sepsis with additional sepsis-induced organ dysfunction or tissue hypoperfusion (Gille-Johnson, Hansson et al. 2013). An even more severe version of sepsis is when the patient develops septic shock. Septic shock is

defined as a sepsis-induced hypotension that persists despite adequate fluid resuscitation (Gille-Johnson, Hansson et al. 2013).

Epidemiology and etiology

The mortality rate in sepsis differs between studies, but is approximately 10-20% in sepsis, 20-50% in sever sepsis and 40-80% in septic shock (Martin 2012). One positive trend in the fight against sepsis is that the mortality rate among patients with sepsis is declining (Martin, Mannino et al. 2003). Unfortunately, this positive trend is completely eradicated when the incidence is taken into account. The incidence of sepsis is increasing worldwide, and taken together with the mortality rate, the total number of deaths has grown tremendously, in some reports nearly tripled (between year 1979 to 2000) (Martin, Mannino et al. 2003; Russell 2006; Martin 2012). This increase in deaths has been attributed to an increase in our aging population, multidrug resistant pathogens, patients under immunosuppressive treatment and more complex surgeries (Russell 2006).

Sepsis can be caused by bacteria, viruses and fungi (Martin, Mannino et al. 2003; Simonsen, Anderson-Berry et al. 2014), and of all these pathogens, bacteria is the most common cause. Bacteria can be divided into gram-positive and gram-negative bacteria based on the properties of their cell wall and membrane. Between these two types, gram-negative bacteria used to be the most common cause of sepsis up until the late 1980s. Since then, the incidence of sepsis caused by gram-positive bacteria has increased and become the most common (Martin, Mannino et al. 2003). Of all gram-positive bacterium, *Staphylococcus aureus* (*S. aureus*) is the one most frequently isolated from blood (Benfield, Espersen et al. 2007). This may not be so surprising, considering that approximately 20% of the population is colonised with *S. aureus* (Tong, Davis et al. 2015). Colonisation, however, is not to be confused with infection, as it just means that bacteria are found on our body without leading to a disease. Nevertheless, if one of the natural barriers of the body is weakened, either by penetration or immune suppression, the colonising bacteria might take the opportunity to infect the body.

Symptoms and pathophysiology of sepsis

Patients diagnosed with sepsis can have a large variation in symptoms and severity. Many patients suffering from sepsis are treated with immunosuppressive drugs, and therefore, they may not develop any fever or changes in white blood cell count, but may still be in a critical condition. Other patients may already be frail from age or other disease, and therefore, sepsis symptoms may go unnoticed. Both mechanisms lead to difficulties in diagnosing patients quickly and correctly. Several studies have investigated different

biomarkers to find one that is specific to sepsis patients in order to facilitate the diagnosis (Marshall, Vincent et al. 2003). So far, no biomarker specific for all patients diagnosed with sepsis has been found (Stearns-Kurosawa, Osuchowski et al. 2011; Biron, Ayala et al. 2015). Two of the reasons for which such a biomarker has not been found is the heterogeneity among sepsis patients and the pathophysiology of sepsis (Biron, Ayala et al. 2015). Biomarkers could also be beneficial for determine where in the disease progression patients are to be able to better modify their treatment (Stearns-Kurosawa, Osuchowski et al. 2011).

Sepsis has a two-phase pathophysiology, meaning that it starts with an initial hyper-inflammatory state followed by a later hypo-immune state (Deitch 1998; Hotchkiss and Karl 2003; Russell 2006; Kotsaki and Giamarellos-Bourboulis 2012). The hyper-inflammatory state is characterised by a cytokine storm that comes after the activation of the innate immune system (Russell 2006). The released pro-inflammatory cytokines include tumor necrosis factor- α (TNF α) and IL-1 β , both of which lead to the up-regulation of adhesion molecules in the neutrophils and endothelial cells. Anti-inflammatory cytokines such as interleukin-10 (IL-10) are simultaneously released as an attempt to dampen the inflammation. Patients in this phase present symptoms such as shock, fever and hyper-metabolism (Hotchkiss, Monneret et al. 2013). The bodies of some patients cannot withstand the cytokine storm and succumb to the disease; however, most deaths occur during the next state of the disease: the hypo-immune state (Hotchkiss, Monneret et al. 2013). The hypo-immune state occurs when the patient's immune system does not manage to clear the pathogen from the body. Then, the patient is in risk of developing hypothermia, paralysis of the immune system followed by nosocomial infections (Russell 2006) and eventually, death.

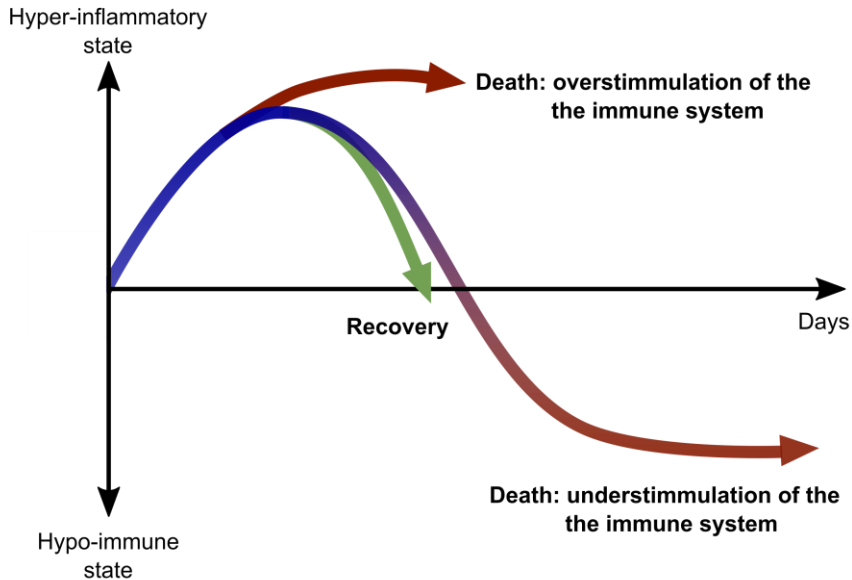


Figure 2. Sepsis has a two-phase pathophysiology with an initial hyper-inflammatory state and a later hypo-immune state. Some patients die in the hyper-inflammatory state, due to over activation of the immune system, but most death occurs in the later hypo-immune state, due to under stimulation of the immune system. Figure modified from Hotchkiss, Monneret *et al.* 2013.

Treatment of sepsis

Today's treatment of sepsis follows the strategy of early-goal directed therapy. This means that the health care giver should promptly administer fluids, broad-spectrum antimicrobials, and oxygen. Of these three actions, the administration of broad-spectrum antimicrobials is the most important (Kotsaki and Giamarellos-Bourboulis 2012). In a retrospective cohort study of patients with septic shock, it was shown that if broad-spectrum antimicrobials are administered within the first hour after the patient is presenting septic shock symptoms, the survival rate was 79.9%. And for each hour that the administration of antimicrobials was delayed, the survival rate decreased with 7.6% over the next 6 hours (Kumar, Roberts et al. 2006).

Since early treatment increases survival, it is of great importance to rapidly find patients with sepsis and to start their treatment as soon as possible. Hence, clinical preliminary diagnoses of sepsis are based on physiological parameters, as there is no time for making any bacteria cultures to determine the origin of the infection nor the pathogen. Treatment often starts with a broad-spectrum

antimicrobial drug, followed by a narrower antimicrobial drug once the pathogen has been identified.

The use of antimicrobial drugs is threatened by increasing antimicrobial resistance. Over the past decades, several new non-antimicrobial drugs have been clinically investigated. Unfortunately, very few, if any, have proven to be effective clinically (Remick 2003). One possible reason for which so many drug candidates have failed might be because of a misguided aim to dampen the cytokine storm during the hyper-inflammatory state. This aim is largely due to misinterpreted results from animal sepsis models where injections of lipopolysaccharide (LPS) have been used to induce sepsis (Hotchkiss and Karl 2003; Remick 2003). Interestingly, it is during the hypo-immune state that patients often succumb to the disease (Hotchkiss, Monneret et al. 2013). Perhaps it is not a question of stimulating or dampening the immune system, but rather modulating it to suit the different states of sepsis.

Antimicrobial resistance

Microbial resistance to available drugs is increasing at an alarming rate worldwide (Levy and Marshall 2004). The world health organisation estimates that within the European Union 25 000 patients die each year from infections caused by multidrug-resistant bacteria, with an associated cost estimated to 1.5 billion Euros every year. (Leung, Weil et al. 2011). Antimicrobial resistance is a naturally occurring phenomenon, but the use of antimicrobial drugs has facilitated the accumulation of antimicrobial resistant pathogens (Levy and Marshall 2004; WHO 2012).

Unfortunately, it is not as easy to get rid of antimicrobial resistance in the world, as it is to obtain it. Bacteria lose their antimicrobial resistance at a very slow pace because the cost of keeping the resistance genes is minimal (Levy and Marshall 2004). Also, the resistance genes are often clustered together with resistance to other antimicrobial or toxic substances on the same plasmid (Summers 2002).

As previously mentioned, gram-positive bacteria are the biggest cause of sepsis (Martin, Mannino et al. 2003) and *S. aureus* is the most frequently gram-positive bacterium isolated from blood (Benfield, Espersen et al. 2007). Unfortunately, *S. aureus* are prone to develop antibiotic resistance. In all isolates of *S. aureus* sent for analysis, approximately 20% are methicillin-resistant *S. aureus* (MRSA) in Europe (Control 2013). In *S. aureus* isolates from the hospitals, 40-60% are MRSA (Weinstein 2001; De Rosa, Corcione et al. 2015).

The combination of increasing antimicrobial resistance, increasing incidence of sepsis and the lack of new, effective pharmacological treatments of sepsis is a deadly cocktail.

Fatty acids

We ingest fatty acids through our food, but they are not only a macronutrient that gives us energy, they also play important roles in our bodies as cell membrane constituents and biologically active substances (Calder 2015). The body can also synthesise fatty acids from molecules like glucose or convert one fatty acid into another fatty acid. Here follows a summary of their structural division and some of their functions in the body.

There are several groups of fatty acids, each of which affect the body in different ways. All fatty acids are built up by a carboxylic acid and an aliphatic chain, and it is the structure of the aliphatic chain that determines which group of fatty acids it belongs to (Figure 3). The crudest division is saturated fatty acids (SFAs) and unsaturated fatty acids, but this division is seldom used. Fatty acids are more commonly divided into SFAs, monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). PUFAs can be further divided into omega-3 fatty acids (ω -3 PUFAs), omega-6 fatty acids (ω -6 PUFAs) and omega-9 fatty acids. Omega-9 fatty acids are rarely found in natural fat sources and will therefore not be further discussed.

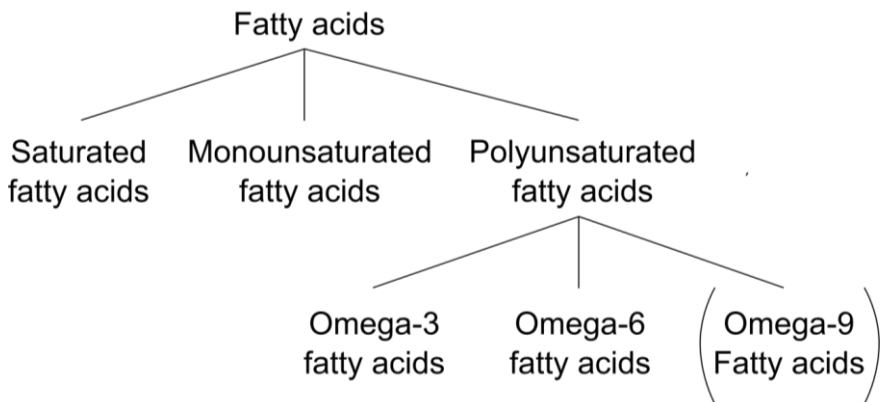


Figure 3. Division of fatty acids.

