Metabolic and immunological interactions between adipose tissue and breast cancer

Implications of obesity in tumor progression

Peter Micallef

Department of Physiology/Metabolic physiology
Institute of Neuroscience and Physiology
Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

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Cover illustration: The adipose tissue – breast cancer interface in inguinal white adipose tissue of mice by Peter Micallef

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peter.micallef@gu.se

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Those who cannot remember the past are condemned to repeat it
George Santayana (1863-1952)

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Triple-negative breast cancers have fewer treatment options than other breast cancers. The overall goal of this research is to identify new pharmaceutical targets for triple-negative breast cancer through studies of the tumor-promoting crosstalk between tumor and surrounding adipose tissue. In **paper I**, we established extracellular flux analyzer-based methodology to evaluate metabolic function of cultured cells, used in **paper II** and **III**. In **paper II**, we identified the C1q/TNF-related protein family member *C1qtnf3* as one of the most upregulated secreted proteins in E0771 triple negative breast cancer-associated mouse adipose tissue – in particular in the obese setting. Antibody-mediated blockage of C1QTNF3 reduced macrophage infiltration in breast cancer-associated adipose tissue in mice. In cultured macrophages, C1QTNF3 decreased oxidative phosphorylation and enhanced M1-polarization. In **paper III**, we demonstrated that E0771 breast cancer tumors grew faster, associated with increased *de novo* lipogenesis from glucose, if transplanted orthotopically into adipose tissue than if transplanted outside adipose tissue. Based on our *in vitro* data, we propose that adipose tissue-produced lactate triggers the observed increase in *de novo* lipogenesis in the tumor. In conclusion, paracrine interactions between adipose tissue and breast cancer involve both immunological and metabolic processes, associated with enhanced tumor progression. In the future, we hope that pharmaceutical targeting of these interactions, in combination with conventional therapy, will improve the survival of breast cancer patients.

Keywords: Breast cancer, Adipose tissue, Macrophage, Metabolism, Paracrine

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SAMMANFATTNING PÅ SVENSKA

Det övergripande målet med vår forskning är att identifiera nya behandlingsstrategier för terapiresistent bröstcancer genom att studera samspelet mellan tumör och omgivande fettväv.

Bröstcancer är den vanligaste cancerformen hos kvinnor. Många botas idag från sin bröstcancer, men trots betydande medicinska framsteg inom detta område utvecklar 10-20% av bröstcancerpatienterna trippelnegativa tumörer som inte svarar på hormonell behandling och därför har sämre prognos. Unga kvinnor med övervikt löper större risk för att drabbas av terapiresistent bröstcancer. Bröstcancer växer i nära anslutning till fettväv, och fettväven har i flera studier visat sig utgöra en miljö som stimulerar tumörtillväxt. Vår hypotes är således att fettväv, och i synnerhet fet fettväv, stimulerar progression av bröstcancer.

I **delarbete I**, fastställdes protokoll och cellodlingsförhållanden för mätning av cellulär respiration och glykolys med en så kallad ”extracellular flux analyzer”. En metodik som vi använde för att studera metabolismen hos makrofager och metabola effekter av fettväv på trippelnegativ bröstcancer i **delarbete II** respektive **III.**

I **delarbete II** analyserade vi genexpressionsmönstret i tumörassocierad och kontrollfettväv i smala och dietinducerat feta möss för att identifiera potentiella nyckelprotein/mekanismer för den stimulerade tumörtillväxten i fettväv. Genom denna analys upptäckte vi att tumörnärvaro leder till kraftigt ökade fettvävsnivåer av adipokinen C1QTNF3 och att denna tumöreffekt förstärktes av fetma. Vidare fann vi att C1QTNF3 bidrar till ökad infiltrering av makrofager i fettväven samt att C1QTNF3 sänker respirationen och stimulerar proinflammatorisk aktivering av odlade makrofager.

I **delarbete III** fann vi att bröstcancer i kontakt med fettväv växer snabbare än bröstcancer utanför fettväven. Den snabbare tillväxten var kopplad till ökad *de novo* lipogenes dvs. ökad omvandling av glukos till fett, vilket är en metabol process som anses essentiell för tumörprogression. Våra *in vitro* data visar att laktat frisatt från fettväven skulle kunna förklara den ökade *de novo* lipogenesen i cancercellerna.

Sammanfattningsvis involverar samspelet mellan bröstcancer och närliggande fettväv både immunologiska och metaboliska processer, vilka är associerade med ökad tumörprogression. I framtiden hoppas vi att denna forskning leder till nya läkemedel som, i kombination med konventionell cancerbehandling som kemoterapi och strålning, förbättrar prognosen hos patienter med trippelnegativ bröstcancer.

LIST OF PAPERS

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

I. Antioxidant treatment induces reductive stress associated with mitochondrial dysfunction in adipocytes. Peris, E., **Micallef, P**., Paul, A., Palsdottir, V., Enejder, A., Bauzá-Thorbrügge, M., Olofsson, C.S., Wernstedt Asterholm, I. *Journal of Biological Chemistry, 294 (7), pp. 2340-2352 (2019).*

II. The adipokine C1QTNF3 is increased in breast cancer-associated adipose tissue and regulates macrophage functionality. **Micallef, P**., Wu, Y., Peris, E., Wang, Y., Li, M., Chanclón, B., Rosengren, A., Ståhlberg, A., Cardell, S., Wernstedt Asterholm, I. *Submitted.*

III. Adipose tissue - breast cancer crosstalk leads to increased tumor lipogenesis associated with enhanced tumor progression. **Micallef, P**., Chanclón, B., Stensöta, I., Wu, Y., Peris, E., Wernstedt Asterholm, I. *Manuscript.*

Publications not included in the thesis

IV. Parabrachial Interleukin-6 Reduces Body Weight and Food Intake and Increases Thermogenesis to Regulate Energy Metabolism. Mishra, D., Richard, J.E., Maric, I., Porteiro, B., Häring, M., Kooijman, S., Musovic, S., Eerola, K., López-Ferreras, L., Peris, E., Grycel, K., Shevchouk, O.T., **Micallef, P**., Olofsson, C.S., Wernstedt Asterholm, I., Grill, H.J., Nogueiras, R., Skibicka, K.P. *Cell Reports, 26 (11), pp. 3011-3026 (2019).*

V. CNS β3-adrenergic receptor activation regulates feeding behavior, white fat browning, and body weight. Richard, J.E., López-Ferreras, L., Chanclón, B., Eerola, K., **Micallef, P**., Skibicka, K.P., Wernstedt Asterholm, I.

American Journal of Physiology - Endocrinology and Metabolism, 313 (3), pp. E344-E358 (2017).

VI. Adiponectin protects against development of metabolic disturbances in a PCOS mouse model. Benrick, A., Chanclón, B., **Micallef, P**., Wu, Y., Hadi, L., Shelton, J.M., Stener-Victorin, E., Asterholm, I.W. *Proceedings of the National Academy of Sciences of the United States of America, 114 (34), pp. E7187-E7196 (2017).*

CONTENT

ABBREVIATIONS

ACC	acetyl CoA
ACLY	ATP citrate lyase
ACSL	acyl CoA synthetase lyase
AMPK	AMP kinase
AR	adrenergic
ATGL	adipose triglyceride lipase
CARKL	carbohydrate kinase-like protein
CPT-1/2	carnitine-palmitoyltransferase 1/2
DAG	diacylglycerol(s)
ETC	electron transport chain
ErbB2	human epidermal growth factor receptor (HER2)
FABP4	fatty acid binding protein 4
FACS	fatty acyl CoA synthetase
FASN/FAS	fatty acid synthase
FATP	fatty acid transport proteins
FFA	free-fatty acids
FGF2	fibroblast growth factor 2
Glut 1/4	glucose transporter 1/4
HIF1A	hypoxia-inducible factor alpha
HK2	hexokinase 2

HSL	hormone-sensitive lipase
IL-10	interleukin 10
IL1B	interleukin 1 beta
LACS	long-chain acyl-CoA synthetase
LDHA/B (LDH)	lactate dehydrogenase A/B
MAG	monacylglycerol(s)
MAGL	monacylglycerol lipase
MCP-1	monocyte chemoattractant protein-1 (CCL2)
M-CSF	macrophage colony stimulating factor
ME1	malic enzyme
MMPs	matrix metallopeptidases
NO	nitric oxide
P	phosphorylation
PDK 1	pyruvate dehydrogenase kinase 1
PFK1	phosphofructokinase-1
PG	prostaglandin
PHD	prolyl hydroxylase
PKA	protein kinase A
PKM1/2	pyruvate kinase isozymes M1/M2
PLIN	perilipin
ROS	reactive oxygen species
TAG	triacylglycerol(s)

TCA	tricarboxylic acid
TGFB	transforming growth factor beta
TNFA	tumor necrosis factor-α
VEGF	vascular endothelial growth factor

INTRODUCTION

Preface: Obesity is an emerging epidemic whilst having implications for cancer prevalence and progression.

Obesity is caused by an increase in adipose tissue mass due to positive energy balance i.e. the caloric intake has over time surpassed the energy expenditure. The estimated prevalence of obesity has, according to the World Health Organization, tripled since 1975. In 2016, it was reported that over 1.9 billion adults worldwide in both developing and developed countries, are overweight, and 650 million were obese. Overweight and obese individuals sum up to 55% of the population in U.S.A, while most countries has a prevalence of 10-30% [1]. Moreover, the global burden of obesity was projected to reach 2.16 billion overweight and 1.12 billion obese by the year of 2030, highlighting that obesity is an emerging challenge to public health as the number of obese people will almost double over the course of 14 years [2]. Furthermore, there is a gender difference in the prevalence of obesity where females are over-represented, although in countries of the Organisation for Economic Co-operation and Development (OECD i.e. high income countries, such as Scandinavia and Finland) there is a higher prevalence of obesity in males [3].

Adipose tissue is the body's main reservoir for energy storage. However, in 1987 the adipose tissue was identified as a major site for the production of sex steroids and adipokines, and thereby also regarded as an important endocrine organ [4]. A compromised endocrine function is often seen in obese individuals contributing to insulin resistance, hyperglycemia, dyslipidemia, hypertension, and pro-thrombotic and pro-inflammatory states, which together often are described as the metabolic syndrome [5]. The metabolic syndrome increases the risk of developing type 2 diabetes and cardiovascular disease, but also other forms of disease such as several forms of cancer including endometrial, colon and breast cancer. In fact, several epidemiological studies indicate that there is a correlation between obesity/metabolic syndrome and breast cancer risk [6-10]. The most well established mechanisms underlying increased cancer risk in obesity are alterations in endocrine function such as increased levels of insulin/IGF-1 and estrogen, and altered levels of adipocyte-derived cytokines (adipokines) [11]. This thesis aims to look beyond these endocrine links and to explore the symbiotic relationship between breast cancer and neighboring adipose tissue to identify new mechanisms underlying tumor progression.

BREAST CANCER

Breast cancer

In women diagnosed with cancer, breast cancer is the most prevalent form alongside cervical cancer, representing 25% of all cancer cases in 2012 [12, 13]. Moreover, one in ten of all diagnosed cancers worldwide are contributable to the female breast, and is one of the most common cancer forms with 1.7 million new cases each year. Increased incidence and improved treatments may partly explain an increase in prevalence and decreased mortality of breast cancer, however, the risk of developing breast cancer also relies on a numerous risk factors [14, 15]. These may e.g. comprise of age, lifestyle, environmental and socioeconomic status, which exerts the most vital role as most breast cancers are sporadic and non-familial, whereas hereditary forms (such as *BRCA1* and *BRCA2*) only constitute to about 5-10%, although presenting a near certain risk (60-80%) of developing breast cancer [16]. Breast cancer, alike many other cancers, show a vast span in histological, molecular and functional heterogeneity that is reflected upon the wide range of treatment options including hormone and targeted treatments such as estrogen receptor inhibitor tamoxifen or human epidermal growth factor receptor 2 (HER2) antibodies. However, several subtypes lack the presence of hormone receptors, thus having fewer treatment options and worse prognosis [17].

Breast cancer heterogeneity

Histological heterogeneity

The subtyping of breast cancer is based on histological, molecular and functional classification. Histological examination assesses the growth pattern (type) and the degree of differentiation (i.e. grade). The growth pattern is used to broadly categorize breast cancer into two major subtypes; *in situ* carcinoma and invasive carcinoma. *In situ* carcinomas are further sub-classified as either ductal or lobular, while the invasive is further divided into seven sub-groups. *In situ* carcinomas are organized into five well known differentiation types; comedo, cribiform, micropapilary, papillary while the invasive carcinomas are defined as grade 1-3. Thus, the histological examination comprises of 17 types [18]. The most common type of carcinoma is ductal invasive carcinomas comprising about 50-80% of all carcinomas, while 5-15% of all cases comprise of invasive lobular carcinoma.

Molecular heterogeneity

In order to better determine the prognosis and to develop targeted therapies, the gene expression profiles of breast cancer tumors have been classified by microarray. This classification work led to seven groups; claudin low, basal like, HER2 enriched, normal breast like (adipose tissue signature) and Luminal A/B. The gene expression analysis added thus increased depth to the immunohistochemical analysis allowing for better separation of previous subtypes; triple negative (basal), luminal (estrogen and progesterone positive) and HER2 positive. This has allowed for personalized therapy, and better prediction of disease free survival and overall survival [18]. Luminal A is the most common subtype and has the best survival rates whereas triple negative breast cancers comprise about 15-20% of all breast cancer cases and are associated with the poorest survival [19-21].

Functional heterogeneity (intra- and inter-tumor heterogeneity)

There are clinical traits, such as disease progression in terms of growth, metastasis and response to treatment which cannot be explained by the subtype. Thus, there is heterogeneity in cell populations within each tumor and/or differences between patients. This heterogeneity is proposed to arise either from cells with equal tumorigenic potential as a consequence of stochastic influences or from cancer stem cells that are different from the remaining tumor cells, and responsible for the initiation and progression of tumors [22, 23]. Genetic and epigenetic factors, stemness and microenvironment heterogeneity as well as the origin of tumor cells are thought to play important roles in the development of functional heterogeneity [24].

Immunological heterogeneity – cold and hot tumors

Immune cell infiltration within the tumor microenvironment can also be used to classify tumors. Tumors can either be defined as immunosuppressed or as immune-activated, and this is also referred to as immunologically cold or hot tumors. Generally, a cold tumor display low infiltration of lymphocytes ($CD4^+$ and cytotoxic $CD8^+$ T cells) and low mutational load (unprovoked), while a hot tumor is defined as the direct opposite [25]. Tumors are in general void of cytotoxic lymphocytes and in most cases often associated with a higher number of immune-suppressive cells such as regulatory T cells (FoxP3), and myeloid derived suppressor cells (MDSCs) [26]. In addition, there is also an accumulation of several other immune cell types such as T helper cell 2 and M2 macrophages which suppress anti-tumor immunity and promote disease progression. In an immunologically hot tumor, anti-tumoral responses are

generally functional and associated with accumulation mature dendritic cells, natural killer cells and type 1 natural T killer cells, also often presenting with a T helper cell 1 response and generation of M1 macrophages posing anti-tumoral responses [27].

In breast cancer, macrophages in many cases, make up 50% of the cellular mass and increased macrophage accumulation is associated with poor prognosis [28]. These macrophages are thought to originate from recruited circulating monocytes from the bone marrow, unlike tissue resident macrophages, which predominately are thought to originate from yolk-sac progenitors [29]. Tissue resident macrophages depend on self-renewal and are in most tissues and organs irreplaceable, however, in organs such as the gut the embryonically derived macrophage pool is over time replaced by circulating monocytes [30]. Similar to the gut, macrophages accumulated in tumors, referred to as tumor associated macrophages, originate from bone marrow derived monocytes and are predominately destined to present with a M2 phenotype [31]. Although most evidence, based on gene profiling data, supports the M2 phenotypical nature of tumor associated macrophages, there are cases where tumors are predominated by M1-type macrophages [32]. Nonetheless, tumors infiltrated by macrophages present with indicative signs of a poor prognostic outcome such as high tumor grade and proliferation, as well as low hormone expression (i.e. triple negative breast cancer) [33]. Clinicopathological features associated with high macrophage infiltration include lymph node metastasis, lack of response to neoadjuvant chemotherapy and overall decreased disease-free survival [34].

Defining the immune landscape of tumors has a high prognostic value and may lead to patient specific treatments. However, current tumor data sets lack information related to cellular proportions and heterogeneity which makes interpretation difficult [25]. Moreover, discrepancy in choice of methodological approaches and definition criteria between laboratories adds an additional level of complexity in the assessment of tumor associated macrophages.

ADIPOSE TISSUE

The endocrine function of adipose tissue

White adipose tissue was for long considered to solely serve as a long-term fuel reservoir; storing energy as triglycerides in the fed state, whereas releasing energy as fatty acids and glycerol in the fasted state. In 1987, this paradigm was challenged upon the discovery of the adipocyte-produced hormone adipsin, and the capability of adipose tissue to produce sex hormones [35, 36]. Subsequently, these findings paved the way for the discovery of many adipose tissue-produced cytokines, which today are known as adipokines or adipocytokines. These adipokines are indeed mainly produced by adipose tissue, but not exclusively. Among these adipokines, it was the discovery of leptin and its role in energy homeostasis that permanently defined the adipose tissue as an endocrine organ back in 1994 [37]. Leptin possesses many effects on energy homeostasis and most prominently leptin reduces energy intake while increasing energy expenditure. These effects are primarily exerted at the level of the brain, but leptin has also effects on peripheral tissues such as muscle and pancreas. Beyond the role of leptin in energy homeostasis, leptin also regulates immune responses, hematopoiesis, angiogenesis [38]. Furthermore, these leptin-mediated processes have been shown to be important in wound healing processes [39]. Tumor progression displays similarities to wound healing and has been described as a wound that never heals [40]. It is therefore not surprising that leptin has been shown to play a role in tumor progression, especially in breast cancer [41-43]. Adipose tissue is not only consisting of adipocytes, but to about 50% also of pre-adipocytes, fibroblasts, vascular endothelial cells and various immune cells such as macrophages. It should be noted that several cytokines/adipokines (such as CCL2, IL-6, IL-1B, TNFA and TGFB and plasminogen activator inhibitor 1) that are produced by adipocytes are also produced at much higher levels by other adipose tissue-resident cells [44, 45]. The adipose tissue secretome is depot-dependent and the functions of many adipokines are not fully investigated.

Adipose tissue and the development of comorbidities

The metabolic syndrome is a constellation of metabolic abnormalities such as central obesity, high blood pressure, high blood sugar, high serum triglycerides, and low serum high-density lipoprotein which subsequently are well known risk factors for the development of obesity related comorbidities such as type 2 diabetes and cardiovascular disease [46-48]. The development of comorbidities occurs in part as a consequence of pathological adipose tissue expansion where the growing adipose tissue

loses its functionality along with increased size. Physiological (normal) adipose tissue expansion during weight gain involves optimal immune responses leading to sufficient extracellular matrix and vascular remodeling that accommodate the growing adipocytes, and is permissive for the formation of new adipocytes (adipogenesis) and a sustained anti-inflammatory state [49]. In contrast, dysfunctional adipose tissue expansion, which frequently is seen in obese individuals, is associated with adipocyte hypertrophy, hypoxia, increased levels of reactive oxygen species (ROS), chronic inflammation, fibrosis and a decreased ability to store excess nutrients. Dysfunctional expansion is also associated with an altered adipokine release [4, 50]. All these pathological changes may ultimately result in ectopic lipid deposition (lipotoxicity) and systemic low grade chronic inflammation that in turn increase the disease risk [51].

C1Q tumor necrosis factor (TNF) family

Adiponectin belongs to the C1Q (complement component 1q) family of proteins and is one of the most well studied adipokines and regulates whole-body metabolism through its insulin sensitizing and anti-inflammatory effects [52]. Adiponectin exists in different multimer forms (trimer, hexamer and high molecular weight multimer) and can also be proteolytically cleaved into a globular form. The structural complexity of adiponectin is a result of extensive post-translational modification which likely is key to the function of adiponectin. The high molecular weight form is suggested to be the most bioactive form of the protein [52]. Within the C1Q family is the C1q and tumor necrosis factor related proteins (C1QTNF1-15), which thus are paralogs with adiponectin, but much less explored and expressed both by adipocytes and stromal vascular cells of adipose tissue. Similar to adiponectin, these C1QTNF proteins undergo extensive post-translational modification and are composed by four distinct domains, a signal peptide at the N-terminal, a short variable region, a collagenous domain and a globular domain (the C1Q-domain) at the C-terminal (**Figure 1**). All C1Q members are mostly arranged as homotrimers, but C1QTNF proteins can also form heterotrimeric proteins by combining with different family members. For instance, C1QTNF2 and 9 have been shown to form heterotrimeric complexes with adiponectin [53]. Relatively little is known about the multimeric structures of C1Q proteins [54].

Based on structure and sequence homology to adiponectin, the C1QTNF family is thought to be engaged in both metabolic and immunological regulation [55]. Indeed, several members (1, 2, 3, 5, 9 and 13) of the C1QTNF family have been shown to affect glucose and fatty acid

metabolism in adipocytes, liver, myocytes and skeletal muscle [54]. Overall, this family of proteins appears to serve to improve glucose metabolism and insulin sensitivity thus having anti-diabetic effects. Moreover, both C1QTNF3 and 12 have been shown to affect inflammatory responses.

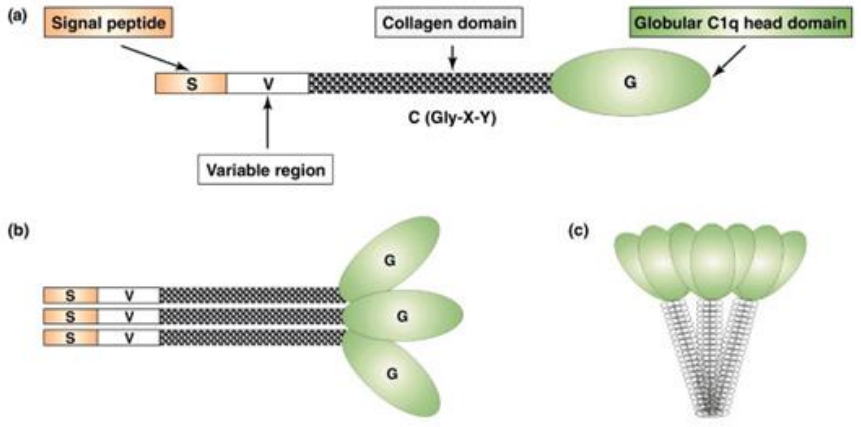


Figure 1. The structural organization of the C1QTNF family. **(A)** Illustrates the monomeric domain structure of C1QTNF consisting of signal peptide at the N-terminal, a short variable region, a collagenous domain and a globular domain at the C-terminal. **(B)** the homotrimeric structure. **(C)** The higher order structures such as nona- and dodecamers [53].

C1QTNF3 is an adipokine secreted from adipose tissue

C1qtnf3 mRNA is predominantly expressed in adipose tissue. Less is known about the protein expression, but C1QTNF3 protein is secreted and found in the circulation.

The effect of obesity on circulating levels of C1QTNF3 is inconclusive. The levels have been reported to increase in genetically obese leptin deficient mice, decrease or not change in dietary obese mice (for which were having an abundance in leptin levels), and decrease or in human obesity and in patients with type 2 diabetes [56-59]. However, the circulating C1QTNF3 levels are about 1000-fold lower than the adiponectin levels, which possibly indicates that endogenous C1QTNF3 exerts auto- or paracrine effects rather than endocrine. [60].

Pharmacological treatment with C1QTNF3 recombinant protein, has been shown to reduce serum glucose levels in mice by suppressing gluconeogenic enzyme expression in hepatocytes, and enhance oxidative phosphorylation (OXPHOS) through expression of peroxisome proliferators activated receptor-γ co-activator-1α (and associated mitochondrial biogenesis) in neonatal rat ventricular myocytes suggesting anti-diabetic effects [59, 61, 62]. Moreover, C1QTNF3 has been shown to exert anti-inflammatory and anti-fibrotic effects in e.g. the context of collagen-induced arthritis in vivo and LPS-induced inflammation in vitro [12, 63-66].

C1QTNF3 is also expressed in metastasis-associated fibroblasts and has been shown to contribute to the cellular proliferation of osteosarcoma cells as well as to promote proliferation and migration of endothelial cells in mice, suggesting an important role in tumor progression [67, 68].

MACROPHAGES

Adipose tissue macrophages in obesity and metabolic disorders

Macrophages are crudely categorized into the classically activated M1 or the alternatively activated M2 type. In reality, these cells encompass a much wider and heterogenic spectrum of phenotypes including M2a (activation: IL-4 or IL-13), M2b (activation: IL-1B or LPS), M2c (IL-10, TGFB or glucocorticoids) and M2d (activation: toll like receptor agonists and adenosine) [69]. In simplicity, the M1 type are pro-inflammatory associated with IL-12 production promoting microbiocidal T helper cell 1 polarization, while the M2 type are anti-inflammatory, and generally associated with IL-10 production promoting T helper cell 2 polarization and wound healing [70].

The M2 subtypes, which are important for tissue repair and angiogenesis, are the predominant resident macrophage type in adipose tissue of healthy individuals. In obese individuals, M1 type macrophages increase associated with improper vascularization, hypoxia and low grade inflammation [71]. Furthermore, studies indicate that there are more macrophages in visceral than in subcutaneous adipose tissue and it may also be a difference in macrophage functionality between men and women [72]. The implications for macrophages in the development of metabolically associated comorbidities are however difficult to fully establish as there is uncertainty whether the inflammatory state is the cause or the consequence of metabolic dysregulation [51]. However, one

hypothesis is that adipocyte death, as result of too severe hypoxia due to adipocyte hypertrophy and insufficient vascularization, attracts macrophages [73]. Furthermore, increased fatty acid levels due to increased lipolysis and reduced storage capacity of obese insulin resistant adipocytes can trigger pro-inflammatory responses via activation of toll like receptors in adipose tissue resident macrophages. Such inflammatory response can be further aggravated through macrophage release of the monocyte chemotactic protein-1 (MCP-1/CCL2) that attracts even more macrophages. Ultimately this results in a vicious cycle of macrophage infiltration and production of pro-inflammatory cytokines that aggravate the insulin resistance [74, 75].

Tumor associated macrophages

One of the major components of a tumor is the leukocyte infiltrate of which macrophages are a major part of [31]. Tumor associated macrophages are thought to originate from circulating monocytes. However, it is still being questioned that within some tissues macrophages originate from resident precursors seeded during fetal and embryonic development (i.e. yolk-sac progenitors) through self-renewal [76]. Regardless of the origin, tumor cells and stromal cells produce several chemoattractants such as the notorious CCL2 (as well as CCL5), but also by cytokines such as the macrophage colony-stimulating factor and members of the vascular endothelial growth factor (VEGF) family [77]. Once recruited, these cells are differentiated into macrophages by various signals in the tumor microenvironment such as Il-10 and TGFB. The macrophages are then generally polarized into an M2-like phenotype, although evidence suggests the existence of a M1-like tumor associated macrophages [78]. M2-like tumor associated macrophages exerts a very versatile role in tumor progression (**Figure 2**). In brief, these cells can exert immunosuppressive effects by inhibiting the cytotoxicity of T and natural killer (NK) cells through the production of Il-10 [79]. Moreover, M2-like macrophages exhibit a poor antigen-presenting capability rendering T cells naïve allowing for immune evasion. This has been shown to be further aggravated by induction of programmed cell death protein 1 (PD-1) via TNFA and Il-10 which reduces proliferation and induces dysfunction of T cells, as well as stimulates the recruitment of other immunosuppressive cells such as myeloid derived suppressor cells and regulatory T-cells [80]. Beyond the immunosuppressive effects, tumor associated macrophages are also able to affect metastasis and invasion by the production of epidermal growth factor, VEGF and several matrix metalloproteinases (e.g. MMP2 and 9), which serve to remodel the

extra cellular matrix and to give rise in new blood and lymphatic vessels allowing for extravasation of the primary site [80].

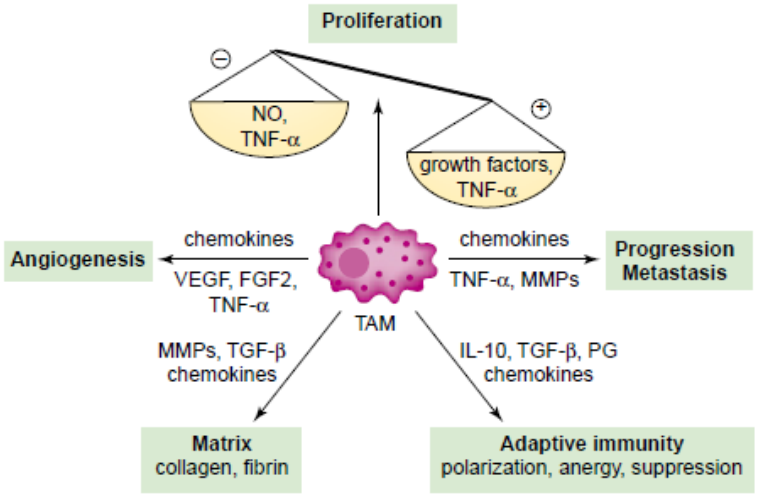


Figure 2. The versatility of tumor associated macrophages in tumor progression [31].

OBESITY, ADIPOSE TISSUE AND THE LINK TO CANCER

Endocrine links between obesity and increased tumor progression

Several epidemiological studies show that obesity increases the risk for several types of cancer. These obesity-associated cancer types are not solely confined to highly metabolic organs such as gallbladder, intestine, liver and pancreas but includes also many other cancers such as breast cancer [81, 82]. For instance, there is a correlation between obesity and increased metastatic burden in breast cancer [6-10]. Obesity-associated endocrine alterations such as increased levels of insulin-like growth factor 1, insulin, estrogen and leptin are the most well-established links between obesity and increased tumor progression [11, 81, 83-86].

Insulin-like growth factor

The insulin-like growth factor system is a signaling system that plays a crucial role in growth and development of tissues and organs and possesses potent mitogenic effects [87, 88]. The role of insulin-like growth factor was first discovered of its ability to stimulate chondrogenesis (formation of cartilage), but the biological significance was rapidly expanded to include stimulation of DNA, proteoglycan, glycosaminoglycan and protein synthesis but also serves to regulate neuronal proliferation, apoptosis and cell survival [89]. The insulin-like growth factor system has been implicated in the development several pathological conditions including tumorigenesis [90]. Epidemiological evidence shows that high circulating levels of insulin-like growth factor 1 constitute as a risk factor for the development of several cancers including breast cancer. However, the validity of the association between insulin-like growth factor 1 and cancer risk has been questioned in some human studies [88, 91]. Moreover, it has been shown that cells with mutated tumor suppressor gene p53 overexpress the insulin-like growth factor 1 receptor leading to increased proliferation [92]. Furthermore, insulin-like growth factor 1 receptor signaling can promote cellular migration in epithelial and certain breast cancer cell lines by alterations in integrin and adhesion complexes (E-cadherin) [93, 94].

Estrogen

Adipose tissue can produce estrogen due to its aromatase activity that converts androgen precursors produced from e.g. adrenal glands and gonads. There are two main alterations that influence sex hormone production by adipose tissue; adiposity and menopause. In postmenopausal women, the ovaries stop producing estrogen and adipose tissue becomes the main production site resulting in decreased estrogen levels. Obesity is associated with increased adipose tissue aromatase activity leading to increased estrogen (E1) and oestradiol (E2) levels [36, 81, 95]. The levels of estrogens and other steroid hormones can be much higher in local tissues such as breast fluids. Several epidemiological studies serve as evidence for a link between sex hormones and increased cancer risk, such as endometrial, breast, uterine, ovarian, and prostate cancers [96, 97]. Estrogen signaling can exert mitogenic effect in both normal and neoplastic mammary tissues and unbound estradiol may cause direct or indirect free-radical-mediated DNA damage, genetic instability, and mutations in cells, all of which are hallmarks for cancer development [96, 98]. Estrogen signaling may however be most relevant for cancers expressing sex hormone receptors (ERα and ERβ) [99]. Furthermore, as cancer cells may also require the expression of aromatase, it is not

improbable that interplay between cancer cells and normal cells in the vicinity, such as fibroblasts or adipocytes can occur both in auto- and paracrine manners to promote disease progression [100].

Adipokines

Recent research highlights the potential role of adipokines in tumor progression via their effects on e.g. angiogenesis, inflammation, proliferation and apoptosis. To date, leptin and adiponectin are the most well-studied adipokines with respect to cancer risk. Adiponectin modulates several important biological responses such as activation of pro-survival pathways, stimulation of angiogenesis and anti-inflammatory cytokine production as well as exerting antagonizing effect of leptin signaling [101]. Leptin regulates food intake and energy expenditure, but also mediates proliferation and inhibition of apoptosis [101, 102]. Adiponectin and leptin regulate both innate and adaptive immunity. Adiponectin suppresses macrophage M1 activation and promotes M2 proliferation, while leptin does the exact opposite inducing M1 activation [103, 104]. More over adiponectin suppresses the activation of other various immune cells involved in innate immunity, such as eosinophils, neutrophils, γ δ T cells, natural killer cells, and dendritic cells [104]. The differences between adiponectin and leptin are also reflected in the adaptive immunity where T helper cells 1 and 2 are promoted accordingly to adiponectin and leptin levels and their respective macrophage polarization [38, 102].

Epidemiological studies have associated low levels of circulating adiponectin with an increased risk for several types of cancer, and likewise for high circulating levels of leptin [105-109]. Tumor promoting effects of adiponectin deficiency has on several levels shown to have tumor promoting effects such as tumor formation and proliferation, and vice versa for adiponectin administration, which also decreases metastatic formation. Interestingly, adiponectin has shown to delay early onset due to decreased vascularization and increased apoptosis in mice suggesting that effects likely are secondary to initiation [101, 110-112].

Both high levels of leptin and leptin receptors have been shown to be associated with increased tumor growth and progression. For instance, leptin receptors and leptin were found in 83% of human breast cancer cases, and this was associated with increased occurrence of distant metastasis [113].

Adipose tissue is a tumor-promoting microenvironment

There are several mechanisms other than the endocrine links (as described above) that can explain the connection between obesity and increased cancer risk. Adipose tissue with its innate ability for extensive vessel and extracellular matrix remodeling can provide a hospitable environment for growing tumors. In line with this assumption, intra-abdominal tumors (e.g. ovarian cancer) often metastasize in an adipocyte-dominated environment suggesting that adipose tissue is a tumor-promoting microenvironment [7-9, 114-116]. Moreover, obese dysfunctional adipose tissue is associated low grade chronic inflammation, fibrosis and hypoxia – pathological processes that can trigger tumor progression even further (as outlined below).

The inflammatory process in obese adipose tissue is mediated by a vast array of cytokines produced by the adipocytes or stroma (such as TGFB, IL-6, TNFA, and CCL2), leading to infiltration and activation of immune cells such as myeloid derived suppressor cells, macrophages, as well as fibrosis that may contribute to tumor progression [117, 118]. The formation of crown-like structures is a common phenomenon of inflamed adipose tissue in obesity. A crown-like structure consists of macrophages recruited as a response to the spewing of cellular contents of dying or already dead adipocytes, such as lipids, cytokines, damage-associated molecular patterns (e.g. fatty acids, ATP, ROS, cholesterol and nucleic acids), for which they encircle [119]. Crown-like structures are also found in certain cancer forms such as breast cancer [119-121]. The consequence of crown-like structures on cancer can at this stage only be hypothesized, but data show that such structures are associated with elevated aromatase levels and increased the breast cancer risk in women with benign breast cancer disease [122]. Moreover, dying adipocytes can be carcinogenic via increased release of ROS leading to increased DNA damage and reduce DNA repair and thereby genomic instability in surrounding cell types (e.g. cancer cell). Furthermore, in breast cancer models as well as in obesity, programmed death-ligand 1 (PD-L1), an immune checkpoint ligand is upregulated in myeloid derived suppressor cells and sets out to diminish the function of $CD8^+$ T cells, which are important in immune surveillance [123]. Furthermore, adipose tissue fibrosis, defined by excess deposition of extracellular matrix components such as fibronectin, laminin and collagens, and desmoplasia [124-127] can trigger epithelial-to-mesenchymal transition of cancer cells [128]. Crown-like structures has been implicated in the formation of desmoplasia.

The formation of blood vessels (angiogenesis) is also dysregulated in obese adipose tissue. Although tumors in some occasions have the ability to become vascularized, vessel function is often aberrant and insufficient similarly to that of obese adipose tissue and may provoke inflammatory alterations and enhance metastasis [119]. Interestingly, obese (hypoxic) adipose tissue display increased lactate production that can stimulate tumor progression (this will be discussed in the next sections) [129].

More recently, attention has been redirected towards the establishment of a metabolic interplay between adipose tissue and cancer, giving rise to the metabolic symbiosis paradigm.

TUMOR METABOLISM

The Warburg effect

The Warburg effect, discovered 1924 by Otto Warburg, refers to the phenomenon where cancer cells increase their ATP-production via aerobic glycolysis rather than the oxidative phosphorylation pathway, despite sufficient oxygen supply [130, 131]. Glycolysis is a 100-fold faster process than oxidative phosphorylation, but generates much less ATP per glucose molecule. The reason for cancer cells undergoing a switch towards aerobic glycolysis is still unknown, but several hypotheses have been proposed. Consolidating pieces of evidence suggest that, although ATP deficiency results in cell cycle arrest or even apoptosis, ATP is not a limited resource during cellular proliferation in multicellular organisms. Instead, rapid proliferation implies a high demand for carbon that is used in macromolecular precursors, such as Acetyl-CoA and NADPH, which are used in fatty acid, amino acid and nucleotide synthesis [132]. Carbon is most quickly generated from glycolysis and from glutamine metabolism.

Atypical tumor metabolism: fatty acid synthesis and glutamine metabolism

Rapidly proliferating cancer cells' have a high reliance on acquiring fatty acids to build membranes, and most tumors rely on *de novo* lipogenesis as the major source of fatty acids [133]. However, a recent meta study shows that less aggressive breast cancer subtypes rely on a balance between synthesis and oxidation of fatty acids, whereas more aggressive type rely on exogenous uptake as indicated by gene expression data [134].

Fatty acid synthesis implies that citrate leaves the mitochondria and generates Acetyl-CoA through ATP citrate lyase (ACLY), and Acetyl-CoA is in turn carboxylated to Malonyl-CoA through Acetyl-CoA carboxylase (ACC, the rate-limiting step for long-chain fatty acid synthesis). Malonyl-CoA and Acetyl-CoA are thereafter used as building blocks for long chain fatty acids in a stepwise process catalyzed by fatty acid synthase (FASN) (**Figure 3**) [133, 135-137].