Saturated fatty acids

SFAs are found in many types of natural fat sources such as lard and palm oil (Calder 2015). Their aliphatic chain does not contain any double bond, which

is what defines them as SFAs. The lack of double bounds makes the aliphatic chains in SFAs straight (Figure 4).

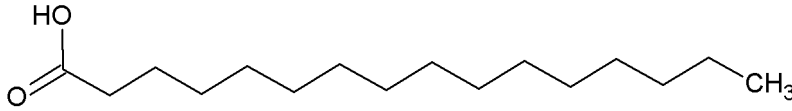


Figure 4. Example of a saturated fatty acid, palmitic fatty acid.

In the body, SFAs are found in the cell membrane (Calder 2015). SFAs are commonly regarded as unhealthy fatty acids (Lee, Sohn et al. 2001). Several studies have shown that replacing SFAs with other types of energy source reduces the risk of cardiovascular diseases (Hu, Mills et al. 2012; Schwab, Lauritzen et al. 2014). An increase of 5% of energy intake from saturated fat, when compared with an equivalent energy intake from carbohydrates, was associated with a 17% increase in the risk of coronary disease (Hu, Stampfer et al. 1997). SFAs may also be involved in the development of type-2 diabetes. Dietary fatty acids affect the composition of serum fatty acids, and a correlation between the percentage of SFAs in serum and the circulating levels of inflammatory cytokine IL-6 has been observed in patients (Fernandez-Real, Broch et al. 2003; Vessby 2003). Since high levels of circulating IL-6 correlate with development of type-2 diabetes (Pradhan, Manson et al. 2001), one could speculate that the intake of SFAs may contribute to the development of type-2 diabetes.

SFAs affect the body by promoting low-grade inflammation. Low-grade inflammation is recognised as a large contributor to the development of cardiovascular diseases (Dessi, Noce et al. 2013). SFAs promote low-grade inflammation by altering gene expression through the activation or inactivation of transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B) and peroxisome proliferator activated receptor gamma PPAR γ (Lee, Sohn et al. 2001; Kennedy, Martinez et al. 2009; Maloney, Sweet et al. 2009). SFAs activate NF κ B, which leads to an up-regulation of pro-inflammatory cytokines (Lee, Sohn et al. 2001). SFAs has the opposite effect on the activation of PPAR γ ; it decreases it (Kennedy, Martinez et al. 2009). However, PPAR γ is regulating anti-inflammatory cytokines, so the effect is an increase in inflammation (Kong, Yen et al. 2010).

Monounsaturated fatty acids

One common dietary source of MUFAs is olive oil, where MUFAs are mostly in the form of oleic fatty acids and, to a small extent, in the form of palmitoleic

fatty acids. Fatty acids that belong to MUFAs have an aliphatic chain that contains one double bond that makes the aliphatic chain have one bend (Figure 5).

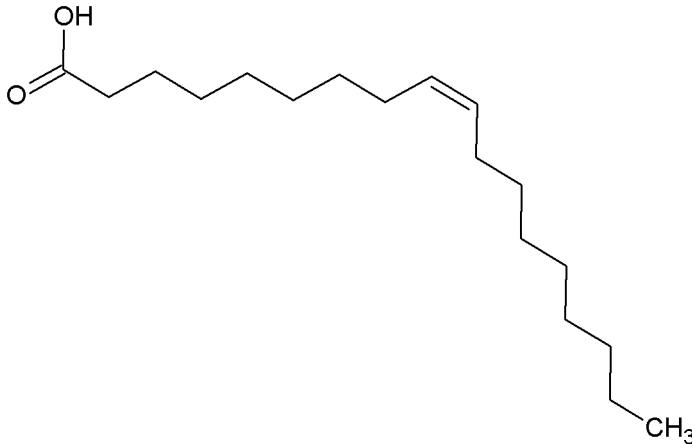


Figure 5. Example of a monounsaturated fatty acid, oleic fatty acid.

The view of the effect of MUFAs on the body and immune system is somewhat divided. MUFAs have been reported to have a small cholesterol lowering effect, when compared to SFAs in diet. However, whether this is effect caused by the consumption of MUFAs or the lower consumption of SFAs is not completely clear (Calder 2015). MUFAs, in the form of oleic fatty acids, have been shown to have a moderate lowering effect on the blood pressure (Bermudez, Lopez et al. 2011).

Polyunsaturated fatty acids

PUFAs are found mostly in fatty fish, nuts and seeds. The aliphatic chain of PUFAs contains several double bonds and has therefore several bends. PUFAs are, as previously mentioned, commonly further divided into ω -3 PUFAs and ω -6 PUFAs depending on where the last double bond is in relation to the omega carbon (Figure 6 and 7).

ω -3 PUFAs

The ω -3 PUFAs that are mostly discussed for their health effects are eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and α -linolenic acid (ALA). EPA and DHA are mostly found in fish oil, whereas ALA is mostly found in plant oil.

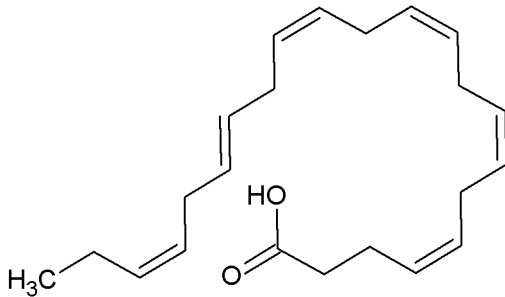


Figure 6. Example of an omega-3 fatty acid, docosahexaenoic fatty acid.

ω -3 PUFAs can be found in cell membranes, where its concentration increases with dietary intake (Calder 2015). ω -3 PUFAs are regarded as anti-inflammatory (Dessi, Noce et al. 2013); therefore, it is not so surprising that dietary intake of ω -3 PUFAs has been reported to have beneficial effects in patients with inflammatory diseases such as rheumatoid arthritis and atherosclerosis (Calder 2012; Miles and Calder 2012). Epidemiology studies have shown that intake of EPA and DHA also protects against cardiovascular diseases (Dessi, Noce et al. 2013).

There are several ways that ω -3 PUFAs mediate their anti-inflammatory effects. For example, it has been found that they affect the transcription factors $\text{NF-}\kappa\text{B}$ and $\text{PPAR}\gamma$ in the opposite way as SFAs (Forman, Chen et al. 1997; Kliewer, Sundseth et al. 1997; Krey, Braissant et al. 1997; Lee, Sohn et al. 2001; Novak, Babcock et al. 2003; Kong, Yen et al. 2010; Dessi, Noce et al. 2013) Another way that ω -3 PUFAs mediate their anti-inflammatory effects is by affecting the production of inflammatory eicosanoids and anti-inflammatory resolvins. Both eicosanoids and resolvins are groups of signal molecules that are mediators and regulators of inflammation but in the opposite ways (Calder 2012; Lim, Park et al. 2015). ω -3 PUFAs reduces the production of inflammatory eicosanoids from arachidonic acid (AA) (a type of ω -6 PUFAs) and increases the production of anti-inflammatory resolvins from EPA and DHA (Calder 2015). Eicosanoids and resolvins will be discussed further below.

ω -6 PUFAs

ω -6 PUFAs are found in different plant oils (Calder 2015). The predominant dietary ω -6 PUFAs found in our diet is linoleic fatty acid and the second most common is AA (Farvid, Ding et al. 2014; Calder 2015).

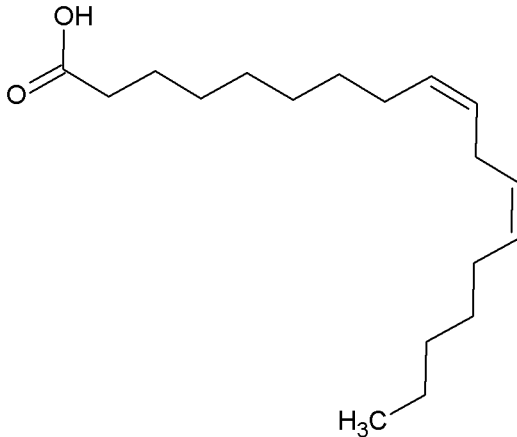


Figure 7. Example of an omega-6 fatty acid, linoleic fatty acid.

The health benefits of ω -6 PUFAs are controversial. For example, linoleic fatty acids have been shown to lower the total cholesterol and low-density lipoprotein concentrations (Mensink and Katan 1992; Mensink, Zock et al. 2003), which may imply a beneficial effect on cardiovascular diseases (Farvid, Ding et al. 2014; Calder 2015). However, linoleic and AA have been shown to activate NF κ B, which leads to an up-regulation of pro-inflammatory cytokines (Camandola, Leonarduzzi et al. 1996; Hennig, Toborek et al. 1996), but at the same time activate PPAR γ , which leads to the opposite effect (Kliwer, Sundseth et al. 1997; Krey, Braissant et al. 1997). Furthermore, AA are also metabolised into inflammatory eicosanoids (Simopoulos 2008). Whether ω -6 PUFAs are pro- or anti-inflammatory remains to be elucidated.

Metabolites from fatty acids

As previously mentioned, ω -3 PUFA and ω -6 PUFA can be metabolised into eicosanoids. ω -3 PUFA can also be metabolised into resolvins. Both eicosanoids and resolvins play roles in the orchestra of inflammation, but with different instruments.

Eicosanoids

Eicosanoids are a group of signal molecules that consist of prostaglandins, thromboxanes and leukotrienes (Simopoulos 2008; Calder 2012). Eicosanoids are generally regarded as pro-inflammatory; however, it is important to keep in mind that this is not the case for all eicosanoids. Prostaglandin E₂ has both pro- and anti-inflammatory effects (Calder 2009), and lipoxin A₄ is anti-inflammatory (Gewirtz, Collier-Hyams et al. 2002).

ω-6 PUFAs, especially AA, are synthesised into inflammatory eicosanoids that play roles in inflammation, inflammatory pain, platelet aggregation, blood clotting and so on (Calder 2015; Dennis and Norris 2015). ω-3 PUFAs are also synthesised into eicosanoids, but to ones with weaker effect than the ones of ω-6 PUFAs. ω-3 PUFAs also decreases the amount of eicosanoids synthesised from ω-6 PUFAs (Calder 2015).

Resolvins

Resolvins are a substance group of metabolites from ω-3 PUFAs (Spite, Norling et al. 2009) that displays anti-inflammatory actions (Lim, Park et al. 2015). DHA is metabolised into resolvin D1 (RvD1) and resolvin D2 (RvD2) (Spite, Norling et al. 2009), whereas EPA is metabolised into resolvin E1 (RvE1) and resolvin E2 (RvE2) (Lee and Surh 2012).

Both RvD1 and RvD2 have been shown to increase survival and decreased bacterial load in blood and peritoneum after cecal ligation and puncture (CLP)-induced sepsis (Spite, Norling et al. 2009; Chen, Fan et al. 2014). Resolvins have also been shown to reduce inflammatory pain (Lim, Park et al. 2015), and have been proposed as a treatment option in cancer (Murray, Hraiki et al. 2015).

The mechanisms behind the effects of the resolvins are not yet fully understood. It has been suggested that RvD1 acts by binding to the lipoxin A₄ receptor and the GPR32 receptor which leads to blocking the activity of NFκB (Krishnamoorthy, Recchiuti et al. 2010), but further investigation is warranted.