In order keep metabolic homeostasis during fatty acid synthesis, citric acid intermediates need to be replenished. Such replenishment is called anaplerosis or anaplerotic flux. Most tumors have an increased glutamine catabolism that feeds α-ketoglutarate into the citric acid cycle. This in turn can either lead to increased ATP-production through oxidative phosphorylation or increase citrate production.

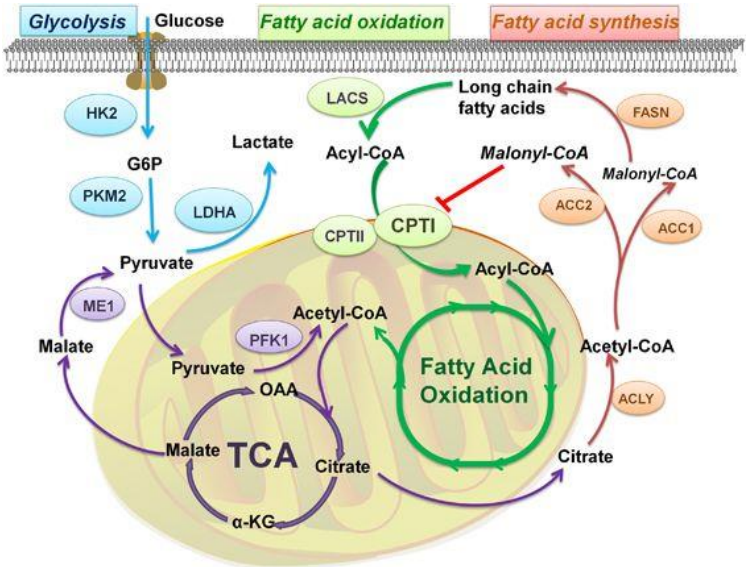


Figure 3. Metabolic pathways used by tumors, highlighting the connection between the Warburg effect (glycolysis) and fatty acid metabolism. Glycolysis, regulated by hexokinase 2 (HK2) and pyruvate kinase isozymes M1/M2 (PKM2), serves to generate substrates such as Acetyl-CoA which are used in the synthesis of fatty acids. The synthesis of fatty acids is catalyzed by ACLY, ACC and FASN. ATP production can be generated from fatty acid oxidation (although likely not when fatty acid synthesis occurs because of malonyl-CoA mediated CPT1 inhibition), which itself can be sustained by either exogenously or endogenously produced fatty acids. Anaplerotic flux in forms of NADPH feeding into the citric acid cycle (TCA cycle) or by α-ketoglutarate (α-KG) from glutaminolysis to maintain cellular energy homeostasis is not depicted in this illustration [138].

Metabolic interactions

Cancer cells have been shown to engage in a more complex metabolic rewiring by interacting with neighboring cells. Pathophysiological interactions include symbiotic nutrient sharing, nutrient competition, and metabolite-mediated signaling through G protein-coupled receptors [139]. These interactions typically serve the tumor in its growth and progression.

One example of symbiotic nutrient sharing is the so called lactate shuttle hypothesis, which implies that lactate generated from glycolytic cancer cells can be taken up and used for ATP production in neighboring oxidative cancer cells [140]. Such a metabolic exchange is well-characterized in non-tumor tissues e.g. in the brain between astrocytes and neurons. Thus, oxygenated cancer cells may use lactate for ATP production and that way glucose are spared for cancer cells in hypoxic regions of the tumor [141]. This lactate shuttle paradigm has been further elaborated on by the Lisanti group. They propose a so called reverse Warburg effect where cancer associated fibroblasts are transformed to engage in aerobic glycolysis, thereby feeding surrounding (oxidative) cancer cells with lactate and pyruvate [142]. This transformation is not only restricted to metabolic changes of fibroblasts, but has also been shown to occur in cancer associated adipocytes and immune cells (e.g. tumor associated macrophages) [115, 143].

Metabolic transformation of cancer associated cells stretches beyond the Warburg and reverse Warburg effects. For example, cancer associated adipocytes can supply the tumor with nutrients such as fatty acids from increased lipolysis (**Figure 4**) [115]. Tumors may use the exogenously derived lipids acquired from adipocytes in e.g. fatty acid oxidation to generate ATP or in membrane synthesis.

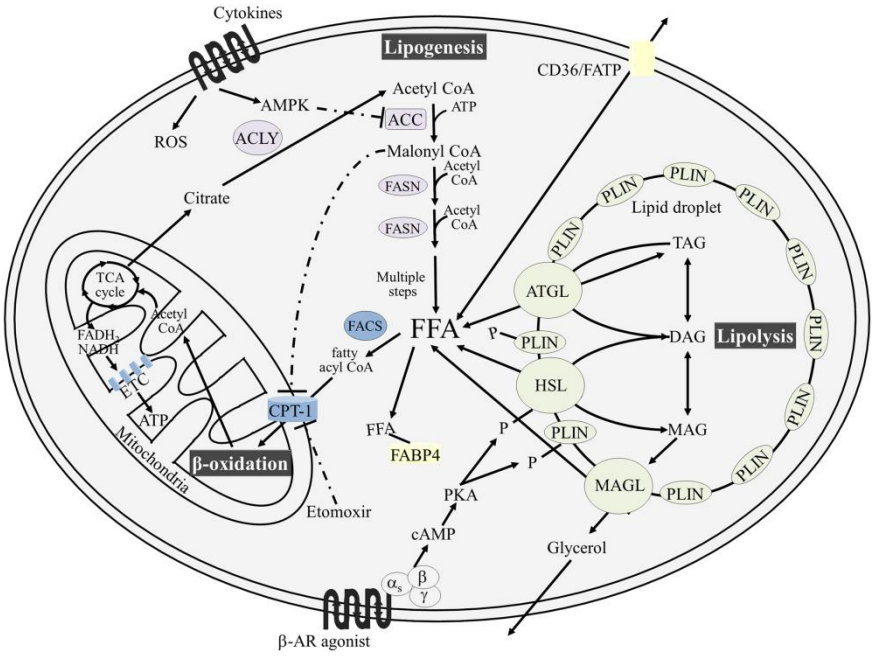


Figure 4. An overview of adipocyte lipid metabolism. Lipolysis is orchestrated by four key enzymes for which perilipin-1 (PLIN) and hormone sensitive lipase (HSL) initiate the process. Adipose triglyceride lipase (ATGL) hydrolyzes triacylglycerol into diacylglycerol which is further hydrolyzed by HSL into monoacylglycerol. Monoacylglycerol lipase (MAGL) removes the final fatty acid from the glycerol backbone. Malignant tumors typically stimulate adipocyte lipolysis. The resultant fatty acids may be used for the cancer cells' energy requirement or for membrane synthesis [115].

IMMUNOMETABOLISM: THE CONNECTION BETWEEN METABOLISM AND EFFECTOR FUNCTION OF IMMUNE CELLS

Cellular metabolism of activated immune cells

The Warburg Effect was initially described as phenomenon solely confined to cancer cells. In fact, Warburg had discounted this effect in white blood cells, describing it as an artefact. Later on, it was however concluded that the Warburg effect also applies to activated white blood cells: activation of immune cells is associated with increased aerobic glycolysis and reduced oxidative phosphorylation [144]. Indeed, within

the last decade, complex interconnections between metabolic pathways during immune cell activation have been discovered and thus giving rise to the concept of immunometabolism. In essence, Warburg metabolism is a key feature of activated immune cells, such phagocytes (i.e. macrophages) and some leukocytes, and occurs as a response to e.g. hypoxia, nutrient alterations but also danger signals and cytokines [145].

There are six key metabolic pathways that are important for survival, proliferation and effector function of immune cells: glycolysis, the citric acid cycle, the pentose phosphate pathway, fatty acid oxidation, fatty acid synthesis and amino acid metabolism [132].

Glycolysis and the citric acid cycle

The main difference between alternatively (M2) and classically (M1) activated macrophages is glycolysis. M1 macrophages enhance their glycolytic rate following activation. Glycolysis converts glucose into pyruvate. Normally, pyruvate is converted in Acetyl-CoA through pyruvate dehydrogenases, and the generated Acetyl-CoA then enters the citric acid cycle. During hypoxic or at pro-inflammatory conditions, HIF1A is activated leading to increased conversion of pyruvate into lactate, rather than Acetyl-CoA synthesis and ATP-production via oxidative phosphorylation. This effect is in part mediated by HIF1A-induced expression of pyruvate dehydrogenase kinase 1 (PDK1) [146, 147].

In parallel with the difference in glycolysis, the citric acid cycle also differs between M1 and M2 macrophages. M1 macrophages present with a “broken cycle” that result in the accumulation of citrate and succinate (**Figure 5**). Citrate can be used in the production of nitric oxide and prostaglandins, key effector molecules of M1 macrophages. The accumulation of succinate inhibits prolyl hydroxylase 1 (PHD1), which in turn stabilizes HIF1A and thereby glycolysis and increased Il-1B expression [148].

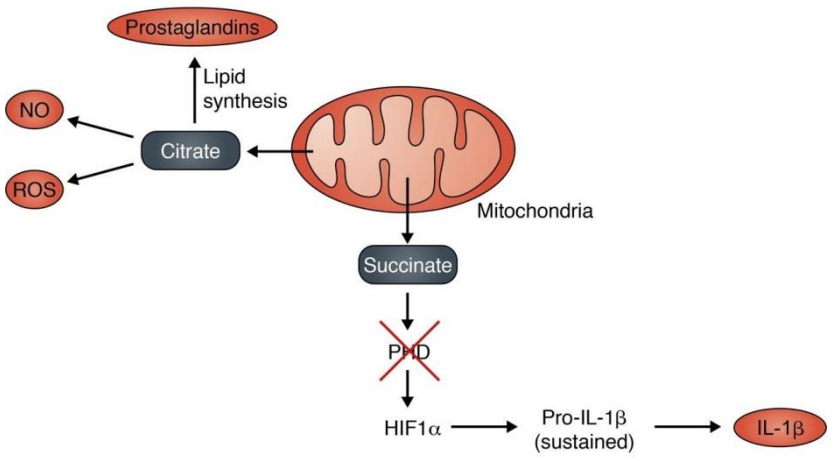


Figure 5. M1 macrophages express a broken citric acid cycle leading to the accumulation of citrate and succinate. The accumulation of citrate mediates the production of nitric oxide, reactive oxygen species and prostaglandins. Whereas, the accumulation of succinate results in the stabilization of HIF1A, which in turn results in a sustained Il-1B transcription. Modified from [145].

The pentose phosphate pathway and the respiratory burst of M1 macrophages

The pentose phosphate pathway, being a part of glycolysis, serve two functions; to divert intermediates from glycolysis into nucleotide and amino acid precursors that are necessary for cell growth and proliferation and the generation of NADPH which is used by NADPH oxidases to generate reactive oxygen species commonly referred to as the respiratory burst (**Figure 6**) [149]. The respiratory burst is essential for the cytotoxic actions of M1 macrophages, and is kept under control by anti-oxidant enzymes that prevent excessive tissue damage during inflammatory responses [150]. NADPH can also be used to synthesize fatty acids which can be used in e.g. cellular signaling processes that are important for effector function of immune cells [151]. Furthermore, reactive oxygen species are also important regulators of cell functions via so called redox signaling (reviewed in [150]).

The respiratory burst is regulated by the carbohydrate kinase-like protein (CARKL), an enzyme that limits substrate entry to the pentose phosphate

pathway from glycolysis and is highly expressed in M2 macrophages and is thus a key regulator of the macrophage phenotype [152].

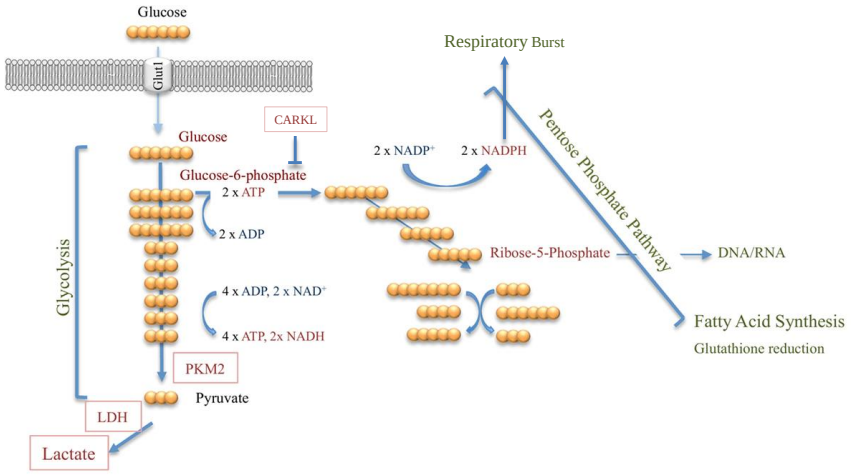


Figure 6, In immune cells such as macrophages, metabolic pathways can be diverted to support effector functions. In this example, the pentose phosphate pathway utilizes glucose to generate intermediates such ribose-5-phosphate used in DNA and RNA synthesis, and NADPH that can be used in fatty acid synthesis and in the respiratory burst. Modified from [153].

Amino acid metabolism

The difference in effector function between M1 and M2 macrophages are reflected on their difference in arginine metabolism. Arginine acts as a substrate for two key enzymes inducible nitric oxide synthase (iNOS/NOS2) and arginase 1. NOS2 is highly expressed in M1 macrophages and converts arginine into nitric oxide (NO) and citrulline. Moreover, the cytotoxic activity of M1 macrophages relies in part on reactive nitrogen species (e.g. N_2O_3, peroxynitrite or nitronium ion) that are generated from NO. Citrulline is recycled into arginine [154, 155]. M2 macrophages express high levels of arginase that generates ornithine and urea and limit the availability of arginine for NOS2. Ornithine is important in downstream pathways for cellular proliferation and tissue repair [154].

METABOLISM AS A THERAPEUTIC TARGET IN CANCER

Targeting tumor metabolism

Cancer cells typically display a high dependence on aerobic glycolysis, fatty acid synthesis and glutaminolysis, although the progression of some cancers depends on fatty acid oxidation. These metabolic pathways are linked to therapeutic resistance and represent thus potential targets for cancer treatment [137].

Indeed, there is a wide range of drugs that target e.g. glycolysis, the citric acid cycle and fatty acid synthesis, that are used in cancer therapeutics [137]. In brief, glycolysis can be inhibited by targeting glucose transporters (Glut 1 or 2) as well as other key enzymes such as hexokinase, pyruvate kinase isozymes M1/M2 and lactate dehydrogenase A (**Table 1**). Inhibition of glucose transporters decreases glucose uptake thus lowering glycolytic rate and generation of ATP and ultimately cellular growth. The use of several drugs targeting the same glucose transporters can result in a higher anti-cancer effect by overcoming a hypoxia-conferred drug resistance that generally occurs in tumors [137]. Hexokinase inhibitors such as 2-DG and 3-BrPa and LND are used in pre-clinical and early phase clinical trials of prostate cancer, intracranial metastases, and benign hyperplasia, respectively for 2-DG and LND. 3-BrPa has been studied in hepatocarcinoma (animal model) [156]. These substances have similar effects on glycolysis to glucose transporters, but have only been shown to have substantial effects in combination of other treatments such as radio- or chemotherapy in various different cancer types *in vitro*. Combinatorial treatments for both 2-DG and LND has been shown to effective in sensitization to cell death of several cancer types including breast cancer cells [156]. PKM2 is another rate limiting enzyme of the glycolytic pathway having four isoforms and being differentially expressed among cell types, whereas PKM2 is predominantly expressed in tumors cells. Changes in PKM2 expression has been correlated with drug resistance in human ovarian cancer and gastric carcinoma cell lines, and in patients with colorectal cancer; a decreased PKM2 protein activity is linked to cisplatin resistance in human gastric carcinoma cells, whereas suppression of PKM2 expression by siRNA can increase the resistance to cisplatin [157]. The therapeutic efficacy has been shown to be improved by targeting PKM2 with shRNA and increasing apoptosis and inhibition of proliferation in a human A549 xenograft lung cancer model, however, the mechanism of action remains to be elucidated and highlights importance of patient specific therapy

[158]. Lastly, targeting lactate dehydrogenase, an enzyme catalyzing the last step of glycolysis (pyruvate and NADPH to lactate and NAD^+) has, as previously discussed, been shown to be a promising therapeutic target. Knockdown of lactate dehydrogenase in human lymphoma (P493 human lymphoma B cells) and pancreatic cancer (P198 human pancreatic cancer cells) xenografts resulted in increased oxidative phosphorylation, decreased ability to withstand hypoxic conditions and decreased proliferation [159]. Overall, this results in increased generation of reactive oxygen species leading to apoptosis and reduced tumor growth [137].

Besides glycolysis, metabolic pathways stimulating fatty acid synthesis are important in proliferating cancer cells and are therefore also subjects for the development of new cancer therapeutics. For instance, there are several compounds that display anti-tumoral effect through inhibition of fatty acid synthase, an enzyme that is overexpressed in many breast cancers and thus a therapeutic target (**Table 1**). Inhibition of fatty acid synthase results in apoptosis by e.g. accumulation of malonyl-CoA, p53 accumulation, induction of endoplasmic reticulum stress and suppression of DNA replication [137]. Inhibition of fatty acid synthase has also been shown to affect the formation of lipid rafts through phospholipid partitioning thus resulting in the internalization and degradation of human epidermal growth factor receptor ErbB2 (HER2) in breast cancer, which is suggested to potentiate the anti-tumoral effects of trastuzumab.

Targeting glutaminolysis, is a two-part process. Glutamine is first converted into glutamate by glutaminase and thereafter glutamate is converted into α-ketoglutarate by glutamate dehydrogenase. Inhibition of glutaminolysis inhibits anaplerotic flux of the citric acid cycle leading to reduced generation of ATP through oxidative phosphorylation and/or reduced biosynthesis (e.g. fatty acid synthesis). Furthermore, the mTORC1 signaling pathway that drives cellular growth is co-induced by glutamine and leucine metabolism. The mTOR pathway has also been shown to be involved in cisplatin resistance in highly malignant gastric cancer cells. Inhibition of glutaminase results in decreased proliferation and increased hypoxia-induced cell death. However, sole inhibition of glutaminase can increase glycolysis. Therefore, simultaneous inhibition of both glutaminolysis and glycolysis is more effective [137].

Metabolic targets in breast cancer

The standard therapeutic targets for breast cancer accounts for the molecular heterogeneity within the subtypes, and thus includes endocrine therapy for estrogen receptor-alpha positive and human epidermal growth factor receptor-2 enriched, and general chemotherapy for triple negative subtypes [160]. The metabolic phenotype of breast cancer is likely dependent on the breast cancer subtype, its metastatic stage (i.e. primary, disseminated and metastatic tumor) and location. Signaling through the estrogen receptor α and estradiol has been shown to being able to reprogram metabolism based on the glucose availability. In circumstances of high glucose availability, estradiol has been shown to enhance glycolysis and suppress oxidative phosphorylation, whereas the opposite during conditions of low glucose availability [161]. The estrogen receptor α itself has been shown to regulate HIF1A and thus suggest an indirect action in activation of glycolysis [162]. In addition, both the estrogen receptor α and estradiol are involved in regulating nuclear and mitochondrial genes encoding proteins involved in mitochondrial function. Meanwhile, the estrogen receptor β has been suggested to exert similar effects on glucose metabolism; knockdown of estrogen receptor β led to a diminished expression of glycolytic genes, while enhancing the expression of genes involved in oxidative phosphorylation [160]. Furthermore, human epidermal growth factor receptor-2 enriched tumors express higher levels of genes involved in oxidation, storage and synthesis of fatty acids in comparison to other subtypes (in addition to their reliance on glycolysis) [163]. On the other hand, the importance of oxidative phosphorylation associated with an increased susceptibility to fatty acid oxidation inhibitors has recently been highlighted in triple negative breast cancer [160]. Furthermore, based on mRNA expression data triple negative breast cancers rely on the utilization of exogenously derived fatty acids as opposed to reliance on de novo lipogenesis [134].