Protein

Another component in our diet that has also been suggested to impact the immune system is protein. Proteins are built up by amino acids. Dietary supplementation of the amino acid glutamine has been shown to increase survival after MRSA infection (Suzuki, Matsumoto et al. 1993). Glutamine has also been shown to increase the phagocytosis rate of neutrophils and macrophages (Parry-Billings, Evans et al. 1990; Calder and Yaqoob 1999), and

to improve the ability to kill *S. aureus* (Ogle, Ogle et al. 1994). In the protein diets that were used in paper I, the amino acid with the highest concentration was glutamic acid, which can be converted into glutamine in the body.

Also, high-protein-to-carbohydrate high fat diets (HFDs) have previously been shown to decrease body weight compared with low-protein-to-carbohydrate HFDs (Raymond, Wang et al. 2012). Since it has previously been suggested that obesity increases the risk of infection (Falagas and Kompoti 2006) we saw it as interesting to investigate if mice fed a high-protein-to-carbohydrate HFDs would have increased survival in *S. aureus*-induced sepsis.

AIM

General aim

The general aim of this thesis was to investigate how different dietary fatty acid composition affect the survival in *S. aureus*-induced sepsis. Since neutrophils are so fundamental for the defence against bacterial sepsis, particular attention was given to them.

Specific aims

- Paper I The aim of “*Dietary Polyunsaturated Fatty Acids Increase Survival and Decrease Bacterial Load during Septic Staphylococcus aureus Infection and Improve Neutrophil Function in Mice*” was to investigate how the differences in dietary fat composition affect survival and bacterial load after experimental septic infection as well as neutrophil function in uninfected mice.
- Paper II The aim of “*The Impact of Dietary Fat Composition on the Transcriptomes of Six Tissues Reveals Specific Regulation of Immune Related Genes*” was to investigate how the dietary fatty acid composition affects the total transcriptome profile, and especially the immune related genes, in the six different tissues important for metabolic and immunological function: Skeletal muscle, bone marrow, white adipose tissue, brown adipose tissue, spleen and liver.
- Paper III The aim of “*Dietary polyunsaturated fatty acids lead to increased G-CSF and subsequent neutrophil expansion*” was to further investigate the impact of polyunsaturated fatty acids on the frequency and distribution of neutrophils in mice.
- Paper IV The aim of “*Dietary omega-3 fatty acids increase survival and decrease bacterial load in mice subjected to S. aureus-induced sepsis*” was to investigate which of two HFDs, one rich in ω -3 PUFAs and one rich in ω -6 PUFAs, was most efficient at increasing survival and decreasing bacterial load after experimental septic infection. An additional aim was to further investigate the neutrophil function in uninfected mice.

METHODOLOGICAL CONSIDERATIONS

The methods used in this thesis are described in detail in each of the appended papers. In this section, general considerations about the methods are presented.

Table 1. Summary of the methods used in this thesis.

Methods	Paper I	Paper II	Paper III	Paper IV
Sepsis model	✓			✓
Diets and treatment	✓	✓	✓	✓
Flow cytometry	✓		✓	✓
Gene expression analysis	✓	✓		
Body composition	✓			✓
Neutrophils characterization	✓		✓	✓
Immunohistochemistry		✓	✓	
Blood analysis			✓	
Protein determination			✓	
Metabolic analysis		✓		

Experimental protocol

The designs of the studies in the four appended papers had a similar set up; however, the readout and diets differed. In all papers, all the animals were male C57BL/6J mice, and they were fed different diets for 8 weeks before the analyses. The C57BL/6J mice were chosen because it is well documented that this strain gains weight and develops an obesity phenotype similar to the one seen in humans (hyperinsulinemia, hyperglycemia, and hypertension) on a HFD (Collins, Martin et al. 2004). The mice included in the survival, bacterial load and

thioglycollate analyses remained on their designated diets throughout the experiment. Male mice, instead of female or mixed genders, were chosen because of the higher incidence of sepsis in men compared with women (Martin, Mannino et al. 2003) and for practical reasons: to exclude hormone-cycle-variations that would be present in female mice.

Animal models

Sepsis models

The first focus of this thesis work was to investigate how different diets affect the survival in sepsis (paper I and IV). In the literature, there are several different sepsis models mimicking different aspects of human sepsis pathology; however, none of them is perfect. Patients with sepsis develop symptoms and organ failure over the course of several days. Therefore, models in which the death of the animals occur within hours do not reflect the situation in humans (Deitch 1998). The sepsis model should also mimic the two-phase pathology that human sepsis exhibits.

Sepsis models are usually divided into three types: injections of LPS into different compartments (e.g. intravenously (i.v.) or intraperitoneal (i.p.)), cecal ligation and puncture (CLP), and injection of pathogen intravenously (Deitch 1998).

LPS, also known as endotoxin, is found in the outer membrane of gram-negative bacteria (Wichterman, Baue et al. 1980). LPS induces a strong immune response in the host (Wichterman, Baue et al. 1980; Deitch 1998). When LPS is used to induce sepsis, it can be administered in different ways of which the most common are intravenously and intraperitoneal injection. This model gives a repeatable effect and is easy to induce. However, LPS is only a small part of gram-negative bacteria, and not a whole living cell. Therefore, no new production of LPS will occur after the injection. Another shortcoming of this model is that LPS leads to death without an infection. LPS overstimulates the immune system of the host, causing an inflammation process that kills it. This effect may lead to the following paradox: mice with a dysfunctional immune system survive LPS-administration, whereas mice with a functional one succumb.

CLP is a polymicrobial sepsis model where the host is subjected to both gram-positive and gram-negative bacteria (Cuenca, Delano et al. 2010). In short, the CLP model consists of opening the peritoneal cavity of an animal, ligating its cecum, and puncturing it with a needle (Rittirsch, Huber-Lang et al. 2009;

Cuenca, Delano et al. 2010). Animals subjected to this model present septic symptoms over a period of days, and therefore, this model is considered to be a very good one for sepsis studies, but it has some disadvantages (Dejager, Pinheiro et al. 2011). Since CPL is a surgical procedure, it is up to the operator to limit the variations between the mice. These variations include opening of the peritoneal cavity, the percentage of the cecum that is ligated (which will induce different amount of necrotic tissue), the gut flora of the mouse, the immune system's ability to close off the infection by abscess formation, and the pressure applied to the cecum, which impacts the leakage of bacteria to the peritoneal cavity (Dejager, Pinheiro et al. 2011).

By choosing intravenously **injection of pathogen**, we were able to choose which bacteria strain to use. Since sepsis caused by gram-positive bacteria has the highest increase in incidence, we chose to use *S. aureus* LS-1 in all the studies. LS-1 is coagulase and catalase positive, and produces large amounts of toxic shock syndrome toxin 1 (Verdrengh and Tarkowski 1997). An important concern with this model is that the animals receive all the bacteria at one time instead of over a longer period. However, since we see a progress in the animals' symptoms throughout the experiment, as well as an accumulation of bacteria in the kidneys with abscesses, we do believe that this model reflects the two-phase pathology in humans.

Neutrophil models

The second focus of this thesis has been to investigate how different diets affect the neutrophils (paper I, III and IV). Many different types of cells can be studied using cell lines in cell cultures. However, for neutrophils, this is not feasible since they are not possible to culture. There are some modified neutrophil cell lines, but they should be used with great cautions since they are not particularly similar to human or mice neutrophils (Amulic, Cazalet et al. 2012).

Diets and treatment

Diets

Ten different diets were used in this thesis, and all were bought from Research Diets, New Brunswick, NJ.

One low fat diet (LFD, D12450B), five HFDs with different fat source and four high or low protein vs. carbohydrate diets with different fat sources. The HFDs were as following; HFD rich in SFAs (HFD-S, D12492), HFD rich in SFAs

with added cholesterol and antioxidants (HFD-S Chol, D09121703), HFD rich in PUFAs (HFD-P, D09020505), HFD rich in ω -3 PUFAs (HFD- ω 3, D09120501) and HFD rich in ω -6 PUFAs (HFD- ω 6, D10031504). The four protein/carbohydrate diets were as following: high-protein-to-carbohydrate ratio (HP/C) HFD rich in SFAs (HP/C HF-S, D13091403), low-protein-to-carbohydrate ratio (LP/C) HFD rich in SFAs (LP/C HF-S, D13091404), HP/C HFD rich in PUFAs (HP/C HF-P, D13091405) and LP/C) HFD rich in PUFAs (LP/C HF-P, D13091406). The composition of these diets is shown in detail in table 2 and 3. Since the fat comes from natural sources instead of synthesized ones, there are some batch variations. The numbers shown in table 1 and 2 are from the most recent batch.

LFD and HFD-S have the same source of macronutrients, but differ in total fat amount. HFD-S is a commonly used HFD to induce obesity. HFD-P was designed to have similar content of macronutrients as HFD-S, but to differ in the fat source so as to obtain another fatty acid composition. In HFD-P, some of the lard was replaced with menhaden fish oil. The cholesterol and antioxidant levels differed between HFD-S and HFD-P, and both, the cholesterol and antioxidant levels have been reported to affect the immune system (Casas, Sacanella et al. 2014; Mangge, Becker et al. 2014; Scheiermann, Frenette et al. 2015). Therefore, another HFD-S diet was used (HFD-S Chol) with supplemented extra cholesterol and antioxidants to match the levels in HFD-P.

HFD- ω 3 and HFD- ω 6 were used to investigate the effect of ω -3 PUFAs and ω -6 PUFAs separately, since they are combined in HFD-P. In HFD- ω 3 and HFD- ω 6, the menhaden fish oil was replaced with ROPUFA 75EE and safflower oil respectively. In HFD- ω 3, the amount of ω -3 PUFAs was similar to the one in HFD-P. For technical reasons it was not possible to increase it without the diet turning into liquid. However, the amount of ω -6 PUFAs in HFD- ω 6 is much higher than in HFD-P.

In the four protein diets, the total amount of fat was kept constant, whereas the source of the fat was changed as well as the amount, but not the source, of proteins and carbohydrates. The fat sources were the same as in HFD-S (soybean oil and lard) and HFD-P (soybean oil, lard and menhaden fish oil). Further, two different amounts of proteins and carbohydrates from the same source were combined with the same amount of fats from two different sources: HFD-S (soybean oil and lard) and HFD-P (soybean oil, lard, and menhaden fish oil). This rendered in one diet with a HP/C with the same fat source as in HFD-S (HP/C-HF-S), one diet with a LP/C with the same fat source as in HFD-S (LP/C-HF-S), one diet with a HP/C with the same fat source as in HFD-P

(HP/C-HF-P) and one diet with a LP/C ratio with the same fat source as in HFD-P (LP/C-HF-P).

Treatment with resolvins

As mentioned previously, ω -3 PUFAs can be metabolized into resolvins. Mice treated with resolvins have been reported to have increased survival in CLP-induced sepsis (Spite, Norling et al. 2009; Lee and Surh 2012). In paper IV, to investigate if mice fed HFD-S could be saved if treated with resolvins, we fed mice HFD-S for 8 weeks, inoculated them with *S. aureus* and treated them with RvD1, RvD2 or vehicle i.v. on day 1-4 after inoculation. The mice were monitored for 17 days for survival study and 6 days for bacterial load analysis.

Table 2. Diets, low fat diet and the corresponding high fat diets.

	LFD	HFD-S	HFD-S Chol	HFD-P	HFD- ω 3	HFD- ω 6
Energy density (kcal/g)	3.9	5.2	5.2	5.2	5.2	5.2
Macronutrients (% kcal)						
Protein	20	20	20	20	20	20
Carbohydrate	70	20	20	20	20	20
Fat	10	60	60	60	60	60
Fat source (% of total fat)						
Soybean oil	55.6	9.3	9.3	9.3	9.3	9.3
Lard	44.4	90.7	90.7	27.8	62.2	27.8
Menhaden oil	-	-	-	63.0	-	-
ROPUFA 75EE	-	-	-	-	28.5	-
Safflower Oil	-	-	-	-	-	63.0
Total antioxidants (wt%)	0.000	0.000	0.0044	0.0044	0.0044	0.0044
Total cholesterol (wt%)	0.000	0.027	0.098	0.097	0.098	0.098
Fatty acids (% by wt of total fatty acids)						
Σ SFA	22.7	32.0	32.0	28.7	23.6	16.0
Σ MUFA	29.8	36.0	36.0	27.5	28.3	19.9
Σ PUFA	47.5	32.0	32.0	43.9	48.1	64.1
Σ ω-3 total fat	5.2	2.1	2.1	22.6	24.7	1.2
Σ ω-6 total fat	42.4	29.9	29.9	18.1	23.4	62.9
ω-6/ω-3	8.2	14.1	14.1	0.8	0.9	51.9

SFA, saturated fatty acids; MUFA; monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ω -3, omega-3 fatty acids; ω -6, omega-6 fatty acids.

Table 3. Diets, high-protein-to-carbohydrate ratio and low-protein-to-carbohydrate ratio, with corresponding source of fatty acids.

	HP/C-HF-S	LP/C-HF-S	HP/C-HF-P	LP/C-HF-P
Energy density (kcal/g)	5.2	5.2	5.2	5.2
Macronutrients (% kcal)				
Protein	30	10	30	10
Carbohydrate	10	30	10	30
Fat	60	60	60	60
Fat source (% of total fat)				
Soybean oil	9.3	9.3	9.3	9.3
Lard	90.7	90.7	27.8	27.8
Menhaden oil	-	-	63.0	63.0
Fatty acids (% by wt of total fatty acids)				
∑ SFA	32.0	32.0	28.7	28.7
∑ MUFA	36.0	36.0	27.5	27.5
∑ PUFA	32.0	32.0	43.9	43.9
∑ ω-3 total fat	2.1	2.1	22.6	22.6
∑ ω-6 total fat	29.9	29.9	18.1	18.1
ω-6/ω-3	14.1	14.1	0.8	0.8

SFA, saturated fatty acids; MUFA; monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ω-3, omega-3 fatty acids; ω-6, omega-6 fatty acids

Flow cytometry

Flow cytometry is an essential element of this thesis. In short, flow cytometry is a technique that enables investigation of cells in single cell suspension. Figure 8 shows the gating strategy for the phagocytosis capacity analysis in blood as an example of data obtained from flow cytometry (see section phagocytosis capacity analysis for more details on this specific method). The first plot is used to single out the singlets (Figure 8A). Cells may clot together during the preparation and staining process and if they are used in the analysis, they will interfere with the results. Therefore, only the singlets are used in the subsequent plots. Blood that has been challenged with pHrodo™ Green *S. aureus* BioParticles™ (pHrodo particles) contain immune cells, but also other, non-immune cells. To exclude them from the analysis, an excluding gate was used (Figure 8B) (for more information, see further down). Then, by measuring the

size and granulation of the cells (Figure 8C) and combining it with labelling extracellular or intracellular protein structures, one can divide the cells into populations (Figure 8D), subpopulations (Figure 8E), expression level of a protein (Figure 8D), etc.

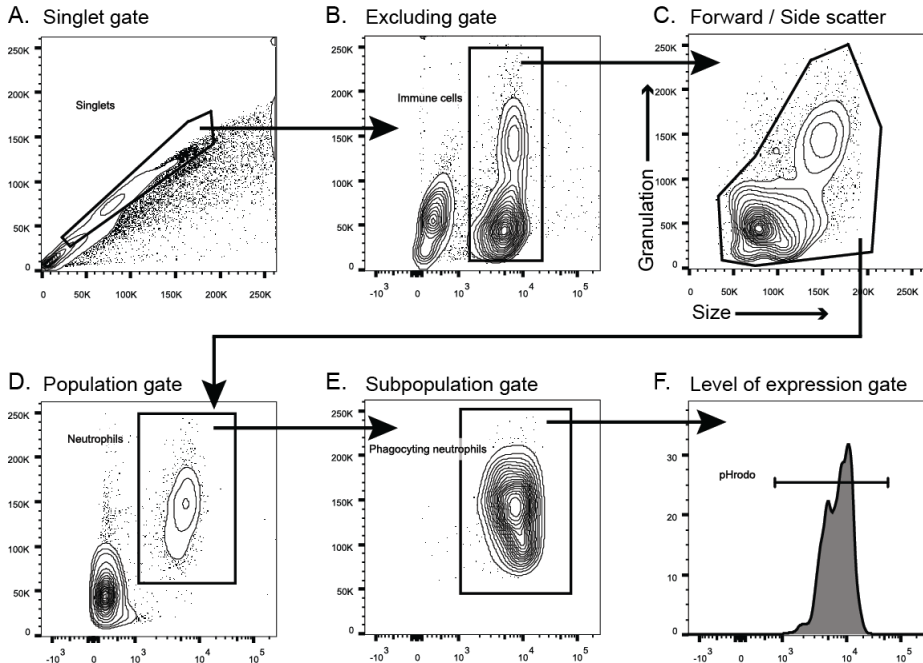


Figure 8. Gating strategy showing an example flow cytometry data. The strategy includes singlet gate, exclusion gate, forward/side scatter (size and granulation separation), population gate (Ly6G⁺ neutrophils), subpopulation gate (Ly6G⁺pHrodo⁺ neutrophils) and level of protein expression histogram (median fluorescence intensity of Ly6G⁺pHrodo⁺ neutrophils).

When flow cytometry data is analysed, it is important remember that the results do not represent the number of cells of a certain type in a population, but their frequency in said population. The frequency is calculated as follows:

frequency of population of interest = n positive cells of interest / n total cells (positive + negative cells)

Therefore, particular regard to how the data is interpreted is required. Figure 9 presents the considerations made regarding the frequency of neutrophil found in bone marrow to exemplify how this issue was handled in this thesis. One could argue that the increased frequency of neutrophils found in mice fed PUFA/ ω -3 PUFAs is in fact just a decrease in the other cell populations.

Immune cells mostly found in bone marrow:

Lymphocytes (B- and T-cells)

Monocytes

Neutrophils

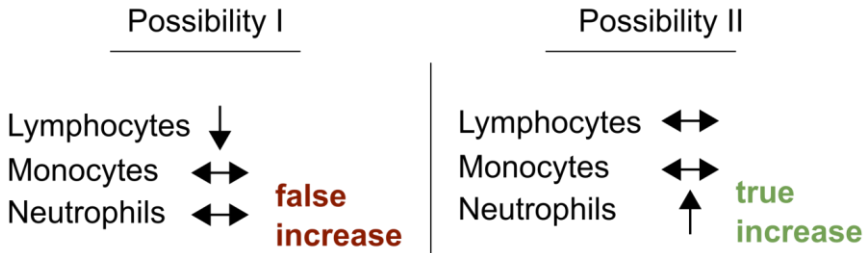


Figure 9. Which of the cell population changes is measured in flow cytometry analysis? Special consideration of how to interpret data from flow cytometry has to be made because the data shows cell frequency: number of cells in relation to other populations.

In the neutrophil analysis, the cell populations found in the denominator (n total cells) are mostly lymphocytes (B- and T-cells), monocytes and neutrophils. Therefore, there are two possibilities on how to interpret the measured increase in neutrophil frequency. Possibility I is that the number of neutrophils did not increase, and that the observed increase in frequency is due to a decrease on the lymphocyte population. Possibility II is that the number of neutrophils did increase, and therefore, that this increase is the reason behind the measured increase in frequency; the populations of lymphocytes and monocytes are unaffected by the diets. This is the statement made in paper I, III and IV. The basis for this consideration was that in previous investigations we had found that LFD, HFD-S, and HFD-P do not affect the response of the adaptive immune system to antigens (data not shown).

Another aspect of importance when analysing flow cytometry data is the type of cells in the denominator of the cell frequency formula. The cells in the denominator are “ n of total cells (positive + negative)”. When analysing a rather homogenous tissue, such as bone marrow, where most cells are immune cells, this detail may not be of great importance. However, when analysing the phagocytosing capacity of the circulating neutrophils, this detail was of great importance. In our analyses, cells that were not immune cells were scattered among the immune cells on the forward side scatter plot. The non-immune cells were disturbing the analysis of the immune cells, so we needed to exclude them

from the analysis. In the phagocytosing capacity analysis of the blood, the exclusion was made through the immune cell marker CD45 (Figure 8B). By defining all CD45⁺ immune cells as the “Number of total cells (positive + negative)”, the non-immune cells were excluded from the analysis.

In flow cytometry, the expression level of a protein in a cell population sample is measured by labelling the protein of interest with appropriate antibody, and then measuring the median fluorescence intensity (MFI). If the protein is highly expressed, the level of MFI will also be high. Figure 8F shows a histogram of the fluorescence intensity of the sample. The higher the MFI is, the more to the right the main body of the histograms is. This analysis was done for the pHrodo particles in paper IV to estimate how many pHrodo particles each neutrophil had phagocytised.

Dead cells are another thing that may disturb flow cytometry analyses, as they can lead to unspecific binding of the antibody and affect the cell population frequency calculations. In the initial studies, this issue was handled by using the dead/alive marker 7AAD. The marker 7AAD is a dye that binds to dead cells, allowing their exclusion from the flow cytometry analyses. We stopped using this technique once it was determined that only a few percent of all cells in the analyses were dead.

As just mentioned, samples that are to be used for flow cytometry analysis can not be dead. An exception is if the cells are fixated. This means that the cells have been treated to not change. In paper III, this was done on the neutrophils from the bone marrow that were analysed based on their CXCR2 and CXCR4 expression. We have previously noticed that the CXCR4 is down-regulated after the cell has been isolated from the bone marrow. To get an accurate measurement of the expression, the bone marrow was fixated in formaldehyde. An important aspect when using fixated cells is that not all antibodies work on fixated cells and must therefore be carefully tested in advance.

Gene expression analysis

Gene expression analysis is a cluster term for techniques to analyse the expression of genes in a tissue or a sample, which include microarray and real-time quantitative polymerase chain reaction (real-time quantitative PCR). Both techniques quantify the amount of messenger ribonucleic acid (mRNA) in a sample as a measurement of the gene expression. In paper I and II, microarray analysis was used, and in paper II, real-time quantitative PCR was used.

Some aspects have to be taken into consideration when working with gene expression analysis. It is important to keep in mind that mRNA is an unstable molecule that degrades fast. To minimise its degradation, one must shorten the extraction time and keep the samples thawed for as little time as possible. In paper I and II, the degradation of the mRNA in the bone marrow samples was minimised by extracting them into RNAlater, which stabilises the mRNA. A final aspect to keep in mind is that measuring mRNA in a tissue is not the equivalent of measuring the protein level. It only gives an estimation of the protein levels.

Microarray analysis

The gene expression in a tissue or a sample can be measured with microarray analysis. Microarray is preferable when a characterisation of the whole genome is wanted, instead of single gene(s). Microarray has this capacity because it analyses thousands of genes at the same time. In short, RNA was extracted from the sample, converted into complementary deoxyribonucleic acid (cDNA), fragmented and biotin-labelled before being loaded to a microarray chip. Microarray chips consist of cDNA sequences attached to a solid phase. When the labelled samples are loaded on the chip, their cDNA hybridizes to their corresponding cDNA sequence, emitting a light that is measured. The intensity of the emitted light corresponds to the amount of RNA in the sample.