Table 1. Therapeutic drugs used to target metabolic pathways in cancer [137].

Targeted metabolism	*Targeted metabolic enzymes*	*Metabolic inhibitors*	*Cancer therapeutics/ other inhibitors*	*Cancer types* (in vitro *and/or* in vivo)
Glycolysis	GLUT1	Phloretin	Daunorubicin	Colon cancer (*in vitro*), leukemia (*in vitro*)
		WZB117	Cisplatin/paclitaxel	Lung cancer (*in vitro*), breast cancer (*in vitro*)
	GLUT4	Ritonavir	Doxorubicin	Multiple myeloma (*in vitro*)
	HK	2-DG	ABT-737/ABT-263	Leukemia (*in vitro*), cervical cancer (*in vitro*), hepatocarcinoma (*in vitro*), breast cancer (*in vitro*), small lung cancer (*in vitro*), lymphoma (*in vitro*), prostate cancer (*in vitro* and *in vivo*)
			Trastuzumab	Breast cancer (*in vitro* and *in vivo*)
			Prednisolone	Leukemia (*in vitro*)
		3-BrPA	Daunorubicin	Leukemia (*in vitro*)
			Doxorubicin	Multiple myeloma (*in vitro* and *in vivo*)
			Oxaliplatin/5-FU	Colon cancer (*in vitro*)
			Prednisolone	Leukemia (*in vitro*)
		LND	ABT-737	Leukemia (*in vitro*), lymphoma (*in vitro*)
			Prednisolone	Leukemia (*in vitro*)
	PKM2	shRNA	Cisplatin	Lung cancer (*in vivo*)
			Docetaxel	Lung cancer (*in vitro* and *in vivo*)
	LDHA	FX11	FK866	Lymphoma (*in vivo*)
		Oxamate	Paclitaxel	Breast cancer (*in vitro*)
			Trastuzumab	Breast cancer (*in vitro* and *in vivo*)
Citric acid cycle	PDK3	siRNA	Paclitaxel	Cervical cancer (*in vitro*)
			Cisplatin/paclitaxel/ oxaliplatin	Colon cancer (*in vitro*)
	PDK	DCA	Omeprazole	Fibrosarcoma (*in vitro* and *in vivo*) colon cancer (*in vitro*)
			Omeprazole + tamoxifen	Fibrosarcoma (*in vitro*)
			5-FU	Colon cancer (*in vitro*)
			Sulindac	Lung cancer (*in vitro*), squamous cell carcinoma (*in vitro*)
			Irradiation	Prostate cancer (*in vitro*)
Fatty acid synthesis	FASN	Cerulenin	Docetaxel	Breast cancer (*in vitro*)
			Trastuzumab	Breast cancer (*in vitro*)
			5-FU	Breast cancer (*in vitro*)
		C75	Trastuzumab	Breast cancer (*in vitro*)
		Orlistat	Adriamycin/ mitoxantrone	Breast cancer (*in vitro*)
			Gemcitabine	Pancreatic cancer (*in vitro*)

Targeting metabolic pathways in immune cells

Metabolic pathways in immune cells serve also as targets in the development of better cancer treatments as well as in many other conditions where regulation of immune responses plays a key role [164]. As previously discussed, the preferred metabolic pathways within an immune cell determine its phenotype and effector function. Moreover, the availability of metabolites may also be crucial for regulating the metabolic and phenotypic fate of immune cells [165]. In essence, there are two strategies that have been used to target metabolic pathways in immune cells; 1) adoptive cellular immunotherapy, and 2) metabolic reprogramming of the host.

Adoptive cellular immunotherapy involves naturally or engineered cells such as lymphocytes originating from the resected tumor itself or by the engineering of T cells derived from peripheral lymphocytes. These cells can then be modified *ex-vivo* and thereafter re-introduced into the patient, which generates a T cell population that has a higher replicative capacity and is apathetic to differentiation thus persisting for longer and increasing the therapeutic efficiency [165]. While the fortitude of metabolic

reprogramming as a therapeutic strategy remains to be elucidated, it has been shown that augmentation of OXPHOS and fatty acid oxidation in $CD8^+$ T cells improved longevity and anti-tumor immunity in-vivo [166].

Metabolic reprogramming was conceived as a viable approach as several pharmaceutical treatments, such as metformin, have been shown to disrupt the electron transport chain (OXPHOS) by inhibiting complex I [165]. Other agents such as CPT-1a inhibitors that inhibits fatty acid oxidation, and rapamycin that lowers mTOR activity, which functions to integrate signaling pathways involved in many pathways affecting cell growth, proliferation, and metabolism. Both metformin and rapamycin has been shown to act as immunosuppressants by reducing the formation of cytotoxic $CD8^+$ effector cells and instead stimulate the formation of $CD8^+$ memory T cells. Collectively, the exerted metabolic effects of metformin, rapamycin and CPT-1a inhibitors (etomoxir or perhexiline) have been shown to affect T cells and may prove useful in in the treatment of several immunity driven diseases such as graft versus host disease and cancer. However, as activated T cells and cancer cells often share similar metabolic traits, targeting cellular metabolism may serve to positively impact tumor progression by means of negatively impact infiltrating effector T cells [165, 167].

Moreover, metabolites conjugated with pharmaceutical agents can potentially be used to target specific immune cell types via transported-facilitated drug uptake [165]. One example is exploiting cells engaging in glycolysis by conjugating a pharmaceutical agent to D-glucose which then is taken up via glucose transporters (such as Glut1). This strategy has so far resulted in a phase III clinical trial using glufosfamide with D-glucose to treat patients with metastatic pancreatic adenocarcinoma which previously had undergone treatment gemcitabine [168]. Similarly, D-glucose has also directly been used to target metabolism by inhibiting lactate dehydrogenase through an N-hydroxyndole-based compound [165].

Tumor associated macrophages have been identified to exist as both M1-like MHC-II^{high} and a M2-like MHC-II^{low} populations, even within the same tumor [78]. However, M2-like tumor associated macrophages are currently the most plausible therapeutic target as they have been shown to affect the efficacy of anticancer treatments, contribute to therapy resistance and to mediate tumor relapse [169]. Inhibition of monocyte recruitment by blocking CCL2 has been shown to reduce tumor progression in many different cancers. However, blocking CCL2 can also result in accelerated metastasis through increased cell motility and

angiogenesis in breast cancer [170]. Therefore, new strategies targeting the repolarization of macrophages through altered metabolism are currently being investigated. There are a couple of key studies on this topic that is briefly presented in this section:

1) Tumor derived lactate has been shown activate G protein-coupled receptor 132 (GPR132) leading to M2 polarization of tumor associated macrophages through stabilization of HIF1A and the induction of *Vegfa*, *Arg1*, *Relma*, *Mgl1* and *Mgl2* gene expression [171]. Silencing of Gpr132 in macrophages reduces cancer growth *in vitro* and E0771 breast cancer tumor growth is reduced in whole-body GPR132 knockout mice [172, 173].

2) The metastasis promoting effect of tumor associated macrophages in an *in vitro* model of pancreatic ductal adenocarcinoma was successfully inhibited by 2-deoxyglucose treatment [174].

3) Inhibition of mTOR activity by rapamycin has been shown to stimulate the M1-phenotype and thus enhance the macrophages' anti-tumoral effects in the Huh-7 hepatocarcinoma cell line in a nude mouse model [175]. Moreover, genetic deletion of REDD1 (an inhibitor of mTOR) in macrophages results in an increased glucose uptake and enhanced glycolysis, further linking the metabolic reprogramming to effector function. This ultimately results in nutrient competition between the tumor associated macrophages and endothelial cells preventing vessel leakiness and metastasis [176].

In summary, altering macrophage phenotypes through metabolic reprogramming appears like a promising therapeutic approach in cancer.

AIM

A growing body of research shows that adipose tissue is a tumor-promoting microenvironment. Furthermore, tumor progression is enhanced in obesity and highly associated with altered metabolic (mitochondrial) function. The overall goal of this research is to identify new targets for the treatment of triple negative breast cancer through studies of the paracrine interactions between lean or obese adipose tissue and tumor in breast cancer progression.

SPECIFIC AIMS

1) **Identify new adipose tissue-derived secreted proteins that are involved in the paracrine interactions between lean or obese adipose tissue and breast cancer.**
    a. Set up breast cancer models *in vivo* and in *vitro* (paper II and III).
    b. Set up methodology to study mitochondrial function in cultured cells (paper I, II and III)
    c. Test whether identified secreted protein is involved in breast cancer progression and/or tumor immunity (paper II).
2) **Proliferating cells require increased amounts of lipids to build new membranes.**
    a. Test whether increased supply of exogenous lipids via breast cancer-induced lipolysis play a role in the tumor-promoting effect of adipose tissue (paper III).
    b. Test whether adipose tissue alters tumor metabolism and whether such alteration is causally linked to the tumor-promoting effect of adipose tissue (paper III).

METHODOLOGICAL CONSIDERATIONS

MOUSE MODELS OF BREAST CANCER

Breast cancer is a heterogeneous disease, and its progression include different stages; initiation, invasion and metastasis. In order to address specific aspects of the disease processes such as elucidating underlying molecular pathways it is critical to generate and/or select the most appropriate model. There are essentially three different types of mouse models for breast cancer research; chemically-induced, genetically-induced and xenograft breast cancer models [177].

The link between chemicals and carcinogenesis was discovered more than 200 years ago and has initially been implemented in animals to assess the possible carcinogenic effects of low-dose exposure of environmental chemicals. Chemical carcinogens, usually agents that directly damages DNA, include e.g. polycyclic aromatic hydrocarbons, aromatic amines, N-nitroso compounds, alkylating agents and radiation. In breast cancer research, N-methyl-N-nitrosourea (MNU) and 7,12-Dimethylbenz(a)anthracene (DMBA) are the most commonly used agents to induce breast cancer because of their relatively high reproducibility and these models are used to study tumor initiation and growth in immuno-competent mice and rats. Treatment with MNU or DMBA leads to hormone dependent tumors, but MNU-induced tumors are largely adenocarcinomas while DMBA-induced tumors frequently are benign fibroadenomas.

Genetically engineered mouse models are used to address early events in carcinogenesis and have, just like chemically-induced models, the advantage of producing tumors in their correct microenvironment. Based on knowledge from human tumors, mice can be engineered to study effects of specific genes/mutations. Gene promoters such as mouse mammary tumor virus (MMTV), whey acidic protein (WAP), C3, B-LG and MT can be used to differently express onco- and tumor-suppressor genes in the mammary tissues. However, incomplete characterization of promoters, and the modification of onco- and tumor-suppressor genes that may not completely represent those observed in human tumors, can lead to breast cancer models that do not fully represent the human setting. In addition, it can be a combination of multiple genes (gene mutations) that contribute to carcinogenesis [178]. Moreover, a key drawback in using genetically engineered mice is in the study of metastatic disease; the penetrance of metastasis is low and requires relatively long time to

develop. Thus, large cohorts of mice are necessary and results that e.g. a new treatment may not reflect the human situation [178]. Furthermore, genetically engineered breast cancer mouse models usually present with lung metastasis whereas in humans metastasis are most commonly seen in the lymph nodes [179].

Unlike chemically induced and genetically engineered breast cancer mice, transplant models are by far the most commonly used models for breast cancer. There are three different types of transplant models; patient-derived cell-line derived xenografts (CDX), patient-derived xenografts (PDX), and mouse-derived syngeneic cell lines. These models are used to study breast cancer genetics, biological processes and metastatic disease, but have a poor record of predicting clinical efficacy [178]. Depending on the site of transplantation different stages and organ specific metastasis can be assessed, e.g. direct implantation orthotopically or ectopically allows for the assessment of metastatic disease as a consequence of primary tumor outgrowth. Whereas, injection into the circulatory system allows for direct assessment in different organs commonly metastasized in humans such as lungs, liver and bone dependent on the choice of entry, i.e. tail vein, portal vein or intracardiac infusion [180].

CDX models originate from highly malignant and metastatic tumors or through plural effusion (drained lung fluid) that are cultured and domesticated in a non-native *in vitro* niche aberrant to symbiotic interactions in the microenvironment, thus losing its heterogeneity. The CDX is then to be transplanted in an immune-suppressed mouse model where it rarely metastasizes making it difficult to assess metastatic disease, immune interaction and therapeutic efficacy. Some of these shortcomings were sought to be addressed with the development of PDXs, which involves transplantation of tumor pieces derived from the patient. Indeed, PDX has been shown to be more effective in modeling metastatic disease. Moreover, human cells are not adapted to grow in a murine environment, but tumor pieces include stromal cells leading to enhanced tumor progression compared to CDX. Nevertheless, the establishment of a PDX is relatively slow and may have limited use for the establishment of personalized treatments [178]. Lastly, in order to overcome the lack of immune responses, cell lines from spontaneously derived mammary tumors of the mouse have been developed. The downsides with syngeneic mouse models are that they do not represent human breast cancer and that there are very few available models (and all are hormone independent); EMT-6, E0771, HCC70 and 4T1 [181, 182]. Thus, human xenografts are indispensable for preclinical testing of drugs.

However, hormone receptor assessment is not a trivial procedure as the occurrence of both hormone negative and positive cells may exist within the same tumor. The overall assessment of the hormone receptor status is achieved by counting (i.e. scoring) the number of hormone expressing cells present in a tumor sample and is expressed as a percentage, and this heterogeneity is why some mouse models of human breast cancer can be considered belonging to one subtype although they do not [183].

The E0771 syngeneic breast cancer model

The E0771 breast cancer cell line originates from a female C57BL/6 mouse harboring a spontaneous mammary adenocarcinoma back in 1951 [184]. The E0771 breast cancer cell line is described as highly aggressive and readily colonizes distant locations such as lungs, intestinal mesentery and pancreas which makes it suitable model in different stages of cancer progression [185]. E0771 breast cancer tumors display a low infiltration of lymphocytes such as CD4 and CD8 T cells accompanied by a high infiltration of MDSCs, and M2 over M1 polarization of macrophages [186, 187]. This leukocyte profile is known to promote tumor growth associated with poor prognosis [188, 189]. It is however difficult to subtype breast cancers based on the immune infiltrate (only few studies are available), but functional and molecular characterization has defined E0771 breast cancer as hormone negative [190]. This is of particular importance since those breast cancer subtypes do not respond to endocrine therapy, resulting in worse outcome and survival for the patients than for other subtypes of breast cancer [191]. This model may therefore be a suitable tool to delineate novel therapy strategies for triple negative cancers – the focus of this thesis.

MACROPHAGE MODELS

In the early development, tissue resident macrophages originate from yolk-sac progenitors whereas in certain tissues are replaced over time by bone marrow derived progenitors [29]. Yolk-sac derived macrophages differ in the sense that replenishment occurs through self-renewal and are specifically presenting with phenotypes adapted for a specific tissue environment, making tissue-resident macrophages a very heterogeneous population. The occurrence of temporal heterogeneity may also result from infiltration of macrophages of hematopoietic derived cells, during e.g. inflammation. There is uncertainty whether or not such cells are retained long term after resolution as there are no reliable markers that can distinguish between yolk-sac and monocyte derived cells [192]. However, it has been shown that ablation of resident macrophages can be

compensated by infiltration of bone marrow derived cells and that these undergo phenotypic adaptations to become tissue resident macrophages [193, 194].

Primary mouse macrophages can be obtained directly from e.g. tissues, or from exfiltration through provocation of the immune system, as well as from differentiation of monocyte precursors. The most common origins used to isolate monocytes and macrophages are bone marrow, spleen and the peritoneum. There are also three commonly used mouse cell lines, IC-21, RAW264.7 and J774.2 along with several human cell lines. The phenotype varies between these models, which warrants for selection of the most appropriate macrophage model for the given research question. Bone marrow-derived macrophages are advantageous by means of representing a homogenous population with a long lifespan and a high proliferative capacity. These macrophages and are thus commonly selected when large numbers of cells are needed [195]. Whereas macrophages derived from tissues are more heterogeneous [29, 195, 196]. Cell lines represent cells of higher maturity (i.e. differentiation) than primary cells, while splenic and peritoneal has a higher grade of maturity than bone marrow-derived macrophages [195]. Bone marrow-derived macrophages share many similarities with RAW264.7 in terms of cell surface receptors and immune responses. These cells are expressing high levels of F4/80 and CD11b, while CD14 expression is higher in the RAW264.7 cell line. In contrast, splenic macrophages express very low levels of F4/80, CD11b and CD14 [196]. CD14 is important in generating T helper cell 1 mediated responses and RAW264.7 cell line may thus be more prone to present a M1 phenotype that other models. Furthermore, phenotypic differences exist between the primary macrophages in the expression of co-stimulatory molecules such as CD80, CD86 and the major histocompatibility complexes I and II (MHC I and II). CD80 is highly expressed in bone marrow-derived macrophages in comparison to splenic and peritoneal macrophages. CD86 is relatively evenly expressed among these three macrophage types while MHC II is highly expressed in peritoneal macrophages, whereas, RAW264.7 hardly express any MHC II. MHC I is relatively evenly expressed among bone marrow-derived, splenic macrophages and the RAW264.7 cell line [195, 196]. This clearly suggests that there is a potential difference between macrophage models in their capability of activating T cells. However, one study suggests that there is no difference in antigen presentation the between macrophages derived from bone marrow, spleen or peritoneum [195].

Bone marrow derived cells have been shown to express significantly higher levels of *Il10* and *Tgfb* than splenic and peritoneal macrophages,

whereas splenic macrophages express higher levels of *Il6, Il12* and *Tnfa*. However, all three macrophage types expression similar levels of pro-inflammatory cytokines in response to lipopolysaccharide (LPS) stimulation [195]. Compared to primary macrophages, the macrophage cell lines IC-21, RAW264.7 and J774.2 display very low response to LPS [197]. In order to motivate the use of mouse derived macrophage models it is important to demonstrate a phenotypic profile that is relevant for the research question, as well as to have an understanding in the difference between human macrophages. Unfortunately, there are a limited number of studies available on this topic. However, Spiller et al. has compared the gene expression profiles between mouse and human bone marrow-derived macrophages, including the human monocyte cell line THP-1 [198]. It is one of very few studies that also include the comparison between different activation states (naïve, M1 and M2). This study shows similarities between mouse and human macrophages as judged by e.g. a similar upregulation of *Cxcl-10*, *Cxcl-11*, *Tnfa* and *Ccl1* in M1-macrophages. Despite the similarities, human macrophages are clustered together away from the bone marrow derived cells of mice. One of the most notorious differences between mouse and human (as well as several other species such as rabbit, goat and Syrian hamster) is the difference in arginase metabolism. Human macrophages has been shown unable to produce NO from NOS2 *in vitro*, while undoubtedly being able to express NOS2, this is thought to be as a consequence of very specific signals that are difficult to reproduce *in vitro*. This depicts that there are interspecies differences in effector function based on metabolic indifferences, within these cells [199, 200].

In mouse adipose tissue, the macrophage pool cannot be replaced solely by bone marrow transplantation, indicating that adipose tissue macrophages originates from both monocytes and yolk-sac progenitors, [201]. The homoeostasis of adipose tissue macrophages are thought to be sustained by self-renewal rather than relying on infiltration of circulating monocytes [201-203]. In contrast, during obesity there ample evidence for increased infiltration of monocytes although self-renewal of adipose tissue resident macrophages also increase [204]. Similar to obese adipose tissue, growing tumors accumulate primarily bone marrow derived macrophages. There is a consensus of the field that the selection of a macrophage model should account for the nature and origin, as well as phenotype of macrophages *in vivo*. Therefore, we believe bone marrow derived macrophages serve as a suitable model in the experiments of this thesis.

ENERGY METABOLISM IN ADHERENT CELLS

The mitochondrial bioenergetics is commonly assessed to identify changes metabolic phenotypes in cultured cells. The assessment usually includes evaluation of ATP synthesis (directly or indirectly), redox or ion homeostasis. These processes are defined by the respiratory steady-state conventions (1-5), which were first introduced in a series of five papers in 1955 [205]. In brief, the main driver of ATP synthesis is the membrane potential spanning across the mitochondrial inner membrane, which is generated by protons. The energy released from this proton gradient is used by the ATP synthase (complex V) to generate ATP. The resulting drop in membrane potential is restored by three electron transport complexes (I, III and IV), which consumes substrates (NADH) and oxygen to transport protons exteriorly to the mitochondrial inner membrane. Electrons received from NADH travels through each complex and results in a drop in redox potential in each step. When the requirement for ATP is low, the proton circuit can instead be uncoupled from ATP synthase through proton uncouplers, which for example can prevent the formation of reactive oxygen species from single electrons [206].

The main methods to evaluate mitochondrial function rely thus on measurements of oxygen consumption, redox or ion homeostasis. The two latter revolves around the use of triphenylphosphonium hydroethidine-based dyes (MitoSOX Red, Invitrogen) and cationic fluorescent probes using either confocal microscopy or flow cytometry.

Oxygen consumption is measured by respirometry, for which there are a number of methods. The most notable are the use of amperometric O_2-sensors and O_2-dependent quenching of porphyrin-based phosphors [207]. The latter is utilized in the XF Extracellular Flux Analyzer (Agilent, Seahorse Bioscience), using fluorophores sensitive to O_2 and H^+ which are connected to fiber optic bundles of the sensor cartridge. This method has a lower resolution than amperometric O_2 sensors and is more prone to variation in each measurement. Furthermore, instruments using amperometric O_2 sensors employ closed air-tight reaction chambers with constant stirring, which leads to more accurate substrate delivery, better oxygen diffusion and therefore quicker response time and reduced background noise. Applications utilizing amperometric O_2 sensors (such as OROBOROS Instruments) are mainly adapted to isolated mitochondria, permeabilized cells and intact cells in suspension, while the XF Extracellular Flux Analyzer are adapted to attached cells or

mitochondria, and has a higher throughput allowing for screening-based applications.

The XF Extracellular Flux Analyzer was chosen for this thesis work because of its high throughput application. The mitochondrial function can be assessed using chemicals targeting different pathways in metabolism such as oxidative phosphorylation, glycolysis and fatty acid oxidation. General mitochondrial function can be measured by the so called "Cell Mito Stress Test Kit" that relies on the sequential addition of the ATP synthase inhibitor oligomycin, the proton uncoupler carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), and rotenone together with antimycin which inhibit complex I and III, respectively. The change in oxygen consumption rate, as required by the electron transport chain (complex IV) to maintain the membrane potential (proton gradient), are then used to calculate non-mitochondrial, basal and maximal respiration as well as ATP turnover, proton leak, coupling efficiency and spare respiratory capacity. Basal respiration represents the basic cellular ATP demand and proton leak. ATP turnover is estimated from the difference between basal respiration and respiration after addition of ATP synthase inhibitor (oligomycin), subtracting non-mitochondrial respiration (observed when the electron transport chain is fully inhibited). During ATP synthase inhibition, the electron circuit is looped by proton uncoupling (UCP-1) in order to maintain the membrane potential [208]. Pharmacologically-induced uncoupling by FCCP resembles an artificial energy demand, and is used to evaluate the maximal respiration rate. Normal/high mitochondrial function is reflected in a high maximal respiration whereas a lower maximal respiration may imply mitochondrial dysfunction and a higher reliance on glycolysis. The spare respiratory capacity is the difference between maximal and basal respiration and indicates how close the cell is to its bioenergetical limit – it is thus a measure of the mitochondria's ability to adapt to a rapid increase in energy demand.

Worth mentioning is that the actual ATP production and proton uncoupling are liable to underestimation and exaggeration, respectively. This is due to the fact that during ATP synthase inhibition, the membrane potential is hyperpolarized which affects the voltage-dependent proton uncoupling. In other words, the ATP-synthesis is underestimated meanwhile the proton leak is overestimated. This brings the coupling efficiency (100 (ATP-production-linked respiration/ basal respiration)) as a more useful parameter for mitochondrial function as it reflects alterations in both parameters as a whole. The coupling efficiency is

therefore likely to change during altered mitochondrial function, and this ratio is also internally normalized [206].

Lastly, but of importance to the experimental work performed on macrophages is the non-mitochondrial respiration. This is a process which historically was discovered in phagocytes, neutrophils and later shown in macrophages and was termed "the respiratory burst" [150, 209]. The respiratory burst is mediated by NADPH oxidases (NOX1-5, DUOX1-2) which consume oxygen to generate reactive oxygen species (e.g. during an infection). Reactive oxygen species can also react with nitric oxide produced from NOS2-expressing macrophages thus producing peroxynitrite. Both nitric oxide and peroxynitrite inhibits several mitochondrial electron transport complexes [210]. The non-mitochondrial respiration may thus reflect on the effector function of macrophages.

LIPID METABOLISM MEASUREMENTS USING RADIOLABELED TRACERS

In this thesis, measurements of lipid metabolism are central. Circulating lipid levels reflect lipogenesis, lipolysis, lipid uptake, and lipid absorption from the diet [211]. To measure changes in any of these parameters separately, one can administrate lipids or glucose labelled with either stable or radioactive isotopes. The labeling of a molecule implies the substitution of a common atom such as carbon, hydrogen, nitrogen or oxygen with an isotope, which results in the formation of a tracer. The original molecule is referred to as the tracee. Stable isotopes are used in the clinic as they possess a minimal risk on health, but require magnetic resonance or mass spectrophotometry. This also allows also for simultaneous measurements of tracer and tracee with high precision as it can be used to distinguish between e.g. individual metabolic reactions (depending on the molecular composition of the tracer) [212]. However, the downside is that magnetic resonance or mass spectrophotometry is time consuming, expensive and requires profound sample preparation. In this work the radioactive isotopes ^{3}H-triolein and ^{14}C-glucose has therefore been used to measure lipid and glucose uptake as well as to estimate fatty acid oxidation and lipogenesis in vivo (^{14}C-glucose was also used to measure *de novo* lipogenesis *in vitro*). These lipid and glucose tracers were mixed with Intralipid and administered orally to mice, upon blood, tumor and adipose tissue were collected for analysis 2h after administration. In general, tissue samples were homogenized in in a mixture of chloroform and methanol to separate the aqueous and organic

phase. The ^{3}H and ^{14}C were detected using a liquid scintillation counter. The aqueous phase contains water-soluble metabolites. The ^{3}H counts in the aqueous phase can thus be used to estimate fatty acid oxidation. The organic phase contains neutral lipids such as triglycerides and the ^{3}H and ^{14}C counts represent, therefore respectively, measures of lipid storage and *de novo* lipogenesis.

RESULTS AND DISCUSSION

EXTRA CELLULAR FLUX ANALYZER: INTERPRETING OXYGEN CONSUMPTION DATA

Changes in cellular metabolism play a role in the development of pathophysiological conditions such as obesity-related comorbidities, autoimmune diseases and cancer. At a cellular level, phenotypic switching and metabolic interactions amongst cells are vital in both normal conditions during tissue development and remodeling as well as in many diseases such as cancer and infection.

Cellular density and nutrient level: key parameters in measurements of mitochondrial function

In vitro models, such as cell culture where one does not need to deal with the complexity of the body, may at a first glance seem to be more straightforward and less prone to variability than animal models. However, also cell culture models require extensive experience and consideration to several confounding parameters such as media composition, cellular density and incubation conditions. In other words, one needs to pay attention to changes in the microenvironment. Moreover, cancer cells are heterogeneous and typically display genomic instability, which add additional complexity to consider when designing the experimental set up.

We have used the Seahorse extracellular flux (XF) analyzer (Agilent Technologies) to assess mitochondrial function in 3T3-L1 adipocytes (**Paper I**), 3T3-L1 pre-adipocytes, E0771 breast cancer cells and bone marrow derived macrophages (**Paper II-III**). The experimental protocols for these measurements had to be defined for each cell type. Agilent Technologies encourage users to submit their cell-specific protocols online and these protocols can then be accessed in an online database. Among the cell lines found in this database are 3T3-L1 adipocytes. Nevertheless, to obtain oxygen consumption rates within the appropriate range we had to optimize the experimental conditions to work in our hands (**Paper I**). Moreover, no protocols for assessing E0771 breast cancer cells or bone marrow derived macrophages were to be found in this database, and these needed thus to be established from scratch.

One of the most critical parameters in these Seahorse XF analyzer experiments is the cellular density that determines the oxygen

consumption rates although this also varies between cell types and other experimental conditions. The recommendation from the manufacturer implies a near confluent monolayer of cells which roughly approximates to 100 000 cells per well. Numbers below this value can result in inconsistency in readings between probes (discussed later). However, numbers beyond this value may lead to nutrient depletion at a faster rate that in turn results in cells entering a dormant state where respiration decreases. Visually, this shows up as an oxygen consumption rate that declines over time and the response to challenges such as oligomycin may be low or even absent. Furthermore, such a behavior is also commonly seen in cells with extreme metabolic demands such as highly proliferative cells. Depletion of nutrients may thus lead to an altered metabolism that is not directly related to the type of treatment.

The optimal cellular density is determined by titration for which the outcome should display a linear relationship (determining the range of the assay) and this is discussed as one of the key points by Dranka et al [213]. In that study, a cell density between 20 and 50000 cells per well generated the most reproducible data when myocytes were studied. 3T3-L1 adipocytes, on the other hand, were seeded at 5000 cells per well to allow for the necessary proliferation during the differentiation steps and reached a number around 100 000 per well. For instance, we tried different β3-adrenergic agonist treatment lengths (20 minutes and 24 hours) and N-acetylcysteine (30 minutes to 48 hours) which led to the obvious and important insight that cells of different types and/or states consume nutrients and proliferate at different rates (mature adipocytes do however not proliferate at all). Occasionally 3T3-L1 pre-adipocytes remain in an undifferentiated and proliferative state after activation and this is associated with a completely different metabolic profile as outlined below. It is thus critically important to assess the degree of differentation prior Seahorse analysis. Moreover, using the same cell density and treatment lengths (e.g. C1QTNF3 recombinant protein treatment – **Paper II**) in different cell types may elicit different results on cellular metabolism simply because some cell types proliferate and/or consume nutrients faster.

3T3-L1 pre-adipocytes displayed a relatively low oxygen consumption rate and low response to oligomycin as well as high inter-plate variance, regardless of tested cell densities. This can be interpreted either as a consequence of too high cellular density resulting in nutrient depletion, which may conclude to re-evaluate the seeding protocol for this particular cell line. Moreover, this data can also be interpreted as an increased reliance on glycolysis to generate ATP than on oxidative

phosphorylation, a phenotype commonly seen in undifferentiated cell types such as stem cells and cancer cells that have underwent so-called mesenchymal stem cell transition [7, 214, 215]. As discussed above, nutrient depletion may however lead to a similar phenotype. Thus, one needs to be cautious when drawing conclusions from this type of oxygen consumption measurements.

To study interactions between E0771 breast cancer cells and adipose tissue (**Paper III**), we used the Seahorse XF analyzer in combination with co-culture. In the co-culture setting, it is likely that nutrients are depleted quicker than in the control (non-co-culture condition). This implies that the resultant metabolic profiles need to be evaluated carefully as the difference between groups could be either due to metabolic interactions (that we aim to study) or to excessive nutrient depletion in the co-cultured cells. Therefore, we have analyzed the effect of chamber volume and co-culture time in these experiments and found that 2 mL and 24h culture in a 12-well plate did not lead to increased nutrient depletion in co-cultured E0771 breast cancer cells compared to E0771 breast cancer cells cultured alone (40 000 cell/well) (**Paper III**).

Metabolic plasticity

The fact that undifferentiated cells typically rely on glycolysis doesn't rule out that they can produce ATP through oxidative phosphorylation. Such capability of switching from one metabolic phenotype to another can be referred to as "metabolic plasticity". Metabolic plasticity is commonly seen in cancer cells. Such metabolic plasticity may manifest as intra- (between wells) and inter-plate (between plates) variation. For which the latter has proven to be of the largest concern in oxygen consumption rate measurements, although intra-plate variation may also be troublesome [216]. Intra-plate variation may seem to be an unlikely problem as established cell lines are supposedly stable. Together with the fact that cell culture proves to be a stable environment sustaining less factors provoking evolution of cancer cells than what occurs in the organism itself. However, this does not out rule that cancer cells constantly evolve regardless, and is demonstrated to be problematic in most immortalized cell lines, being explained expressed as genetic drift sustained by sub-culture [217].

Indeed, during this work (**Paper I-III**) inter-plate variation has been of similar concern as intra-plate variation. Even the slightest difference in cellular number between wells may have large effect on respiration since E0771 breast cancer cells proliferate extremely fast (**Paper II**).

Furthermore, some cells did not respond to the compounds used in the mito stress test, although some cells did. Unresponsiveness to the uncoupler FCCP can be interpreted as having a cellular metabolism already close to maximal capacity and/or already an uncoupled electron transport chain (depending on the basal oxygen consumption rate). However, if the basal oxygen consumption rate is low meanwhile not responding to FCCP this may infer to at a mitochondrial dysfunction and/or increased reliance on glycolysis. A reliance on glycolysis may be tested more directly through measurements of the extracellular acidification rate (and the glycolysis stress test). Meanwhile unresponsiveness to oligomycin may reflect that ATP-production from ATP synthase (mitochondria) is not responsible for the observed oxygen consumption rate (the electron transport chain may be uncoupled). Indifferences in the basal respiration (or ratios in oxygen consumption rate between oligomycin and FCCP treatments) is generally interpreted as an altered mitochondrial function, however non-responsiveness could also be interpreted as a reduced mitochondrial density rather than diminished mitochondrial function. Changes in mitochondrial density may be of greater concern in certain experimental conditions where mitochondrial dysfunction is induced, as this ultimately allows for the removal of non-functional mitochondria through mitophagy [218]. In certain experimental conditions such as co-culturing adipocytes with cancer may inflict such changes. Treatment of C1QTNF3 reduced the mitochondrial respiration in macrophages and this is generally interpreted as reduced mitochondrial function, but at least in theory the reduced respiration could be a consequence of reduced number of mitochondria (**Paper II**).

The third cell type we have analyzed with Seahorse technology was bone marrow derived macrophages (**Paper II**). For this cell type, we found that this methodology gave reproducible results. M1- and M2-activated bone marrow derived macrophages displayed their expected phenotype; the energy production of M1-like depended primarily on glycolysis whereas M2-like used oxidative phosphorylation (**Figure 7**) [153]. Furthermore, C1QTNF3-induced reduction in mitochondrial respiration was associated with increased glycolysis i.e. the reduced respiration in the experimental setting is unlikely to result from e.g. increased proliferation associated with nutrient deprivation in C1QTNF3-treated cells (**Paper II**). Nevertheless, inter-plate variation is of great concern also when studying macrophages. Bone marrow derived macrophages are prone to become more M2-like during the differentiation (and activation phase) as reflected by their gene expression profile where M1-like activated cells also display M2-markers [219]. In this thesis, the metabolic profile was,

however, clearly different between M1- and M2-activated macrophages. We believe that the response to C1QTNF3 varies dependent on the degree of macrophage differentiation. Such scenario is unfortunately difficult to control for since cells from different batches may reach slightly different level of differentiation despite the same treatment protocol, leading to inter-plate variation. For instance the degree of macrophage differentiation may affect metabolic plasticity leading to different response to C1QTNF3 treatment [220]. In general, the criteria which was used to determine successful or adequate macrophage differentiation was based on M2-like macrophages having a slightly higher basal metabolism and spare respiratory capacity than naïve, as described elsewhere [221].

Research indicate that cancer cells are able to quickly re-adapt to nutrient deprivation by changing gene expression and metabolic profile to compensate the metabolic needs, similarly such variation is seen in the tumor microenvironment, and thus is of high consideration when comparing different cell lines and primary derived cells [222]. However, E0771 breast cancer cells did not seem to have this ability *in vitro*: In the fatty acid oxidation assay of E0771 breast cancer cells (**Paper III**), the basal respiration decreased with time likely as a consequence of the very low glucose levels in the fatty acid oxidation assay media which were 10 times lower than the glucose levels in the Mito stress test buffer. Thus, cellular metabolism in cell culture is sensitive to media composition where different media elicit rewiring of metabolic pathways and alternated metabolomic profiles or simply leads to nutrient deprivation (which is the most likely scenario in this case).

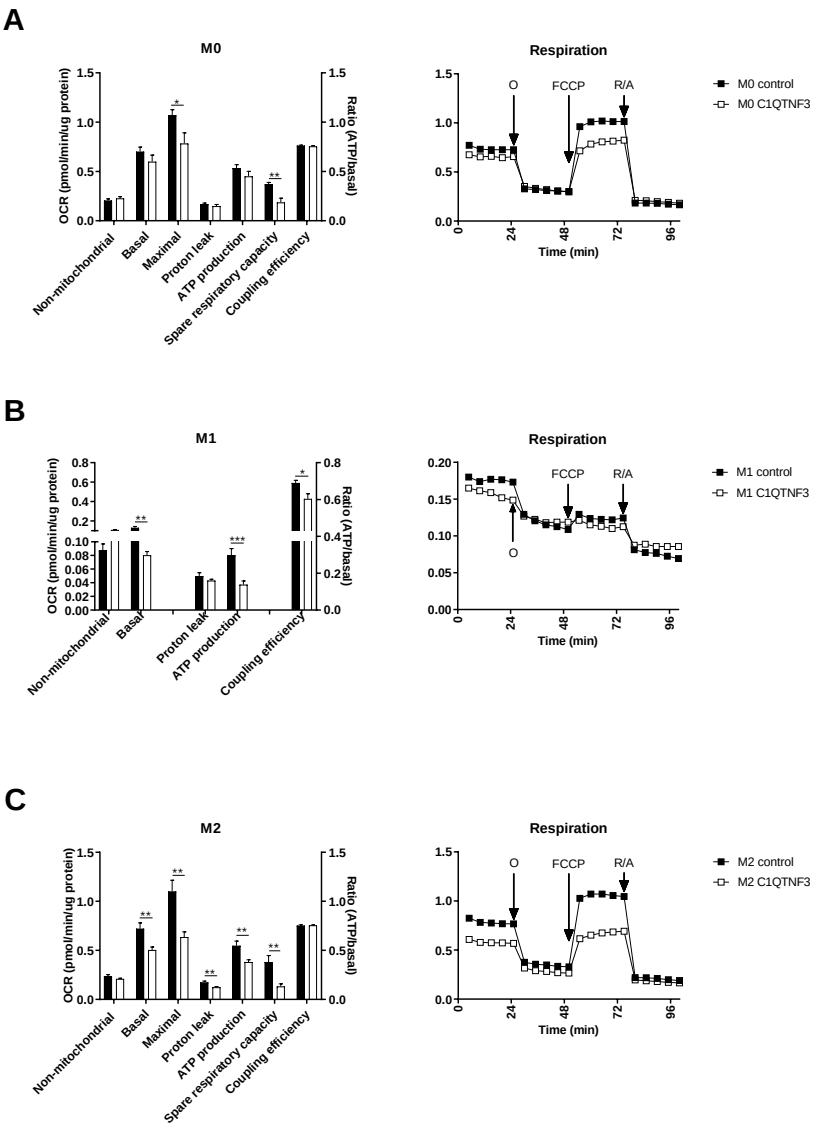


Figure 7. The metabolic profiles of naïve, classically and alternatively activated macrophages in normal conditions and when challenged by C1QTNF3 recombinant protein for 24 hours. As depicted by the graphs, M1 (i.e. classically) activated macrophages (B) presented with an energy profile void of mitochondrial respiration, unlike naïve and M2 (alternatively activated) macrophages, (A) and (C) respectively, which displayed normal mitochondrial function. C1QTNF3 led to a decreased mitochondrial respiration.