In paper II, we wanted to investigate the effect of diets on the global gene expression in six different tissues. Microarray was the technique of choice since thousands of genes are analysed at the same time, and is therefore an unbiased way of looking at gene expression. This approach generated huge amounts of data that allowed us to use GO-term analysis. With GO-term analysis genes that control the same process are clustered together under a GO-term (e.g. immune responses). This technique enables a better overview of the processes affected in a tissue rather than just single genes.

Real-time Quantitative Polymerase Chain Reaction analysis

Real-time Quantitative PCR is a suitable method when one, or a few, genes are to be analysed. There are two main subtypes of real-time quantitative PCR divided based on the method of detecting the copies of the gene DNA-binding dyes reporter method and fluorescent reporter probe method. In paper I and II, DNA-binding dyes reporter method was used, with SYBR Green dye as the dye. In short, the RNA was extracted from the sample and converted into cDNA. Then, copies of the gene of choice were made and subsequently detected by adding: a set of primers (chosen to match the gene of choice), deoxyribose

nucleotide triphosphates (building blocks to make the copies), DNA polymerase (to put the building blocks together) and SYBR Green dye (to detect the copies).

The extracted data was relative gene expression. To be able to compare the gene expression between samples, a reference gene was used. A good reference gene is a gene that has a stable expression level and is not affected by experimental setups. Beta-actin was used as the reference gene in paper II.

Body composition measurement

It is well established that C57BL/6J mice fed HFDs develop obesity (Collins, Martin et al. 2004). Therefore it was natural to investigate the body composition of the mice in our experiments. Dual energy X-ray absorptiometry (DXA) is a technique used to analyse the body composition. This technique gives information about bone density, fat mass and lean mass. In short, in this technique, x-rays were divided by a filter into high- and low-energy photons. Bone density, fat mass and lean mass were calculated based on the amount of different photons that each tissue absorbed. The absorbance is proportional to the density of the tissue; therefore, of all the tissues in the body, bone is the one with the highest absorbance. (Pietrobelli, Formica et al. 1996).

The benefit of using this technique to determine body composition is that it is non-invasive, and can therefore be performed on living animals. To prevent the animals from moving during the measurements, they need to be anaesthetised. However, it is important to consider which anaesthetic agents to use. Several studies have shown that different anaesthetic agents affect cells in the immune system differently (Lee, Kim et al. 2007; Zhang, Liu et al. 2013). Our mice were anesthetized with isoflurane. While isoflurane has been reported to affect neutrophils (Lee, Kim et al. 2007), the effects are considered to be smaller than the ones of other types of anaesthetic agents. Furthermore, since the effects of isoflurane on neutrophils were only seen during the first 24 hours after anaesthesia (Lee, Kim et al. 2007), the mice in our studies were left to rest and recover for at least 5 days after undergoing isoflurane anaesthesia during DXA measurements. No measurements were carried out during the resting period, and all groups were subjected to the same amount of anaesthesia.

A final aspect of DXA that is of importance when using this technique for determining body composition is that the obtained body fat represents total body fat mass and not total adipose tissue mass (Goodpaster 2002). It cannot distinguish different fat depots from each other. In paper II, this problem was addressed by dissecting the different fat depots and weighting them individually.

Neutrophils characterization

Since the flow cytometry analysis revealed that the diet had an effect on the neutrophil frequency, we wanted to further investigate other effects that the diets could possibly have on the neutrophils. Not only the total amount of neutrophils is of importance for an effective immune response to an infection, other factors such as migration capacity, phagocytosis capacity and neutrophil apoptosis/necrosis are also important.

Migration capacity analysis (peritoneal lavage)

To investigate the migration capacity, peritoneal lavage was performed and the peritoneal fluid was collected (paper I). In short, thioglycollate (irritant substance) was injected into the peritoneal cavity to produce a local inflammation. The local inflammation is induced since thioglycollate is insoluble in saline and is therefore crystallized in the body of the mice. The inflammation results in recruitment of immune cells, and depending on the time span after the injection, the composition of the immune cells found in the peritoneal lavage will change. Neutrophils are the first to arrive at site of inflammation. The highest concentration of neutrophils in the peritoneal lavage is found after approximately 4 hours. If the analysis would have been performed after 24 hours, the concentration of neutrophils would have decreased and the concentration of macrophages increased. The measurement of the frequency of neutrophils found in peritoneal lavage was performed with flow cytometry.

Phagocytosis capacity analysis (pHrodo)

To investigate the phagocytosis capacity of the circulating neutrophils, the blood from mice fed LFD, HFD-S, HFD- ω 3 and HFD- ω 6 were challenged with pHrodo particles (paper IV). This is a method that makes it possible to calculate the frequency of neutrophils that have phagocytosed these particles and how many particles each neutrophil has phagocytosed. pHrodo particles are modified *S. aureus*. They are inactivated, unopsonised *S. aureus* that are conjugated to a particle that is only fluorescent when it is in an acidic environment, as it is in the phagosome. Other phagocytosis kits also contain bacteria/particles that neutrophils can phagocytise, but require quenching of the bacteria/particles stuck on the extracellular part of the neutrophils. No quenching step is required for the pHrodo particles since only phagocytosed pHrodo particles emit fluorescent light, which is a benefit of the pHrodo-kit. However, even though the manufacturer of the pHrodo-kit claims that only phagocytosed pHrodo particles emit fluorescence, they still recommend the use of the anti-phagocytosis agent, cytochalasin D, as a control of the background fluorescence when MFI is calculated. This was done for the samples in paper IV; the MFI

value for the sample with cytochalasin D was subtracted from the MFI value for the sample (MFI sample – MFI negative control).

Neutrophil apoptosis and necrosis analysis

In paper III, we wanted to investigate if the time that the neutrophils were alive, *in vitro*, differed between the groups. Therefore, we performed a neutrophil apoptosis and necrosis analysis. In short, the blood was collected and the neutrophil fraction was extracted, incubated (0 hours, 3hours or 14hours) and labelled with AnnexinV-Fluos, 7AAD and Gr-1. AnnexinV-Fluos is a marker for apoptotic cells, 7AAD a marker for dead cells and Gr-1 is a granulocyte marker, and therefore, apoptotic neutrophils were identified as 7AAD-Gr-1⁺AnnexinV-Fluos⁺ and necrotic neutrophils as 7AAD⁺Gr-1⁺AnnexinV-Fluos⁺. The percent of apoptotic neutrophils was calculated as apoptotic neutrophils / total Gr-1⁺ cells, whereas the percent of necrotic neutrophils was calculated as necrotic neutrophils / total Gr-1⁺ cells. The analysis was performed using flow cytometry. Ly6G is a more specific marker for neutrophils than Gr-1, which also is a marker for eosinophils and basophils. Ly6G have been the marker of choice for the other analyses on neutrophils throughout this thesis. However, Gr-1 was considered to be a satisfactory marker for analyses conducted in blood since eosinophils and basophils only constitute 0.084% and 0% respectively of the total immune cell in blood (Hedrich 2006).

Immunohistochemistry

Immunohistochemistry was used to investigate the location of different cell types in the tissues. Immunohistochemistry is a method used to visualize cells or structures based on their protein expression. In contrast to flow cytometry, immunohistochemistry is performed on sections of tissue rather than single cell suspensions. In short, thin sections of the tissue were stained with a primary antibody that binds to the structure of choice. A secondary antibody that contains a substrate that can be visualised, and thereby detected, was thereafter added. To elucidate the morphology and facilitate the orientation in the section, a nuclear staining was also added. After the staining, a subsequent quantification was performed for the crown-like-structures (CLS) (paper II). The quantification was performed as follows: WAT from 8 mice per group was stained, 1-3 areas from each animal's WAT were randomly selected, and the number of CLS in each area was counted. The numbers of CLS were then adjusted for the total area selected.

The other immunohistochemistry analysis included in this thesis did not require any quantification. In paper III, only the localisation of the neutrophils found in

the spleen was investigated with the immunohistochemistry staining. It was preferable to quantify the neutrophils in spleen using flow cytometry instead of immunohistochemistry because of the morphology of the spleen. The spleen contains both red and white pulp, but the neutrophils were found only in the area of the red pulp close to the white pulp. Therefore, the size and shape of the white pulp influence the distribution of the neutrophils to a large extent. This influence is not something that has to be taken into consideration when quantification is performed using flow cytometry, and therefore, flow cytometry was the method of choice for quantification of the neutrophils in spleen, but not the location analysis.

Another consideration required when performing immunohistochemistry is unspecific binding of the antibody. Many immune cells, including neutrophils contain fc-receptors on their surface. If these receptors are not blocked, they might bind the antibody used for staining and will therefore lead to unspecific binding. In our studies, the receptors were blocked. To control that the blocking was efficient, an isotype control was used. An isotype control is an antibody that has the same fc-region as the antibody used for staining, but is lacking an antigen-binding site. If not all fc-receptors are adequately blocked the sections with the isotype control will also render staining.

Histological staining

A third visualisation assay for sections was used in paper II, histological staining. To visualise the fat in the liver, the sections were stained with Oil red O (ORO) (paper II). ORO is a dye that binds to fat and does not require antibodies. ORO stains the fat in a section with bright, red colour; therefore, a quantification based on the intensity of the red stain could be used. Since this can be automated, the analysis was made in an unbiased way.

Blood analysis

Changes in the concentration of immune cells in the blood can be a sign of disease, e.g. an increase in the number of neutrophils is regarded as a sign of inflammation. Therefore, we wanted to investigate the blood composition in mice that had been fed different diets. Human blood is routinely analysed in the clinic. However, it is not as easy to analyse mouse blood. Analysis of mouse blood performed using flow cytometry usually has a large sample spread and day variations; therefore, it is hard to draw strong conclusions from the data. Another commonly used method for analysing blood is to use blood analysing machines developed for human blood. This is not a good option either since human immune cells differ considerably from mouse immune cells, both in size

and morphology. In paper III and IV, a VetH5 blood analyser was used. This is a blood-analysing machine developed for veterinarian use, and therefore, it is adapted to handling the smaller size of mouse immune cells. Unfortunately, it can not discriminate between neutrophils, eosinophils and basophils. However, as mentioned previously, eosinophils and basophils only constitute 0.084% and 0% respectively, of the total immune cell in mouse blood and can therefore be disregarded.

Protein determination

Since the neutrophils frequency in bone marrow was affected by the diet, we wanted to investigate if this effect was due to changes in G-CSF protein levels. Therefore, the bone marrow from mice fed different diets was collected and analysed to determine the G-CSF protein levels (paper III). The measurement of G-CSF protein levels was performed using multiplex analysis. Bone marrow is hard to pipette to an exact volume because it is not a liquid. Therefore, bone marrow from one whole femur was used and the G-CSF protein levels were normalized to the total protein concentration.

The protein levels of several cytokines in serum were also determined through a multiplex analysis. Serum, however, is easy to pipette to exact volumes, so in this case the samples could be compared to each other without normalization to the total protein concentration.

Metabolic analysis

Obesity affects the metabolic status of the body. Therefore, in paper II, the following different metabolic parameters were investigated: fasting B-glucose, homeostasis model assessment of insulin resistance index (HOMAR-IR index) and oral glucose tolerance test (OGTT).

Fasting B-glucose gives a value of how high the normal B-glucose levels are in the animals.

HOMA-IR index gives an estimation of the insulin-sensitivity in the animals. The HOMA-IR index is calculated as fasting blood glucose (mmol/l) x fasting serum insulin ($\mu\text{U}/\text{ml}$) / 22.5 (Matthews, Hosker et al. 1985). A high value of the HOMA-index indicates insulin-insensitivity.

OGTT is a technique where the subject is challenged with a specific amount of glucose orally and the plasma glucose level is monitored at several time points.