Technical variance

Intra- and inter-plate variance is not only reflected upon the experimental conditions as defined by the user but also by technical issues. During each experiment there have been a number of wells that don't respond to oligomycin and FCCP. These wells are typically located on the edges of the cell culture plate. This phenomenon is well known and may be a consequence from temperature fluctuations as there are fewer neighboring media-filled wells. Essentially this reduces the number of replicates in each treatment group; therefore, in order to maximize the number of replicates, the number of treatment groups was limited.

Similar non-responsiveness has been observed in wells that initially were responding to treatment (oligomycin) but then failed to respond to FCCP. This was interpreted as the sensor being blocked by air bubbles or cellular debris that may be generated during running of the assay (and not as a consequence of a retracting sensor between each measurement as it never leaves the media). Although the sensor continues to measure oxygen this does not out rule that the sensor was faulty from the beginning, which makes it sometimes difficult to distinguish between biological and technical outliers. The technical aspects affecting reproducibility and reliability of every probe (of each sensor) can be indulged elsewhere, and may partly serve as an explanation to the data variability [223].

Conclusions

Seahorse XF analyzers are more and more used to assess mitochondrial function and glycolysis of cultured cells, isolated mitochondria and even of tissue pieces. The method allows for comprehensive drug screening in different scenarios where long- and short-term treatments effect of compounds can be studied. However, great cautiousness has to be kept in mind while designing studies, as cells can display metabolic plasticity leading to different results due to timing, cellular density and treatment strategy. Cells not displaying the expected response to oligomycin and FCCP may be considered as technical outliers, but such unresponsiveness may originate from starvation, preference for glycolysis and/or a redirected metabolite flow into pathways required for biosynthesis due to *in vitro* treatment conditions. This suggests that it may be easier to analyze differentiated cell with this technology than undifferentiated stem cell-like cells or stem cells. Thus, using extracellular flux analyzers for metabolic phenotyping requires deep knowledge about the properties of the cells of interest including relevant confounding factors.

C1QTNF3 IN TUMOR PROGRESSION: MACROPHAGE IMMUNITY IN THE ADIPOSE TISSUE-TUMOR INTERFACE

During the past decade, cancer treatment has had a crude appearance, essentially relying on the utilization of chemo- and radiation therapy. The direction of cancer treatments was revolutionized by the idea of targeting immunity. This was demonstrated by the first generation of immune-checkpoint inhibitors, which act by blocking certain receptor and ligand interactions involved in pathways such as activation and effector function of immune cells. These pathways are in turn crucial for the development of self-tolerance allowing the tumor to remain undetected by the immune system (i.e. immune evasion). The first generation of antibody-based immunotherapies, CTLA4, PD-1 and PD-L1, have been used to inhibit de-activation and programmed cell death of T cells [25]. Immune cell activation and effector function exhibit a high level of complexity as well as diversity, which to large extent is defined by the regulation of cellular metabolism [30]. Cellular metabolism is a crucial aspect, not only for activation and effector function but also for immune regulation including antigen presentation, clonal expansion of T cells, differentiation and wound healing [30]. Local cues in the microenvironment act as signals to determine the immune landscape, however, immunity itself can be regulated by cellular metabolism by means of forced reprogramming (i.e. tumor associated cell types such as cancer associated fibroblasts, macrophages and lymphocytes) or metabolite restriction (nutrient competition) as a consequence of tumor interaction. Similarly, excess of nutrients such in obesity-related disorders is suggested to exert a role in immunity via cellular metabolism. Immune cell infiltration has been used to define the tumor microenvironment into different immune infiltrate classes, although this classification is based on low-resolution sources such as bulk tissue microarrays and immunohistochemistry, whereas tumor-derived metabolic intermediates can be used to associate the immune cell infiltrate based on the metabolic status [25, 224]. In obesity, the breast cancer microenvironment can be dysregulated both as a consequence from systemic alterations such as hyperinsulinema and hyperglycemia and due to local changes in adipose tissue that involve low grade chronic inflammation associated with an altered release of adipokines/cytokines. In general, three major links between obesity and increased tumor progression have been proposed; systemic endocrine alterations that involve 1) the insulin-like growth factor 1 axis, and 2) altered adipokine levels (leptin and adiponectin) [225] and 3) increased estrogen levels in postmenopausal obese women. Furthermore, obesity is

associated with an increased risk of developing hormone negative (aggressive subtype) in premenopausal women [8, 226]. There is however knowledge gap as for how lean and obese adipose tissue can affect tumor progression through local interactions.

Finding proteins in the adipose tissue-tumor interface

In order to identify novel adipose tissue-derived factors potentially important in tumor progression and/or adipose tissue functionality, we compared the gene expression profiles between E0771 breast cancer-associated and control mammary/inguinal adipose tissue in syngeneic lean and high fat diet-induced obese female mice (**Paper II**). This approach revealed a marked upregulation of *C1qtnf3* along with several chemokines and macrophages makers in tumor associated adipose tissue, and this upregulation was enhanced by obesity (**Figure 8**). Importantly, increased *C1qtnf3* mRNA expression was associated with increased inguinal adipose tissue release of C1QTNF3 protein although serum C1QTNF3 levels (about 100 ng/ml) were similar between groups. Moreover, the serum levels were approximately 6-10 times lower than the amount secreted from 30 milligram adipose tissue in 1 mL media (after 6 hours), suggesting a more prominent role as an auto- or paracrine mediator than as a classical hormone. C1QTNF3 may thus be similar to inflammatory cytokines that typically are released in response to tissue damage and/or infection and act locally, although low levels can be found in the circulation. At this stage, it was difficult to interpret what the measured C1QTNF3 levels mean, but most cytokines are very potent and act at low concentrations. Unlike most cytokines however, C1QTNF3 has previously been shown to exert anti-diabetic and anti-inflammatory effects as well as mediating effects on oxidative phosphorylation [59, 61, 62]. Whereas, very little is known about its potential role in tumor progression or macrophage regulation.

Figure 8. The *C1qtnf3* gene expression was upregulated in tumor-associated inguinal white adipose tissue (IWAT) and this effect was enhanced by diet-induced obesity.

C1QTNF3 regulation in mouse adipose tissue

The gonadal adipose tissue expression and circulating levels of C1QTNF3 are increased in genetically obese *Ob/Ob* mice whilst circulating levels are reduced in diet induced obese mice [58-60]. However, very little is known about *Ciqtnf3* gene expression in different adipose depots. We have shown that gonadal and mesenteric adipose tissues have a higher *C1qtnf3* expression than inguinal adipose tissue. However, the major part of the gene expression was not attributable to the adipocytes, but rather originating from cells of the stroma vascular fraction and this expression was enhanced by obesity (at least in inguinal white adipose tissue, whereas other depots were not examined). Moreover, the *C1qtnf3* expression was relatively high in 3T3-L1 pre-adipocytes, while very low levels were detected in peritoneal macrophages and E0771 breast cancer cells. Interestingly, 1-week high fat feeding of male mice led to a dramatic upregulation of the inguinal adipose tissue expression of *C1qtnf3* and the pan-macrophage marker *F4/80*. This is a time point where significant tissue remodeling occurs in inguinal adipose tissue as shown by loss of collagenous septa [227] associated with recruitment of $CD206^{+}$-macrophages (Vujičić et al, unpublished observation in the lab). Based on these observations in breast cancer- and high fat diet-challenged conditions, we hypothesized that C1QTNF3 played a role in macrophage recruitment to inguinal adipose tissue. Such effect on macrophages may be at the level of chemotaxis, proliferation, differentiation and/or activation as discussed in the following section.

C1QTNF3 in macrophage metabolism

C1QTNF3 has been shown to regulate metabolism and to be expressed by metastasis-associated fibroblasts. These observations, in combination with our findings that the inguinal adipose tissue expression of *C1qtnf3* is induced by breast cancer and by 1-week high fat diet feeding, and is positively correlated with macrophage markers, suggest that C1QTNF3 is involved in macrophage regulation through metabolic re-programming (**Paper II**). Macrophages play a key role both in adipose tissue during remodeling (e.g. expansion) and in tumor immunity. Thus, we hypothesized that the outcome of such a mechanism can be involved in tumor rejection or is exploited by the tumor itself for immune evasion. Alternatively, the increased C1QTNF3 levels are unrelated to tumor immunity and instead they may play a role in the adaptive adipose tissue remodeling that occurs in response to high fat diet-induced adipocyte expansion or increased pressure from a growing tumor. Immune evasion refers to one of the Three E's of immunoediting; to avoid elimination, achieve equilibrium or to escape that occurs at different stages of tumor progression, for which the immune evasion (escape) most often relate to later stages [228]. Tumor-associated macrophages, which typically are M2-like, are in general associated with enhanced tumor progression (as outlined in the introduction). Furthermore, data have also shown that tumors can be recognized by the immune system, and that the accumulation of tumor infiltrating lymphocytes in fact serves to prevent tumor progression as opposed to serve the tumor [229].

In order to evaluate this hypothesis and to thus elucidate if C1QTNF3 play a role in macrophage regulation and tumor progression, both *in vivo* and *in vitro* techniques were employed. C1QTNF3 recombinant protein was given to E0771 breast cancer cells, 3T3-L1 (pre- and mature adipocytes) and bone marrow derived macrophages (naïve, M1 and M2-like) to investigate whether C1QTNF3 affects metabolism. C1QTNF3 did not affect oxidative phosphorylation of either E0771 breast cancer cells or 3T3-L1 adipocytes. There was however a small increase in ATP-production and coupling efficiency in C1QTNF3-treated 3T3-L1 pre-adipocytes, but the biological significance of such a small effect can be questioned. These data do not exclude that there are changes in fatty acid oxidation or glycolysis, i.e. substrate specificity or pathway rerouting (including anaplerotic reactions), but these parameters were not measured. Interestingly, C1QTNF3 diminished several parameters of the oxidative phosphorylation in all three macrophages subtypes (naïve, M1- and M2-like), where the most prominent effect was seen in M2-like macrophages (**Figure 7**). These data suggest that C1QTNF3 enhances

M1-like macrophage polarization or activation. To evaluate this assumption, the glycolytic rates (extracellular acidification rates, ECAR) were measured in in naïve and M2-like macrophages. Indeed, C1QTNF3 treatment led to increased glycolysis. In further support of this metabolic/immunological reprogramming, C1QTNF3 treated M2-like macrophages displayed an increased expression of M1-like macrophage markers (*Nos2* and *Il-1b*). The modulation of effector function through metabolism after activation (metabolic plasticity) was also studied; 1h C1QTNF3 treatment of M2-like macrophages reduced the maximal respiration and the spare respiratory capacity indicating that C1QTNF3 has an impact on metabolism even after M2-activation. As judged by Western blot analysis, this effect appeared to involve ERK- and possibly Akt-signaling, but not NFκB-activation (**Paper II**).

There have been several reports on tumor associated macrophages showing the ability of M2-like to repolarize into M1-like whereas, a non-reversible mitochondrial dysfunction in M1 macrophages has been identified as a mechanism preventing M2 repolarization [220, 230]. Based on our *in vitro* data, C1QTNF3 may be involved in a tumoricidal response through its M1-polarizing effect on M2 macrophages.

C1QTNF3-mediated immunity and role in tumor progression

Bone marrow derived macrophages undergo phenotypical changes towards a pro-inflammatory phenotype when subjected to C1QTNF3 recombinant protein. This was evident from reduced mitochondrial metabolism and increased glycolysis of naïve and M2-like activated macrophages, as well as from an M1-like gene expression profile. In order to test whether the increased local C1QTNF3 levels affects tumor progression *in vivo*, obese E0771 breast cancer implanted mice received either C1QTNF3 or isotype control antibodies. We hypothesized that blockage of C1QNTF3 inhibits the formation of M1-like macrophages in the tumor-adipose tissue interface possibly increasing tumor progression. We found that antibody-mediated neutralization of C1QTNF3 protein decreased tumor-induced infiltration of macrophages in inguinal adipose tissue (**Figure 9**). However, no effect was seen on neither tumor weight, nor on the percentage of tumor-associated macrophages, macrophage polarization in tumor, tumor associated inguinal white adipose tissue, and spleen (**Paper II**). As judged by FACS analysis of CD206 and NOS2 expressing macrophages ($CD11b^{+}F4/80^{+}$ cells), the effect of C1QTNF3 on macrophage polarization observed *in vitro* could thus not be reproduced *in vivo*. One possibility is that the variability between mice was too large to allow for detection of a possible decrease in the already

very low percentage of $NOS2^+$-macrophages in the C1QTNF3 antibody-treated group. Another explanation for this discrepancy between *in vitro* and *in vivo* conditions is that certain molecules can exert different functions on macrophage infiltration and polarization as a consequence of temporospatial effects such as heterogeneous microenvironment depending on tumor size and stage. An altered microenvironment may also induce post-translational modification that affects the biological function of a given cytokine [231]. For instance, CCL2 has been shown to act both as a chemoattractant and a regulator of macrophage polarization in tumors and in obese adipose tissue. Removal of CCL2 results in increased M1-like polarization of human macrophages, and similarly as shown by another study, CCL2 stimulation caused peripheral blood cells to polarize towards and M2 phenotype [232, 233]. However, neutralization of CCR2 (the receptor for CCL2) reduces the recruitment of M1-like macrophages to obese gonadal adipose tissue [234]. Furthermore, macrophage recruitment and polarization towards M1 in adipose tissue during obesity is attributable not only to the CCL2-CCR2 axis, but also to e.g. the CCL5-CCR5 axis. Inactivation of the latter resulted in a reduced number of adipose tissue macrophages, whereas, recruited macrophages expressed M2-like phenotype [235]. Thus, there are many factors that can affect macrophage polarization and some factors may even display different function dependent on the (patho-) physiological setting. It is therefore not improbable that the effect of C1QTNF3 on macrophages depends on the microenvironment.

In most mouse models of obesity, there is an increased infiltration of macrophages in adipose tissue and these macrophages adopt a pro-inflammatory profile (M1) [236]. In this study (**Paper II**), diet-induced obesity alone did however not have any effects on macrophage infiltration and polarization in inguinal adipose tissue in females, which may reflect both the studied adipose tissue type and sex differences. Macrophage infiltration is less frequent in inguinal than in gonadal adipose tissue and females display in general a lower infiltration of macrophages than males [237]. Unlike healthy lean conditions, obesity was however linked to an increased macrophage infiltration in tumor associated inguinal adipose tissue compared to control inguinal adipose tissue (**Paper II**). Moreover, tumors in obese animals showed a trend towards increased accumulation of macrophages compared to lean animals and these macrophages displayed an increase M2 and M1-M2 polarization. Given the large body of research showing that obesity causes an M1-like polarization, this effect may appear surprising. However, more recent studies show that obese adipose tissue macrophages are both of M1 and M2 type, associated with a metabolic

profile expressing both glycolysis and oxidative phosphorylation which can be interpreted as more M2-like than strictly M1-like [238-240]. Moreover, our data are however in line with a study by Galván et al. showing that obesity increases the infiltration and M2-polarization of tumor associated macrophages in the context of androgen-sensitive prostate cancer [241]. Thus, obesity may promote tumor progression through increased M2-like polarization of tumor associated macrophages although a potential causal link between obesity, M2-polarization and increased tumor size remains to be established.

Conclusions

Meta-analysis has suggested that obesity is correlated with a higher incidence as well as more aggressive cancer types. Moreover, adipose tissue itself has been shown to serve as an environment of which cancers prefer to metastasize. The results presented in **paper II** suggest that breast cancer-induced increase in inguinal adipose tissue C1QTNF3 levels increase macrophage recruitment. Moreover, we observed that C1QTNF3 exert potent effects on macrophage metabolism and polarization in vitro; upregulating the M1 phenotype characteristics such as decreased oxidative phosphorylation and increased glycolysis. In contrary to the main hypothesis, there was however no effect of antibody-mediated C1QTNF3 neutralization on macrophage polarization and tumor progression *in vivo*. We believe that the differences in the microenvironment between the *in vitro* and the *in vivo* condition may serve as explanation for the different outcomes. While possible anti-metastatic effects of C1QTNF3 remain to be elucidated, the current data suggests that C1QTNF3 only plays a modest role in tumor immunity. On the other hand, C1QTNF3 may play a key role in the physiological remodeling of subcutaneous adipose tissue in the context of diet-induced weight gain – a phenomenon that is currently being investigated in our laboratory.

Sex-mediated differences in the immune response play a role in both human and murine models of disease. In our experimental setting, *C1qtnf3* gene expression in adipose tissue was however comparable between male and female mice. Given the sex differences in fat distribution, metabolism and immune responses, the effects of C1QTNF3 is likely to be different between male and females, but this remains to be further elucidated in models of both of obesity and tumor progression.

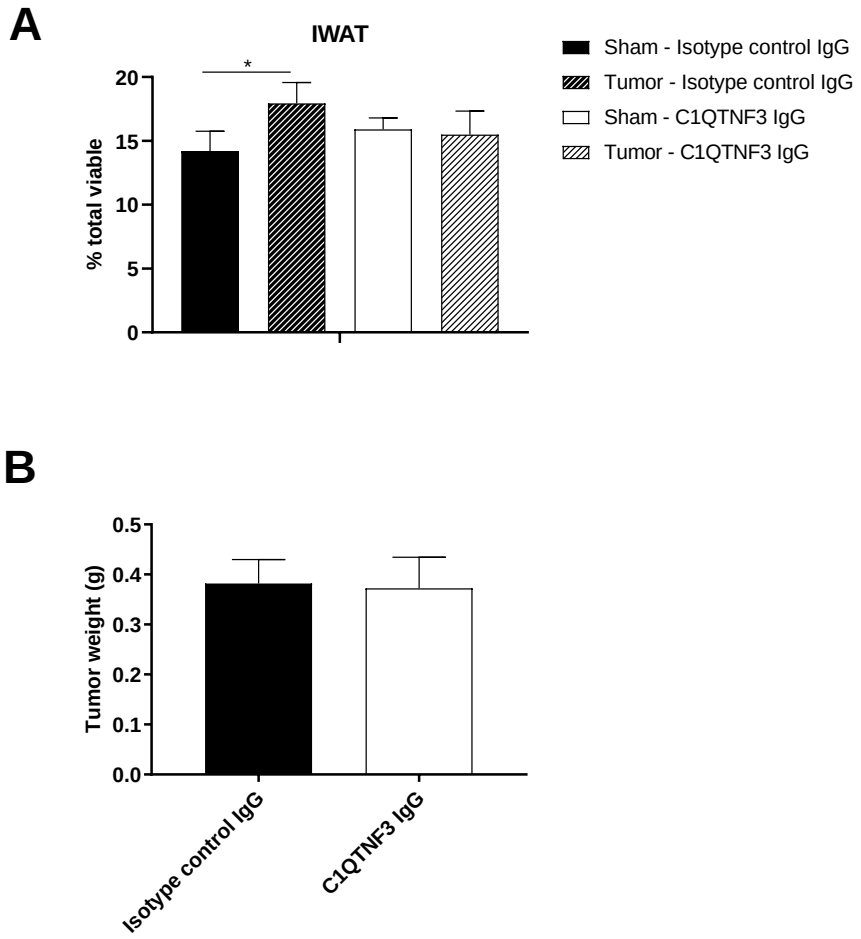


Figure 9. Obese mice orthotopically implanted with E0771 breast cancer were treated with an antibody to neutralize the effect of C1QTNF3 protein or isotype control antibody. Removal of C1QTNF3 resulted in a reduction of **(A)** total ($CD11b^{+}F4/80^{+}$) macrophages in tumor associated adipose tissue, but there was no effect of C1QTNF3 neutralization on **(B)** tumor size.

THE TUMOR GROWTH PROMOTING EFFECT OF ADIPOSE TISSUE IS ASSOCIATED WITH INCREASED DE NOVO LIPOGENESIS IN THE TUMOR

The stimulating effect of adipose tissue on tumor progression is not only a consequence from e.g. alterations in the immune response, but may also take root from metabolic symbiosis between cancer cells and adipocytes/adipose tissue. Altered energy metabolism has been described as an emerging hallmark of cancer [242]. In brief, this refers to reprogramming of the cellular energy metabolism to sustain e.g. the rapid proliferation of cancer cells. The Warburg effect is one example of such metabolic reprogramming: in the 1920s Otto Warburg demonstrated how cancer cells take up large amount of glucose and converts it to lactate even under aerobic conditions. Although the Warburg effect often refers to the pathological condition of tumors, it occurs also in normal tissue and activated immune cells, where there is a great need to generate a rapid bursts in energy for functionality [243, 244]. The Warburg effect is also described in the so-called lactate shuttle hypothesis, in which cells within a tissue display a symbiotic relationship; cells that increase aerobic glycolysis shuttle lactate to cells that rely on oxidative phosphorylation. Nowadays, it is known that metabolic reprogramming in cancer (as well as in e.g. immune cells) also includes other pathways such as increased glutaminolysis and lipogenesis.

Adipose tissue is a metabolically active tissue that may provide tumors with fatty acids that are essential for growth, proliferation and survival of cancer cells [245]. Indeed, tumors have been proposed to engage in such metabolic relationships to access lipid content of adipose tissue, highlighting both the existence of cancer associated adipocytes, as well as fibroblasts mediating such a communicative relationship [114, 124, 125, 142, 246, 247]. Mechanistically, there is little evidence on how this relationship is established, and our aim (**Paper III**) was to elucidate the local effect of adipose tissue on tumor metabolism and progression (metabolic programming) focusing on tumor uptake of exogenous lipids (adipocyte lipolysis and from the diet) and *de novo* lipogenesis from glucose.

Metabolic characterization: fatty acid metabolism in E0771 breast cancer

E0771 breast cancer would, based on its triple-negative subtype, be associated with a profile towards increased utilization of exogenous lipids and reduced *de novo* lipogenesis [134]. Although molecular classification has been shown to be a promising element, it is important to realize that such a classification is not trivial and may not accurately reflect the functionality of all breast cancers within a given subtype. Thus, E0771 breast cancer was characterized with respect to its ability to mobilize and use exogenous fatty acids, as well as for *de novo* lipogenesis using both *in vitro* and *in vivo* models. E0771 breast cancer cells were transplanted both ecto- and orthotopically (under the skin outside adipose tissue and within mammary/inguinal adipose tissue where breast cancer naturally grows) as opposed to the experimental design used in **paper II** where only orthotopic tumors were studied. Tumors within adipose tissue grew 4.5-fold larger than ectopic tumors and the progression was further enhanced by obesity; orthotopic and ectopic tumors of obese mice grew respectively, 2- and 3-fold larger than in lean mice (**Figure 10**). These data suggest that adipose tissue plays in important role in tumor progression, at least for this type of tumor. Furthermore, the enhanced growth in obesity suggests that disrupted adipose tissue functionality further enhances tumor progression although we cannot disregard the pro-tumorigenic effect of e.g. the dietary lipids, hyperglycemia and hyperinsulinemia.

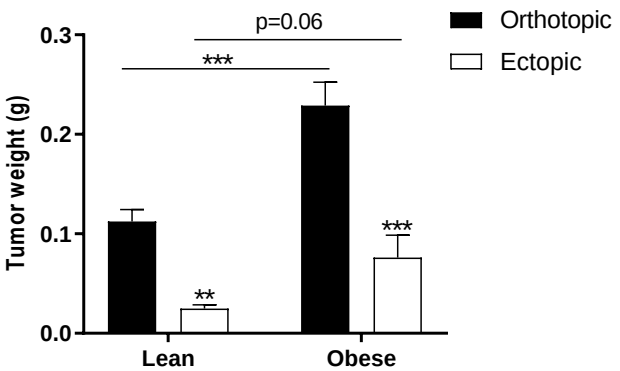


Figure 10. Illustrates the tumor growth of ortho- and ectotopically transplanted tumors. Orthotopic tumors were implanted in adipose tissue, associated with enhanced tumor growth compared to ectopic tumors. Obesity increased the tumor growth in both orthotopic and ectopic tumors.

Animals harboring tumors for two weeks displayed increased lipolysis as determined by their serum free fatty acid levels. In general, increased lipolysis is a sign of cancer-associated cachexia, but mice harboring E0771 breast cancer tumors did not display any weight or fat mass loss. Cachexia in humans, is a phenomenon that usually occurs at later stages of chronic disease and in cancer types other than breast cancer such as gastric and pancreatic cancer, whereas, affecting 50% of all patients suffering from cancer [248, 249]. Thus, it is possible that E0771 breast cancer, albeit being able to induce lipolysis, requires a longer period than two weeks in order to induce measurable cachexia. No metastatic lesions in lungs or other peripheral organs were found, henceforth the model could thus be considered to represent early stages of breast cancer.
To further characterize the extent of the lipolytic capability of E0771 breast cancer cells, we compared the ability to induce lipolysis between different breast cancer cell lines *in vitro*. To this end, 3T3-L1 adipocytes were treated with cancer-conditioned media for 24 hours upon the resultant glycerol levels in the media was measured. In line with our *in vivo* data, E0771 breast cancer cells secrete soluble mediators that induce lipolysis to a larger extent than other breast cancer subtypes. These data are in agreement with a model where triple negative breast cancers, such as E0771 breast cancer, thrive on exogenously derived fatty acids although it is not necessarily so that a lipolysis-inducing tumor is able to take up and use exogenous lipids.

Next, radiolabeled lipid and glucose tracers were used to compare lipid and glucose uptake, fatty acid oxidation and lipogenesis between orthotopic and ectopic tumors. We found that orthotopic tumors take up more lipids and glucose (although the total glucose uptake difference didn't reach significance, $p=0.06$) and that this increased uptake largely was explained by increased lipogenesis as judged by the [^{3}H]-triolein and the [^{14}C]-glucose counts in the organic phase (**Figure 11**). This indicated that orthotopic tumors both take up more exogenous lipids and synthesize more (neutral) lipids from glucose (*de novo* lipogenesis). Orthotopical tumors also displayed an increased expression of *Fasn*, which further supports increased *de novo* lipogenesis in orthotopic compared to ectopic tumors (**Paper III**).

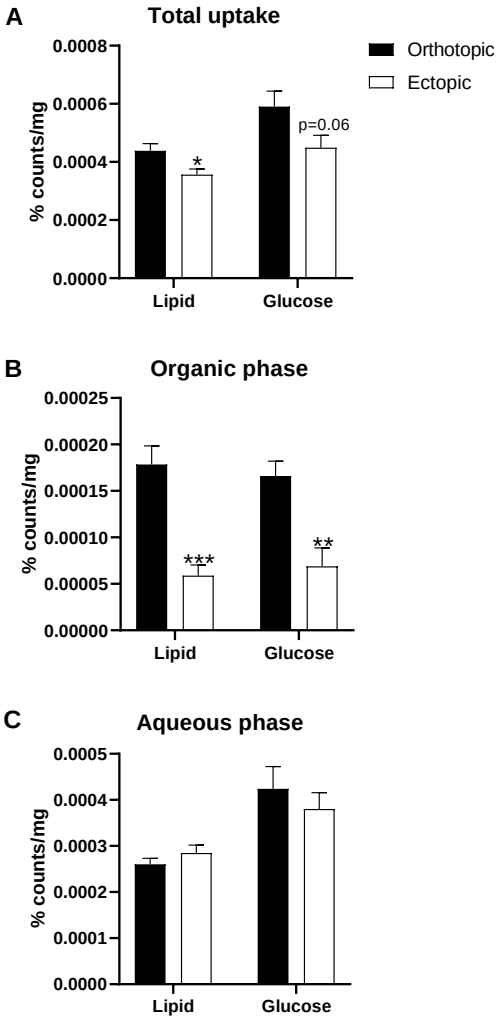


Figure 11. Radiolabeled tracers (glucose and triolein) administered by force feeding was used to determine glucose and lipid utilization in ortho- and ectopic tumors. The separation of the sample using organic phase separation allows for the quantification of neutral lipids storage and water-soluble metabolites. **(A)** The lipid uptake was higher in orthotopic than in ectopic tumors, and there was a similar strong trend for glucose uptake. **(B)** The counts in organic phase represent neutral lipids; orthotopic tumors have a higher lipid capacity and a higher glucose-dependent *de novo* lipogenesis than ectopic tumors. **(C)** The similar lipid and glucose counts in the aqueous phase of orthotopic and ectopic tumors indicated that catabolic processes, such as fatty acid oxidation, was not affected by the anatomical location of the tumor.

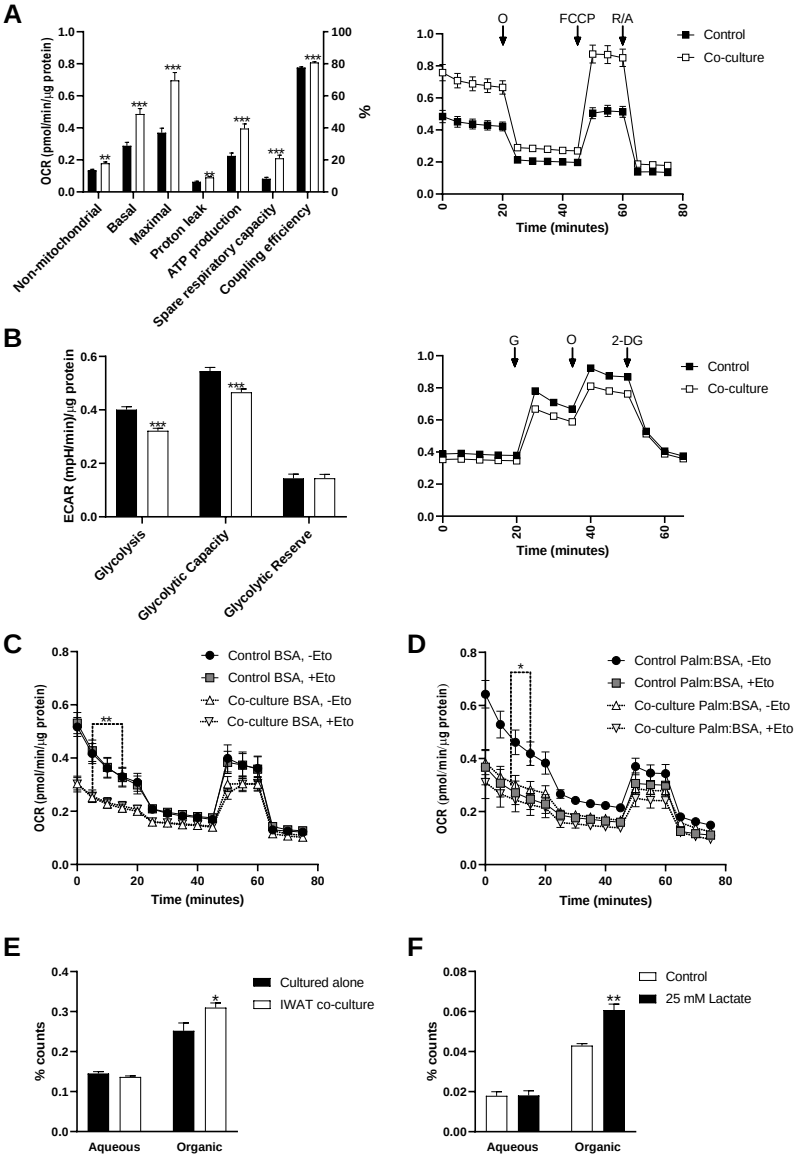
Fatty metabolism in vitro

Thus, adipose tissue-associated E0771 breast cancer tumors displayed increased uptake of lipids and increased *de novo* lipogenesis compared to ectopic E0771 breast cancer tumors. Whether this difference can cause or simply is a consequence from the enhanced tumor progression of orthotopically growing tumors is unknown. Therefore, the possible effects of adipose tissue on tumor metabolism in vitro were investigated. In a series of Seahorse XF Analyzer experiments in combination with co-culture of E0771 breast cancer cells and inguinal adipose tissue, we determined that tumor cells co-cultured with adipose tissue display an increased oxidative phosphorylation whereas the preference for glycolysis was reduced (i.e. diminished Warburg effect) (**Figure 12**). Elevated lactate levels can inhibit glycolysis in T-cells and in alveolar epithelial type II cells [250, 251] and we may have a similar mechanism at play here: adipose tissue releases large amounts of lactate that can be taken up by the tumor and thereby block the conversion of pyruvate to lactate through feedback inhibition and/or to serve as substrate for energy production or lipogenesis. Moreover, a reduced extracellular acidification rate may not necessarily mean that ATP-production from glycolysis is reduced.

A reduction in glycolysis could possibly also be mediated by increased fatty acid oxidation as consequence from increased fatty acid levels in the presence of adipose tissue (although our *in vivo* tracer data did not suggest such a mechanism). Utilizing the Palmitate-BSA Fatty acid oxidation Substrate kit, we determined that the use of endogenously derived fatty acids was not of a preference, whereas the use of exogenous derived fatty acids was reduced in the interface of adipose tissue. In contrast to *in vivo* results where there was no difference in catabolism of exogenously derived fatty acids. However, we believe that these *in vitro* data do not accurately reflect the metabolic symbiosis between adipose tissue and cancer cells in a more physiological setting. Rather the low glucose levels in this assay may put these rapidly dividing cells in a dormant state (as discussed previously). It is also possible that the rapid proliferation seen *in vitro* relies on high *de novo* lipogenesis, and fatty acids oxidation and synthesis are unlikely to occur in the same cell at the same time. *In vivo*, a tumor may well be heterogeneous where some cells engage in lipogenesis while others in fatty acid oxidation dependent on e.g. their stage and oxygenation.

In line with the *in vivo* data, adipose tissue co-culture triggers increased *de novo* lipogenesis from glucose in E0771 breast cancer cells (**Figure**

12E). Acetyl-CoA is an essential substrate for *de novo* lipogenesis and can be generated from the citric acid cycle. Lactate has been proposed to be utilized directly in lipogenesis, in both bovine and rat adipose tissue in 1981 and 1974 respectively. Thus it is possible that adipose tissue derived lactate can be utilized to generate Acetyl-CoA possibly feeding into the citric acid cycle and thereby increasing oxidative phosphorylation and/or feeding into the lipogenic pathway [252, 253]. Such effect of lactate was not measured in this study, but could be an interesting aspect to analyze in the future. However, the effect of lactate treatment on glucose-mediated lipogenesis was examined, and we found that lactate is a possible mediator of the adipose tissue-induced increase in *de novo* lipogenesis of E0771 breast cancer cells (**Figure 12**). Thus, we have proposed that exogenous lactate reduces the conversion of pyruvate to lactate and thereby increases the use of glucose for lipogenesis. Alternatively, E0771 breast cancer cells is able to oxidize lactate via mitochondrial lactate dehydrogenase (a possible metabolic pathway, reviewed in [254]), thereby glucose is spared and can be used for lipogenesis. Based on these data, we have hypothesized that E0771 breast cancer tumors associated with adipose tissue refrains from the Warburg effect and instead thrive on lactate produced by adipose tissue. However, this hypothesis remains to be fully evaluated using e.g. lactate tracers and additional Seahorse extracellular flux analyses.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OCR (pmol/min/µg protein)
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IWAT co-culture
Aqueous
Organic
F
% counts
Control
25 mM Lactate
Aqueous
Organic

Figure 12. Extracellular flux analysis was performed to characterize changes in the metabolic profile of E0771 breast cancer cells with or without 24h inguinal white adipose tissue co-culture. Mito stress test was performed by the sequential addition of oligomycin (O), FCCP, rotenone and antimycin (R/A). Similarly, the fatty acid oxidation utilizes the mito stress test with the exception of pre-treatment with etomoxir (Eto, carnitine palmitoyl transferase-1) inhibitor and acute addition of either BSA control and palmitate:BSA. The glycolysis stress test was performed by the sequential addition of glucose (G), oligomycin (O) and 2-deoxyglucose (2-DG, which is phosphorylated into 2-deoxyglucose-6-phosphate and trapped intracellularly). **(A)** Co-culture resulted in a marked increment in oxidative phosphorylation associated with **(B)** reduced glycolysis. **(C)** Oxidative phosphorylation can be associated with increased usage of fatty acids via fatty acid oxidation, but in this experimental setting there was no response to etomoxir in BSA control conditions suggesting zero oxidation of endogenous fatty acids. However, co-cultured E0771 breast cancer cells presented with a lower basal respiration suggesting that the competition for nutrients makes co-cultured E0771 cells more "dormant" than E0771 breast cancer cells cultured alone at these low-glucose conditions. **(D)** As judged by the etomoxir response, E0771 breast cancer cells co-cultured with inguinal adipose tissue did not oxidize exogenous fatty acids while E0771 breast cancer cells cultured alone appears to oxidize a small amount of exogenous lipids. However, this fatty acid oxidation is likely on expense of a reduced glucose oxidation since the respiration with palmitate is not higher than without palmitate. E0771 breast cancer cells either **(E)** co-cultured with adipose tissue or **(F)** treated with 25 mM lactate for 24h uses more glucose for *de novo* lipogenesis than control cells as indicated by glucose counts in the organic phase.

Inhibition of fatty acid metabolism and tumor progression

E0771 breast cancer tumors induced lipolysis and adipose tissue-associated E0771 breast cancer tumors displayed increased uptake of lipids. We therefore tested the effect of the lipolysis inhibitors (acipimox or atglistatin) on E0771 breast cancer tumor growth, but found no significant effect on tumor growth. This lack of effect is perhaps not surprising given the high capacity for *de novo* lipogenesis of E0771 breast cancer cells. Somewhat surprisingly, however, treatment with the lipogenesis inhibitor Fasnall led to an initial increase in E0771 breast cancer tumor volume in vivo and increased E0771 breast cancer proliferation *in vitro*.

Tumors are suggested to show a high metabolic plasticity during stress, for example the propensity for high lactate production from glucose can be alleviated by increased glutaminolysis when glucose availability is scarce [255]. Moreover, cancer cells are known to fine-tune their lipid metabolism under stressful conditions to sustain proliferative capacity and to overcome nutrient competition with other cells such as immune cells [256, 257]. Similarly, inhibition of lipolysis may lead to increased lipogenesis, and vice versa. This is not to be confused with anaplecrotic

flux as with glutaminolysis, but rather as metabolic plasticity. To test this hypothesis, radiolabeled tracers can be used to investigate the effect of lipolysis or lipogenesis inhibitor in mice on tumor metabolism as a part of determining the metabolic plasticity in lipid metabolism.