To analyse the ability to restore the plasma levels after the challenge, the area under the curve is calculated. Metabolically healthy subjects restore their plasma glucose levels faster than metabolically unhealthy subjects.

Statistics

In the appended papers in this thesis, several different types of statistical methods have been used. Statistical methods must be chosen carefully to accurately describe the data. It is equally wrong to miss a true difference, as it is to show a false one.

In this thesis, the most commonly used statistical model to analyse the data was a one-way analysis of variance (ANOVA) followed by a *post hoc* test. However, not all the data was analysed this way because in some cases it was not appropriate. For example, a flow cytometry analysis has to be made on fresh cells (unless they are fixed). If the number of animals in an experiment is too big, it is not always possible to analyse all the animals in one day. This limitation introduces a day variation that has to be accounted for in the statistical model. For these cases, the data was analysed with a two-way ANOVA with a day factor followed by a *post hoc* test. Two-way ANOVA was also used when other factors (such as multiple experiment), were included in the analysis.

In paper IV, the bacterial load for the mice treated with resolvins was analysed with a two-way ANOVA with contrast as the *post hoc* test. Contrast was used because the hypothesis was that resolvins reduce the bacterial load after *S. aureus*-induced sepsis, and we wanted to analyse two resolvins (RvD1 and RvD2) as one group without pooling them. With contrast the two resolvins' average were compared with the vehicle's average. Pooling both resolvins into one group would have been to regard them as having the same effect, whereas with contrast they were regarded as two individual treatment groups, but with the similar type of effects.

The experimental data was not always normally distributed. In such cases, a log-transformation was used to normalize it. If the normalization was successful, the normalized data was analysed and then back-transformed to be presented as geometrical mean (e.g. G-CSF protein level in paper III). If the normalization was not successful, a non-parametrical Kruskal-Wallis test followed by a Mann-Whitney test was used. These two tests were used when some samples in an analysis were set to zero (e.g. bacterial load in paper I).

Survival analysis were performed in paper I and IV. The method of choice was the non parametric log-rank test, and the data was presented with Kaplan-Meier

curves. In this model no covariate can be taken into consideration; therefore, to investigate if weight at infection affected survival, instead a cox-regression was used. Cox regression is a semi-parametric method for survival analysis.

SUMMARY OF RESULTS AND DISCUSSION

In the following section, the results from the appended papers are summarised and discussed.

Dietary Polyunsaturated Fatty Acids Increase Survival and Decrease Bacterial Load During Septic *Staphylococcus aureus* Infection and Improve Neutrophil Function in Mice (paper I)

Prior to the studies included in this thesis, our group had shown that mice fed HFD-S had decrease survival and increase bacterial load in *S. aureus*-induced sepsis compared with mice fed LFD (Strandberg, Verdrengh et al. 2009). There were three hypotheses for the effect seen in these animals:

1. Mice fed HFD-S were obese compared with mice fed LFD. It could be argued that it was the obesity that led to the decreased survival in mice fed HFD-S (Falagas and Kompoti 2006).
2. The dietary recommendation for a healthy diet has for a long time been to reduce the total fat amount. Therefore, it could also be argued that the survival was affected by the total fat amount in the diet.
3. As mentioned previously, there are several different types of fatty acids, which have been shown to have different effects on the body. Therefore, it could be argued that it was the type of fat that affected the survival.

To investigate these questions further, a third diet, HFD-P, was included. The macronutrients in HFD-P were matched in source and amount to HFD-S except for the fat source, which was rich in polyunsaturated fatty acids (Table 2). The mice were fed the diets during 8 weeks before they were inoculated with *S. aureus* i.v. to induce sepsis. Interestingly, mice fed HFD-P survived *S. aureus*-induced sepsis at least as good as mice fed LFD, and also had increased survival and decreased bacterial load compared with mice fed HFD-S (Figure 10 A and B).

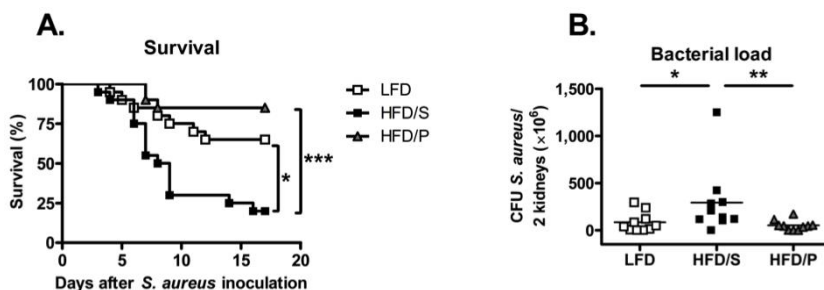


Figure 10. (A) Survival 0-17 days after inoculation and (B) bacterial load 6 days after inoculation. Dietary groups: low fat diet (LFD), high fat diet rich in saturated fatty acids (HFD-S) and high fat diet rich in polyunsaturated fatty acids (HFD-P). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ From Svahn, Grahnemo *et al.* Infect Immun. 2015 Feb;83(2):514-21

The mice fed HFD-P were obese compared with mice fed LFD, but less obese than mice fed HFD-S. Despite these differences, a cox-regression analysis of the survival with body weight as a covariate showed no correlation between survival and body weight ($p=0.779$). This result refutes the first and second hypotheses. Obesity does not affect survival, and neither does total fat amount in the diet. HFD-S and HFD-P contained the same amount of fat, and both made the mice obese; however, mice fed HFD-P had increased survival. Since the only difference between these two diets were the types of fatty acids, we concluded that it was the type of fatty acids that affected the survival in *S. aureus*-induced sepsis. This conclusion is strengthened by several studies that highlight the importance of the type of fatty acid in the development of different inflammatory diseases, such as coronary heart disease and type 2 diabetes (Hu, Stampfer *et al.* 1997; Flock and Kris-Etherton 2011; Schwab, Lauritzen *et al.* 2014).

Since SFAs are generally regarded as unhealthy fat (Lee, Sohn *et al.* 2001), it could be argued that the observed effects were do to the loss of SFAs in the HFD-P diet, rather than positive effects of the PUFAs. This explanation, however, is very unlikely. The HFD-P had a large amount of SFAs compared with HFD-S (28.7% and 32% respectively). It is more likely that the large increase in survival is induced by positive effects of the PUFAs, rather than the small changes in the amount of SFAs.

Cholesterol and antioxidants are two other dietary components that have been reported to affect the immune system (Casas, Sacanella *et al.* 2014; Mangge, Becker *et al.* 2014; Scheiermann, Frenette *et al.* 2015). The HFD-S contained less cholesterol and antioxidants than the HFD-P (Table 2). To determine whether the cholesterol and antioxidants affected the survival of mice, a

modified version of HFD-S was produced. The modified diet, named HFD-S Chol, had the same amount and source of macronutrients as the HFD-S, but with cholesterol and antioxidants to the same concentration as the HFD-P. HFD-S Chol was used in a bacterial load assay, which showed that mice fed HFD-S Chol had increased bacterial load 6 days after inoculation compared with mice fed LFD and HFD-P. This result indicated that cholesterol and antioxidant levels might affect the bacterial load, but to a negligible extent compared with the one of the fatty acid composition (Figure 11).

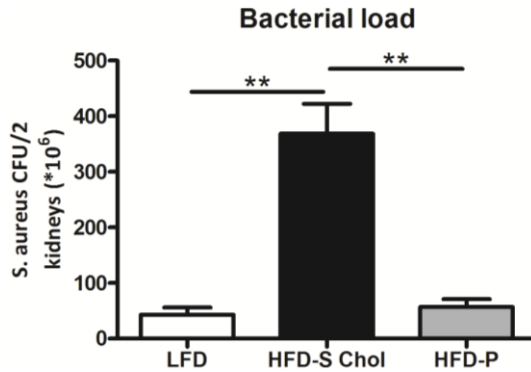


Figure 11. Bacterial load 6 days after *S. aureus* inoculation. Dietary groups: low fat diet (LFD), high fat diet rich in saturated fatty acids with added cholesterol and antioxidants (HFD-S Chol) and high fat diet rich in polyunsaturated fatty acids (HFD-P). HFD-S is supplemented with extra cholesterol and antioxidants to the same concentration as found in HFD-P. **, $p < 0.01$.

We postulated that the mechanism behind this strong effect on survival was somewhere in the immune system. Therefore, different parts of the immune system were investigated. Mice fed HFD-P had increased frequency of Ly6G⁺ neutrophils and CD117⁺ precursor cells in their bone marrow at uninfected state. This result implied that the HFD-P affected the immune system before the infection. This hypothesis was strengthened by the fact that once the mice were inoculated with *S. aureus*, their food consumption was minimal.

To further investigate the effects of PUFAs on the neutrophils, their migration capacity was investigated as a functional assay. The assay showed that mice fed HFD-P had increased frequency of neutrophils in their peritoneal lavage 4 hours after injection of thioglycollate compared with mice fed HFD-S. This result indicated that not only the frequency of neutrophils in the bone marrow, was affected by the diet, but also their migration capacity.

The effect of dietary fatty acids on the frequency of Ly6G⁺ neutrophils in the mice's blood was also investigated. Unfortunately, the result was unclear. Three repetitions of the experiment gave conflicting results: two showed no difference between the groups, and one showed a strong difference. To try reaching a conclusion, the data of the three experiments was pooled ($n=39-40$ mice per group) in a statistical analysis with the experiment as a factor. The analysis showed that mice fed HFD-P had a tendency of increased frequency of Ly6G⁺ neutrophils in blood compared with mice fed HFD-S ($p=0.056$). It is unclear if this difference would persist after a fourth experiment or not.

Increased leukocyte concentration in blood is regarded as a sign of infection. To rule this out as an explanation for the increased frequency of Ly6G⁺ neutrophils in both bone marrow and possibly in blood, pro-inflammatory cytokines in serum were analysed. The analysis showed that no pro-inflammatory cytokines in serum were affected by the diets (Table 4). Also, if mice fed HFD-P had a systemic inflammation, the frequency of monocytes should also be affected. However, there was no difference in monocyte frequency in bone marrow between the mice.

Table 4. Cytokines in serum, at uninfected state.

	LFD	HFD-S	HFD-P
IL-1β	928.4 \pm 106.6	950.5 \pm 130.8	855.3 \pm 132.7
IL-6	29.5 \pm 5.6	34.3 \pm 7.3	36.9 \pm 10.2
IL-10	164.0 \pm 24.6	249.9 \pm 45.2	179.7 \pm 34.5
IL-17	983.3 \pm 153.8	1262.3 \pm 134.3	737.0 \pm 196.7
IFNγ	280.9 \pm 38.1	334.7 \pm 61.2	273.9 \pm 66.2
MCP-1	1050.3 \pm 150.4	1024.3 \pm 152.4	942.4 \pm 161.0
TNFα	24646.1 \pm 3943.7	28269.9 \pm 5427.7	23046.9 \pm 6339.8

As mentioned previously, diets with high protein content might have beneficial impacts on the immune system (Parry-Billings, Evans et al. 1990; Suzuki, Matsumoto et al. 1993; Ogle, Ogle et al. 1994; Calder and Yaqoob 1999). Therefore, four diets with different protein concentration and fat sources were produced: HP/C-HF-S, LP/C-HF-S, HP/C-HF-P and LP/C-HF-P. Table 3 shows the details of the composition of these diets.

Mice fed the HP/C-HF-S, HP/C-HF-P and LP/C-HF-P seemed to have an increased survival during the first week after inoculation compared with mice fed LP/C-HF-S (Figure 12). However, after the first week, mice fed HP/C-HF-

S also started to die at a faster rate and at day 17 after inoculation, there was no difference in survival between mice fed HP/C-HF-S and LP/C-HF-S. Thus, the amount of protein in the diet did not affect the overall survival in *S. aureus*-induced sepsis. However, giving a diet with high amount of protein might perhaps create a window for initiating treatment. Furthermore, also in these four new diets, we could demonstrate that dietary fatty acid composition was important for predicting survival, which further strengthened our conclusion about the beneficial effects of polyunsaturated fatty acids.

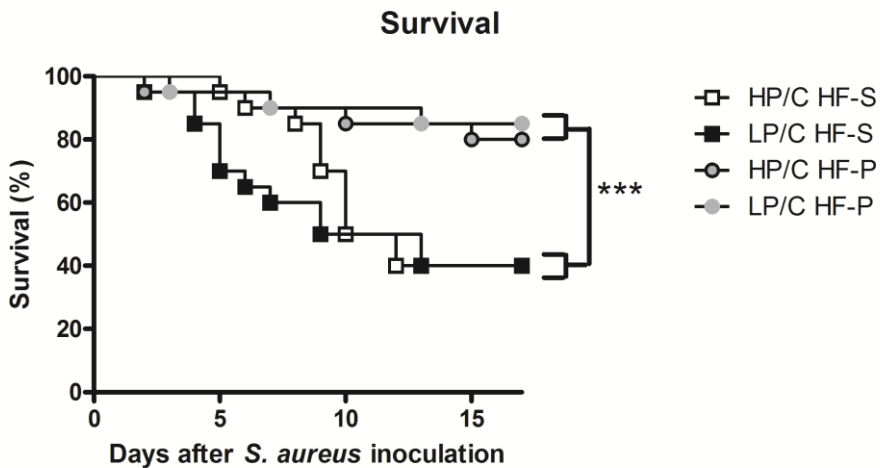


Figure 12. Survival 0-17 days after inoculation. Dietary groups; high protein-to-carbohydrate ratio and saturated high-fat diet (HP/C HF-S), low protein-to-carbohydrate ratio and saturated high-fat diet (LP/C HF-S), high protein-to-carbohydrate ratio and polyunsaturated high-fat diet (HP/C HF-P) and low protein-to-carbohydrate ratio and polyunsaturated high-fat diet (LP/C HF-P). ***, $p < 0.001$ From Svahn, Grahnmö et al. Infect Immun. 2015 Feb;83(2):514-21

The Impact of Dietary Fat Composition on the Transcriptomes of Six Tissues Reveals Specific Regulation of Immune Related Genes (paper II)

To get a better understanding of how dietary fat composition affects the body in general, and the immune system in particular, we investigated the total transcriptome profile in six different tissues: bone marrow, spleen, skeletal muscle, white adipose tissue, brown adipose tissue and liver.

This analysis showed that the dietary fat composition affected the tissues differently. Interestingly, the HFDs (HFD-S and HFD-P) induced a specific

gene profile in the white (visceral) adipose tissue (WAT) and the brown adipose tissue. The visceral WAT was hypertrophied in both mice fed HFD-S and HFD-P. Hypertrophied WAT has been shown to secrete pro-inflammatory cytokines (Weisberg, McCann et al. 2003; Xu, Barnes et al. 2003). This seem to be true when it comes to the WAT from mice fed HFD-S since they had increased numbers of crown-like structures (CLS) in their WAT compared with mice fed LFD. However, we could not see an increase in CLS in WAT from mice fed HFD-P, indicating that PUFAs, even though they lead to hypertrophy of the WAT, induce a healthier phenotype in WAT compared with SFAs. On the other hand, brown adipose tissue was mostly affected by HFDs *per se*, and there was no clear difference between mice fed HFD-S and HFD-P.

In liver, HFDs *per se*, induced a particular gene profile not observed in mice fed LFD. Interestingly, a second gene profile was induced by HFD-P on top of the HFDs effect. After further investigations, we determined that mice fed HFD-S had significant liver steatosis, whereas the liver from mice fed HFD-P completely lacked steatosis.

Spleen was almost exclusively affected by HFD-P. Both genes involved in the adaptive as well as in the innate immune system were affected by the HFD-P. Interestingly, HFD-P led to a down-regulation of these genes in spleen compared with mice fed HFD-S and LFD. This result goes in line with PUFAs' anti-inflammatory properties.

Both skeletal muscle and bone marrow were rather inert to the dietary fat composition. This was particularly surprising for the bone marrow, since we showed in paper I that the dietary fat composition had a great effect on survival in *S. aureus*-induced sepsis as well as the frequency and function of the neutrophils. This might, however, be explained by the high cut-off for significant difference ($q < 0.001$). If the cut-off would have been lower, more genes would have been regarded as significant. We chose to have this high cut-off to compensate for the high number of comparisons (six different tissues).

While the effects of dietary fat on the gene expression in single tissues have been previously studied (Richards, Wood et al. 2014; Kolehmainen, Ulven et al. 2015), our study, to our knowledge, is the first one where such a large number of tissues have been analysed at once to allow for a more complete analysis of the transcriptome data.

When this paper is published, the total transcriptome data from our experiments will be made publicly available. As previously mentioned, the aim of this paper was to determine the effects of dietary fat composition on the immune system;

however, other researchers may find the data useful for other types of analyses or purposes.

Dietary Polyunsaturated Fatty Acids Lead to Increased G-CSF and Subsequent Neutrophil Expansion (paper III)

Since neutrophils are such an important immune cell when it comes to fighting off bacterial infections, and since dietary PUFAs had such a large effect on them, the aim of this third paper was to answer two main questions:

1. How does PUFAs increases the frequency of neutrophils in the bone marrow?
2. Are neutrophils in other tissues in the body also affected by dietary fatty acids?

The frequency of a cell population in tissue depends on four parameters: the cell flows to and from the tissue, the production (in e.g. bone marrow) and the degradation of the cells in the tissue (Figure 13). To determine which of these parameters is affected by the PUFAs when it comes to the frequency of neutrophils in the bone marrow, a series of investigations were undertaken.

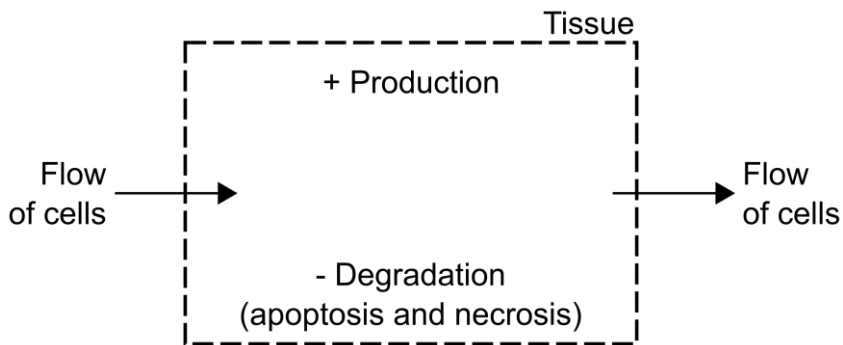


Figure 13. Schematic image showing the parameters that control the cell population in a tissue.

To investigate the **production** of neutrophils in bone marrow, we measured the G-CSF protein levels in mice fed LFD, HFD-S and HFD-P since, as previously mentioned, G-CSF is essential for the production of neutrophils (Kim, De La Luz Sierra et al. 2006; Rankin 2010; Strydom and Rankin 2013). Mice fed HFD-P had higher levels of G-CSF protein in their bone marrow compared with mice fed HFD-S ($p=0.051$).

To investigate the **apoptosis** and **necrosis** of neutrophils, we measured the frequency of neutrophils that had been stained with AnnexinV (apoptosis marker) and 7AAD (marker of dead cells) in blood at 0 hours, 3 hours and 14 hours after harvesting the blood. Blood was used instead of bone marrow for practical reasons. The percent of apoptotic and necrotic neutrophils did not differ between the diet groups at any of the time points.

The neutrophils' egress (**outflow**) out from the bone marrow is dependent on their expression of CXCR2 (Strydom and Rankin 2013). To investigate the egression of neutrophils from bone marrow, the frequency of Ly6G⁺CXCR2⁺ neutrophils was measured. There were no differences between any of the groups.

Investigating the **inflow** of neutrophils into the bone marrow is harder. Neutrophils that are called back to the bone marrow for degradation have increased expression of CXCR4 (Martin, Burdon et al. 2003); however, newly differentiated neutrophils, which are retained in the bone marrow, also have increased CXCR4 expression (Kim, De La Luz Sierra et al. 2006), making it hard to discriminate between these two populations of neutrophils. In an attempt to determine whether or not diet affects the inflow, the frequency of Ly6G⁺CXCR4⁺ neutrophils was measured. There was no difference in the frequency of Ly6G⁺CXCR4⁺ neutrophils between mice fed HFD-S and HFD-P. Therefore, we concluded that despite the lack of discrimination between neutrophils that had been called back to the bone marrow and neutrophils that were retained in the bone marrow, neither the inflow nor the retaining of neutrophils contributed to the total frequency of neutrophils in the bone marrow. Consequently, we further concluded that the increased frequency of Ly6G⁺ neutrophils in bone marrow from mice fed HFD-P was in large part due to an increase in production stimulated by G-CSF.

The second question that we wanted to answer was whether or not the HFD-P affected the frequency of neutrophils in tissues other than bone marrow. Flow cytometry analysis revealed that mice fed HFD-P had increased frequency of Ly6G⁺ neutrophils in spleen and liver compared with mice fed HFD-S. Both of these tissues filtrate large quantities of blood, and therefore, one could argue that the neutrophils had passively ended in these two tissues instead of actively recruited there. Since kidneys also filtrate large quantities of blood, they were also included in the analysis. No, or very few neutrophils were found in the kidneys of the mice in any of the dietary groups; therefore, we concluded that the increased frequency of Ly6G⁺ neutrophils in spleen and liver was due to recruitment. How and why the neutrophils are recruited there is a matter of speculation. While one could argue that the neutrophils found in spleen and

liver are there to be destroyed, the gene expression analysis of the mice spleens included in paper II revealed several neutrophil related genes that were up-regulated in mice fed HFD-P. mRNA is quickly degraded and dead or dying cells do not have up-regulated genes. Therefore, the neutrophils found in spleen were not there to be degraded, but rather seem to have a purpose.

The location of the neutrophils in the spleen also indicates that they are there to interact and not to be destroyed. It has previously been shown that neutrophils interact with B-cells in the marginal zone of the spleen (Puga, Cols et al. 2012); the same location as we find the neutrophils in our mice.

Dietary Omega-3 Fatty Acids Increase Survival and Decrease Bacterial Load in Mice Subjected to *S. aureus*-induced Sepsis (paper IV)

As previously mentioned, PUFAs can be divided into ω -3 PUFAs and ω -6 PUFAs. In paper I, both types of fatty acids were mixed in the HFD-P, so, in this paper, we wanted to investigate which type of fatty acids was the one with the beneficial effects on the immune system seen in paper I-III. Two new HFDs were investigated: one rich in ω -3 PUFAs (HFD- ω 3) and one rich in ω -6 PUFAs (HFD- ω 6) (Table 2). The HFD- ω 3 contained a rather significant amount of ω -6 PUFAs (for technical reasons), whereas the HFD- ω 6 had a very small amount of ω -3 PUFAs. The protocol was the same as in paper I. Mice fed HFD- ω 3 had increased survival compared with both mice fed HFD-S and HFD- ω 6. Furthermore, mice fed HFD- ω 3 had decreased bacterial load compared with mice fed HFD-S (Figure 14A and B).

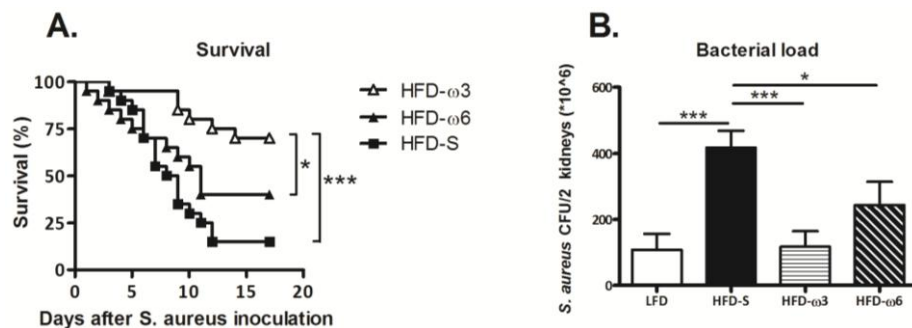


Figure 14. (A) Survival 0-17 days after inoculation and (B) bacterial load 6 days after inoculation. Dietary groups; low fat diet (LFD), high fat diet rich in saturated fatty acids (HFD-S), high fat diet rich in ω -3 PUFAs (HFD- ω 3) and high fat diet rich in ω -6 PUFAs (HFD- ω 6). *, $p < 0.05$; ***, $p < 0.001$.

To further strengthen our conclusion from paper I, that the body weight did not affect survival, we once again did a cox-regression analysis of the survival with body weight as a covariate. The result was the same as in paper I: the body weight did not affect survival ($p=0.56$).

In agreement with the findings of paper I, the frequency of Ly6G⁺ neutrophils and CD117⁺ precursor cells were increased in mice fed HFD- ω 3 compared with mice fed HFD-S. This was not a general increase of immune cells caused by a general inflammation because there was no difference in frequency of monocytes in bone marrow or pro-inflammatory cytokines in serum between mice fed HFD- ω 3, HFD- ω 6 and HFD-S.

In paper I, we also showed an increased frequency of neutrophils recruited to the site of inflammation in mice fed HFD-P compared with mice fed HFD-S. To further investigate the effect of dietary fatty acids on the function of the neutrophils, we measured the phagocytosing capacity of circulatory neutrophils from mice fed LFD, HFD-S, HFD- ω 3 and HFD- ω 6, and found that mice fed HFD-S had decreased frequency of phagocytosing neutrophils in circulation.

Our results show that two mechanisms can explain the increased survival of mice fed HFD- ω 3 compared with mice fed HFD-S: mice fed HFD- ω 3 had increased frequency of Ly6G⁺ neutrophils and CD117⁺ precursor cells in their bone marrow, and mice fed HFD-S had neutrophils with lower phagocytising capacity.

As mentioned previously, ω -3 PUFAs can be metabolised into resolvins. Because resolvins have been shown to improve survival in CLP-induced sepsis, we investigated if mice fed HFD-S could be saved through treatment with RvD1 and RvD2. Unfortunately, the treatment did not increase the overall survival. The survival in mice treated with RvD1 or RvD2 did not differ from mice treated with vehicle on day 17 after inoculation. However, there was a potential beneficial survival effect of the resolvin treatment on day 8 after inoculation, and there was a difference in bacterial load on day 6 after inoculation. The discrepancy between our results and previously published data might be due to the sepsis model used. Both Spite, Norling *et al.* and Chen, Fan *et al.* used CLP-induced sepsis. For Spite, Norling *et al.* death occurred within the first 2 days after induction, while for Chen, Fan *et al.* death occurred within 6 days (Spite, Norling *et al.* 2009; Chen, Fan *et al.* 2014). This is early in the disease development and most likely during the hyper-inflammatory state, which might not mimic the clinical settings particularly well, where most deaths occur during the later hypo-immune state (Hotchkiss, Monneret *et al.* 2013). The mechanisms for resolvins have been suggested to be anti-inflammatory and

limitation of the neutrophils' migration. These proposed mechanisms for resolvins fit well with treatments for the hyper-inflammatory state, but not for hypo-immune states. Our survival experiments are conducted over 17 days and therefore include both the hyper-inflammatory and the hypo-immune state.

CONCLUDING REMARKS

Taken together, the results from the appended papers show that dietary fatty acids affect the immune system in a substantial way. The increased survival in sepsis due to the consumption of PUFAs/ ω -3 PUFAs, seen in paper I and IV, is most likely due to diet induced changes in the immune system that take place before the infection. These changes range all the way from changed expression level of the genes, to frequency of neutrophils in different tissues and neutrophil function.

The studies presented in this thesis suggest that ω -3 PUFAs affect the immune system positively. ω -3 PUFAs are generally regarded as anti-inflammatory (Dessi, Noce et al. 2013). Perhaps it is time to start regarding them as immunomodulators? If ω -3 PUFAs only had anti-inflammatory properties, they would not increase the frequency of neutrophils or precursor cells in the bone marrow under a non-inflammatory state. It seems like ω -3 PUFAs act as anti-inflammatories when dampening of the immune system is warranted, and boosters of the immune system when needed. This dual action is very beneficial against diseases like sepsis that have a two-phase pathophysiology.

A considerable number of drugs that treat sepsis by suppressing the inflammatory response have failed in clinical trials (Hotchkiss and Karl 2003; Remick 2003). While the reason for their failure is not clear, it seems appropriate to explore other mechanisms for treating sepsis. Despite our results, resolvins may still be a candidate for treating sepsis. However, to determine whether they are an effective treatment or not, further testing is necessary. The tests should be carried out with a model other than CLP, unless CLP is performed with a lower bacterial burden, *i.e.* to allow the animals to live longer and reach the hypo-immune state.

FUTURE PERSPECTIVES

Although the results published in this thesis increase our understanding of the effects of dietary fatty acids on the immune system, they inevitably lead to additional questions.

Which ω -3 PUFA(s) is the one that causes the positive effects on the immune system? In paper I and throughout paper II and III, where we showed that dietary PUFAs have positive effects on the immune system, we started to untangle the effects of different types of HFDs. In paper IV we managed to dig one step further and show that the positive effects are mediated by ω -3 PUFAs. The next natural step would be to clarify which ω -3 PUFA(s) cause the positive effects. The two most abundant fatty acids in natural sources and in the diets presented in this thesis, EPA and DHA, are the two most likely candidates.

For technical reasons, we were not able to fully exclude the ω -6 PUFAs from HFD- ω 3. A second possible concern with the HFD- ω 3 diet is that it is produced from ROPUFA 75EE oil, which is squeezed fish. It is possible that there are other components in the fish-oil that also cause positive effects on the immune system. To work around these two technical issues and strengthen the evidence supporting ω -3 PUFAs as the one causing the positive effects, it would be interesting to use *fat-1* mice. *Fat-1* mice contain the *fat-1* gene from *Caenorhabditis elegans* and can therefore convert ω -6 PUFAs into ω -3 PUFAs (Woo, Lim et al. 2015). By feeding one group of *Fat-1* mice and another group of conventional mice HFD- ω 6, it would be possible to both eliminate the possibility that it is something else in the ROPUFA 75EE oil that has the positive effects and also get a cleaner distinction between ω -3 PUFAs and ω -6 PUFAs effects.

How does it look during inflammatory state? We show that dietary fatty acids have an effect on the immune system under non-inflammatory state, an interesting next step is to investigate the effects after infection during the inflammatory state. Since infection/inflammation is a dynamic process, it is impossible to draw any conclusions from one time point. However, some of our preliminary data, where we have looked on different cytokines in serum at different time points after infection, indicates that there is a time dependent cytokine profile. Further investigations of this time dependent cytokine profile would give us more insight to this dynamic process and how dietary fatty acids affect it. Another parameter that would be interesting to investigate during the inflammatory state is the neutrophilic activation marker CD11b. Are neutrophils from mice fed ω -3 PUFAs more activated after an inflammatory stimulus? If

this is the case this would further strengthen our hypothesis that ω -3 PUFAs is more immune-modulating, rather than exclusively anti-inflammatory.

In paper III, we show that dietary PUFAs affect the G-CSF production in the bone marrow. However, further investigations are required to show how dietary PUFAs increase the production. This is a major task since the origin of G-CSF in bone marrow is not fully understood, even though macrophages phagocytosing apoptotic neutrophils have been proposed as a source (Furze and Rankin 2008).

Finally, it would also be interesting to be trying to discriminate between the neutrophils that have been called back to the bone marrow for degradation and the ones that are newly differentiated but have a high CXCR4 expression as a retention signal. To our knowledge this has not been done. An assay on apoptotic Ly6G⁺CXCR4⁺ neutrophils in the bone marrow might be able to shed some light on this issue. This would give us better understanding of which parameter it is that gives the increased frequency of Ly6G⁺ neutrophils in the bone marrow.

ACKNOWLEDGEMENT

Jag vill börja med ett stort tack till min huvudhandledare, **Maria Johansson**, för att du alltid stöttar, lyssnar, är engagerad och för att du låter mig gå min egen väg. Det är inte svårt att hitta motivationen till att arbeta hårt när jag vet att du arbetar minst lika hårt för att vi ska nå dit vi vill med projektet. Jag vet ingen annan som skickar mail på så obskyra tider som du!

Mina bihandledare **John-Olov Jansson**, **Staffan Nilsson** och **Louise Grahnemo**. John-Olov för att du tog emot mig som ex-jobbare och senare som doktorand. Staffan för att du alltid svarar på mina statistikfrågor, även om jag frågar samma sak om och om igen. Du har lyckats få mig till en av få som tycker det är roligt med statistik. Louise för att du introducerade mig till projektet. Jag använder fortfarande ditt system för mina filer!

Johannes för att du förgyllde min sista tid som doktorand. Det har varit oslagbart att ha en kompanjon som har varit ett steg för mig hela tiden, som jag har kunnat ta snålskjuts på.

Marcus för att du alltid är så sjukligt glad och positiv. Var får du din energi ifrån? Du ska veta hur mycket jag har uppskattat att du har kollat till mig innan du gått hem när jag suttit långa kvällar framför FACS:en.

Vilborg för att du alltid ställer upp och har sådan otroligt bred kunskap. Du väljer aldrig den lätta vägen ut, utan har inga problem med att ta av din egen tid för att hjälpa någon annan.

Fredrik, Erik, Sansan samt alla andra på kontoret för alla skratt och galna samtalsämnen som jag har fått njuta av under alla dessa år.

Alla **medförfattare** på de olika delarbetena för ett trevligt och produktivt samarbete.

Susanne och **Pernilla** på EBM för att jag har vetat att om det är någonting fel där ute så har ni superkoll på det. Hör jag inget ifrån er så kan jag vara helt lugn.

Julia och **Rached** för att jag har fått bjuda in mig själv på middagar när jag inte orkat laga mat själv och för alla skratt över antingen en kopp te eller ett glas vin.

Mamma och **pappa** för att jag alltid, och då menar jag alltid, har låtit mig gå min egen väg, men sett till att jag har rätt förutsättningar för att klara mig. Jag undrar hur många frågor som pappa har fått svara på om hur saker runt omkring mig fungerar. Det är att jag har fått peka och fråga om allt som gjort att jag har blivit nyfiken på världen och hur den fungerar.

Frijolita för att du har hållit mig sällskap under de sömnlösa nätterna. Dock tog du 6 veckors arbete ifrån mig, så jag undrar om du verkligen ska tackas... Maria har nog ett och annat att säga till dig när du kommer ut.

Last, but the absolute biggest thanks of them all to my favourite **Luis**. You keep me sane and happy. You are always on my side and I know I can always count on you. You have been my English teacher, chef, psychologist, graphical designer and incubator technician. Without you, this thesis would not exist. I would have given up a long time ago. You are mine, forever, moahahaha!

Now this baby is out, let the next one come out as well!

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