Conclusions

The elegance of Otto Warburg's paradigm, where he described increased aerobic glycolysis in cancer cells, was later described (in the lactate shuttle hypothesis) to occur as part of metabolic interplay between cells of the same tissue. Thus, the theory of metabolic symbiosis was born and serves also as a model for how such interactions can occur between normal and malignant cells [258]. It has been proposed that tumors can be characterized based on their metabolic preference but also on their metabolic interactions with the microenvironment. Bases on gene expression analysis, hormone receptor negative breast cancer subtypes have been suggested to upregulate their exogenous uptake of fatty acids. The data presented here demonstrate however that the triple-negative E0771 breast cancer displayed increased lipogenesis when implanted orthotopically, associated with enhanced growth, compared to ectopically implanted tumors. This increase in lipogenesis occurs despite significant uptake of exogenous lipids. Thus, the microenvironment is critically important for the metabolic behavior of the tumor. It is possible that obese adipose tissue is an even more tumor-promoting environment than lean adipose tissue. For instance, the increased lactate production of obese adipose tissue may be utilized in metabolic pathways of the tumor and also promote tumor progression indirectly through e.g. immunosuppressive effects by compromising the function and assembly of immune responses against tumors [259]. For instance, lactate stimulates the formation of immunosuppressive myeloid derived suppressor cells, stabilize HIF1A to drive M2-like macrophage polarization and inhibiting the cytolytic activity of natural killer cells. However, the exact impact of obesity on the tumor-promoting effect of adipose tissue warrants further investigations.

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I wish to express my capital gratitude to **Ingrid Wernstedt-Asterholm**, my main supervisor. Your supervision was lightsome giving me sturdy directions on the dark sea of science. On a vast sea I rowed through turbulent water and fearsome storms between the devil and the deep blue sea. Finally, after a "ball play" of doubt in-between us, to harbor safe port and acquiring the essential and vital skills for conducting research. We did it! Brain overboard! Or shall we say onboard this time.

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The gym at EBM has served to accommodate as stress relief during the very last moments during my PhD work, however, it wouldn't have been possible without my training buddy and physicist **Ahmed Alrifaiy**. I would also like to thank you for sharing all your house building projects as well as helping me build some things on our spare time.

I am not the only person who has a good sense for research ethics, **Kim Eerola**, I am very sad that we only got to know each other in the very last months before you left to Finland. Nonetheless, the moments and thoughts we shared are priceless and I will never forget you; I sincerely hope we meet soon again. I would like to give an advice, if you are lucky enough to encounter Kim, make sure to get to know him; he is one of a kind, the good kind; upright and noble, a very decent person.

By a certainty, during my PhD I have encountered people outside the fourth floor (a secret and hidden domain). On the first floor, there is a man who goes by the name **Linus Ruijin Shao**, he has given me the best and most funny advice on research, as well as career advice. This man is truly a professor, one of a kind, the university and the world of science is lucky to have him aboard.

Reza Motalleb, what is there to say, actually there is much to say, but let's keep it simple since we both simple men. I have followed you throughout your academic career and I'm proud of what you have become. I am glad to find you as friend after a long journey, however, there is another journey we have not yet undertaken and that is to reach 7k MMR, изи +25, still winning.

Lastly on the fourth floor, some particular characters that I would like to pay my respects to are **Saliha Mušović**, **Ali Komai**, **Birgit Linder** and **Ann-Marie Alborn**, your mere presence, and uplifting conversations regarding life has also been motivating me to finish my PhD work. **Birgit** and **Ann-Marie** I cannot emphasize more that your work at the fourth floor deserves to be recognized more than it has been; you have been a real corner stone to the entire research unit.

To my parents, **Arthur** and **Linda** thank you for giving birth to me, although I never asked for it. Just kidding, lol. By knowing where I came from helps me to understand where I need to be, thank you for being you.

REFERENCES

1. Hruby, A. and F.B. Hu, *The Epidemiology of Obesity: A Big Picture.* Pharmacoeconomics, 2015. **33**(7): p. 673-89.

2. Kelly, T., et al., *Global burden of obesity in 2005 and projections to 2030.* Int J Obes (Lond), 2008. **32**(9): p. 1431-7.

3. Kanter, R. and B. Caballero, *Global gender disparities in obesity: a review.* Adv Nutr, 2012. **3**(4): p. 491-8.

4. Kershaw, E.E. and J.S. Flier, *Adipose Tissue as an Endocrine Organ.* The Journal of Clinical Endocrinology & Metabolism, 2004. **89**(6): p. 2548-2556.

5. Grundy, S.M., et al., *Definition of metabolic syndrome: report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition.* Circulation, 2004. **109**(3): p. 433-438.

6. Cornier, M.-A., et al., *The Metabolic Syndrome.* Endocrine Reviews, 2008. **29**(7): p. 777-822.

7. Capasso, I., et al., *Metabolic syndrome-breast cancer link varies by intrinsic molecular subtype.* Diabetol Metab Syndr, 2014. **6**(1): p. 105.

8. Pierobon, M. and C.L. Frankenfeld, *Obesity as a risk factor for triple-negative breast cancers: a systematic review and meta-analysis.* Breast Cancer Res Treat, 2013. **137**(1): p. 307-14.

9. Bianchini, F., R. Kaaks, and H. Vainio, *Overweight, obesity, and cancer risk.* The Lancet Oncology, 2002. **3**(9): p. 565-574.

10. Hauner, D. and H. Hauner, *Metabolic syndrome and breast cancer: is there a link?* Breast Care, 2014. **9**(4): p. 277-281.

11. Samani, A.A., et al., *The Role of the IGF System in Cancer Growth and Metastasis: Overview and Recent Insights.* Endocrine Reviews, 2007. **28**(1): p. 20-47.

12. Torre, L.A., et al., *Global Cancer in Women: Burden and Trends.* Cancer Epidemiology Biomarkers & Prevention, 2017. **26**(4): p. 444.

13. Ferlay, J., et al., *GLOBOCAN 2012 v1. 0, Cancer incidence and mortality worldwide: IARC CancerBase No. 11. 2013*. 2014.

14. Bertucci, F. and D. Birnbaum, *Reasons for breast cancer heterogeneity.* Journal of biology, 2008. **7**(2): p. 6.

15. Bray, F., P. McCarron, and D.M. Parkin, *The changing global patterns of female breast cancer incidence and mortality.* Breast cancer research, 2004. **6**(6): p. 229.

16. Nathanson, K.N., R. Wooster, and B.L. Weber, *Breast cancer genetics: what we know and what we need.* Nature medicine, 2001. **7**(5): p. 552.

17. Collignon, J., et al., *Triple-negative breast cancer: treatment challenges and solutions.* Breast cancer (Dove Medical Press), 2016. **8**: p. 93-107.

18. Malhotra, G.K., et al., *Histological, molecular and functional subtypes of breast cancers.* Cancer biology & therapy, 2010. **10**(10): p. 955-960.

19. Fallahpour, S., et al., *Breast cancer survival by molecular subtype: a population-based analysis of cancer registry data.* CMAJ open, 2017. **5**(3): p. E734-E739.

20. DeSantis, C.E., et al., *Breast cancer statistics, 2017, racial disparity in mortality by state.* CA: A Cancer Journal for Clinicians, 2017. **67**(6): p. 439-448.

21. Kumar, N., et al., *Prevalence of molecular subtypes of invasive breast cancer: A retrospective study.* Medical journal, Armed Forces India, 2015. **71**(3): p. 254-258.

22. Tu, S.-M., S.-H. Lin, and C.J. Logothetis, *Stem-cell origin of metastasis and heterogeneity in solid tumours.* The lancet oncology, 2002. **3**(8): p. 508-513.

23. Koren, E. and Y. Fuchs, *The bad seed: Cancer stem cells in tumor development and resistance.* Drug Resistance Updates, 2016. **28**: p. 1-12.

24. Liu, J., H. Dang, and X.W. Wang, *The significance of intertumor and intratumor heterogeneity in liver cancer.* Experimental &Amp; Molecular Medicine, 2018. **50**: p. e416.

25. Binnewies, M., et al., *Understanding the tumor immune microenvironment (TIME) for effective therapy.* Nature medicine, 2018. **24**(5): p. 541-550.

26. Nagarajan, D. and S. McArdle, *Immune landscape of breast cancers.* Biomedicines, 2018. **6**(1): p. 20.

27. Gu-Trantien, C., et al., *CD4⁺ follicular helper T cell infiltration predicts breast cancer survival.* The Journal of clinical investigation, 2013. **123**(7): p. 2873-2892.

28. Obeid, E., et al., *The role of tumor-associated macrophages in breast cancer progression (review).* International journal of oncology, 2013. **43**(1): p. 5-12.

29. Ivanov, S., et al., *Biology and function of adipose tissue macrophages, dendritic cells and B cells.* Atherosclerosis, 2018. **271**: p. 102-110.

30. Hobson-Gutierrez, S.A. and C. Carmona-Fontaine, *The metabolic axis of macrophage and immune cell polarization.* Disease models & mechanisms, 2018. **11**(8): p. dmm034462.

31. Mantovani, A., et al., *Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes.* Trends in Immunology, 2002. **23**(11): p. 549-555.

32. Laoui, D., et al., *Tumor-associated macrophages in breast cancer: distinct subsets, distinct functions.* International Journal of Developmental Biology, 2011. **55**(7-8-9): p. 861-867.

33. Volodko, N., et al., *Tumour-associated macrophages in breast cancer and their prognostic correlations.* The Breast, 1998. **7**(2): p. 99-105.

34. Qiu, S.Q., et al., *Tumor-associated macrophages in breast cancer: Innocent bystander or important player?* Cancer Treat Rev, 2018. **70**: p. 178-189.

35. Cook, K.S., et al., *Adipsin: a circulating serine protease homolog secreted by adipose tissue and sciatic nerve.* Science, 1987. **237**(4813): p. 402-405.

36. Siiteri, P.K., *Adipose tissue as a source of hormones.* The American journal of clinical nutrition, 1987. **45**(1): p. 277-282.

37. Zhang, Y., et al., *Positional cloning of the mouse obese gene and its human homologue.* Nature, 1994. **372**(6505): p. 425.

38. Lord, G.M., et al., *Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression.* Nature, 1998. **394**(6696): p. 897.

39. Margetic, S., et al., *Leptin: a review of its peripheral actions and interactions.* International journal of obesity, 2002. **26**(11): p. 1407.

40. Desmoulière, A. *Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing*. in *2nd Scar meeting*. 2008.

41. Garofalo, C. and E. Surmacz, *Leptin and cancer.* Journal of cellular physiology, 2006. **207**(1): p. 12-22.

42. Zheng, Q., et al., *Leptin deficiency suppresses MMTV-Wnt-1 mammary tumor growth in obese mice and abrogates tumor initiating cell survival.* Endocrine-related cancer, 2011. **18**(4): p. 491-503.

43. Park, J. and P.E. Scherer, *Leptin and cancer: from cancer stem cells to metastasis.* Endocrine-related cancer, 2011. **18**(4): p. C25-C29.

44. Weisberg, S.P., et al., *Obesity is associated with macrophage accumulation in adipose tissue.* Journal of Clinical Investigation, 2003. **112**(12): p. 1796-1808.

45. Tilg, H. and A.R. Moschen, *Adipocytokines: mediators linking adipose tissue, inflammation and immunity.* Nature Reviews Immunology, 2006. **6**(10): p. 772.

46. Kusminski, C.M., P.E. Bickel, and P.E. Scherer, *Targeting adipose tissue in the treatment of obesity-associated diabetes.* Nat Rev Drug Discov, 2016. **15**(9): p. 639.

47. Han, T.S. and M.E.J. Lean, *A clinical perspective of obesity, metabolic syndrome and cardiovascular disease.* JRSM Cardiovascular Disease, 2016. **5**: p. 2048004016633371.

48. Eckel, R.H., S.M. Grundy, and P.Z. Zimmet, *The metabolic syndrome.* The Lancet, 2005. **365**(9468): p. 1415-1428.

49. Asterholm, I.W., et al., *Adipocyte inflammation is essential for healthy adipose tissue expansion and remodeling.* Cell metabolism, 2014. **20**(1): p. 103-118.

50. Rajala, M.W. and P.E. Scherer, *Minireview: The Adipocyte—At the Crossroads of Energy Homeostasis, Inflammation, and Atherosclerosis.* Endocrinology, 2003. **144**(9): p. 3765-3773.

51. Bulló, M., et al., *Inflammation, obesity and comorbidities: the role of diet.* Public health nutrition, 2007. **10**(10A): p. 1164-1172.

52. Liu, M. and F. Liu, *Regulation of adiponectin multimerization, signaling and function.* Best Practice & Research Clinical Endocrinology & Metabolism, 2014. **28**(1): p. 25-31.

53. Schäffler, A. and C. Buechler, *CTRP family: linking immunity to metabolism.* Trends in Endocrinology & Metabolism, 2012. **23**(4): p. 194-204.

54. Seldin, M.M., S.Y. Tan, and G.W. Wong, *Metabolic function of the CTRP family of hormones.* Reviews in Endocrine and Metabolic Disorders, 2014. **15**(2): p. 111-123.

55. Shapiro, L. and P.E. Scherer, *The crystal structure of a complement-1q family protein suggests an evolutionary link to tumor necrosis factor.* Curr Biol, 1998. **8**(6): p. 335-8.

56. Wolf, R.M., et al., *Lower Circulating C1q/TNF-Related Protein-3 (CTRP3) Levels Are Associated with Obesity: A Cross-Sectional Study.* PLoS One, 2015. **10**(7): p. e0133955.

57. Ban, B., et al., *Low Serum Cartonectin/CTRP3 Concentrations in Newly Diagnosed Type 2 Diabetes Mellitus: In Vivo Regulation of Cartonectin by Glucose.* PLOS ONE, 2014. **9**(11): p. e112931.

58. Deng, W., et al., *Serum C1q/TNF-related protein-3 (CTRP3) levels are decreased in obesity and hypertension and are negatively correlated with parameters of insulin resistance.* Diabetology & metabolic syndrome, 2015. **7**(1): p. 33.

59. Peterson, J.M., Z. Wei, and G.W. Wong, *C1q/TNF-related Protein-3 (CTRP3), a Novel Adipokine That Regulates Hepatic*

Glucose Output. Journal of Biological Chemistry, 2010. **285**(51): p. 39691-39701.

60. Wong, G.W., et al., *Molecular, biochemical and functional characterizations of C1q/TNF family members: adipose-tissue-selective expression patterns, regulation by PPAR-γ agonist, cysteine-mediated oligomerizations, combinatorial associations and metabolic functions.* Biochemical Journal, 2008. **416**(2): p. 161.

61. Zhang, C.-L., et al., *Globular CTRP3 promotes mitochondrial biogenesis in cardiomyocytes through AMPK/PGC-1α pathway.* Biochimica et Biophysica Acta (BBA)-General Subjects, 2017. **1861**(1): p. 3085-3094.

62. Feng, H., et al., *CTRP3 promotes energy production by inducing mitochondrial ROS and up-expression of PGC-1α in vascular smooth muscle cells.* Experimental Cell Research, 2016. **341**(2): p. 177-186.

63. Murayama, M.A., et al., *CTRP3 plays an important role in the development of collagen-induced arthritis in mice.* Biochemical and Biophysical Research Communications, 2014. **443**(1): p. 42-48.

64. Hofmann, C., et al., *C1q/TNF-related protein-3 (CTRP-3) is secreted by visceral adipose tissue and exerts antiinflammatory and antifibrotic effects in primary human colonic fibroblasts.* Inflammatory bowel diseases, 2011. **17**(12): p. 2462-2471.

65. Weigert, J., et al., *The adiponectin paralog CORS-26 has anti-inflammatory properties and is produced by human monocytic cells.* FEBS letters, 2005. **579**(25): p. 5565-5570.

66. Kopp, A., et al., *C1q/TNF-related protein-3 represents a novel and endogenous lipopolysaccharide antagonist of the adipose tissue.* Endocrinology, 2010. **151**(11): p. 5267-5278.

67. Akiyama, H., et al., *Elevated expression of CTRP3/cartducin contributes to promotion of osteosarcoma cell proliferation.* Oncol Rep, 2009. **21**(6): p. 1477-81.

68. Akiyama, H., et al., *CTRP3/cartducin promotes proliferation and migration of endothelial cells.* Mol Cell Biochem, 2007. **304**(1-2): p. 243-8.

69. Mantovani, A., et al., *The chemokine system in diverse forms of macrophage activation and polarization.* Trends in immunology, 2004. **25**(12): p. 677-686.

70. Martinez, F.O., et al., *Macrophage activation and polarization.* Front Biosci, 2008. **13**(1): p. 453-461.

71. Sun, K., C.M. Kusminski, and P.E. Scherer, *Adipose tissue remodeling and obesity.* The Journal of Clinical Investigation, 2011. **121**(6): p. 2094-2101.

72. Beigh, S.H. and S. Jain, *Prevalence of metabolic syndrome and gender differences.* Bioinformation, 2012. **8**(13): p. 613-616.

73. Lee, M.-J., Y. Wu, and S.K. Fried, *Adipose tissue remodeling in pathophysiology of obesity.* Current opinion in clinical nutrition and metabolic care, 2010. **13**(4): p. 371.

74. Suganami, T., J. Nishida, and Y. Ogawa, *A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor α.* Arteriosclerosis, thrombosis, and vascular biology, 2005. **25**(10): p. 2062-2068.

75. Scott, T. and M.D. Owens, *Thrombocytes respond to lipopolysaccharide through Toll-like receptor-4, and MAP kinase and NF-κB pathways leading to expression of interleukin-6 and cyclooxygenase-2 with production of prostaglandin E2.* Molecular immunology, 2008. **45**(4): p. 1001-1008.

76. Mantovani, A., et al., *Tumour-associated macrophages as treatment targets in oncology.* Nature reviews Clinical oncology, 2017. **14**(7): p. 399.

77. Solinas, G., et al., *Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation.* Journal of leukocyte biology, 2009. **86**(5): p. 1065-1073.

78. Geeraerts, X., et al., *Macrophage metabolism as therapeutic target for cancer, atherosclerosis, and obesity.* Frontiers in immunology, 2017. **8**: p. 289.

79. Guruvayoorappan, C., *Tumor versus tumor-associated macrophages: how hot is the link?* Integrative cancer therapies, 2008. **7**(2): p. 90-95.

80. Purushoth, E., L. Tholcopiyan, and A. Santhosh, *Cancer Progression Related with Tumor-associated Macrophages.* Cancer and Oncology Research, 2016. **4**(4): p. 53-63.

81. Calle, E.E. and R. Kaaks, *Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms.* Nature Reviews Cancer, 2004. **4**(8): p. 579-591.

82. Wolk, A., et al., *A prospective study of obesity and cancer risk (Sweden).* Cancer Causes & Control, 2001. **12**(1): p. 13-21.

83. Renehan, A.G., J. Frystyk, and A. Flyvbjerg, *Obesity and cancer risk: the role of the insulin–IGF axis.* Trends in Endocrinology & Metabolism, 2006. **17**(8): p. 328-336.

84. Grimberg, A., *Mechanisms by which IGF-I may promote cancer.* Cancer biology & therapy, 2003. **2**(6): p. 630-635.

85. Rothenberger, N.J., A. Somasundaram, and L.P. Stabile, *The Role of the Estrogen Pathway in the Tumor Microenvironment.* International journal of molecular sciences, 2018. **19**(2): p. 611.

86. Taniguchi, K. and M. Karin, *IL-6 and related cytokines as the critical lynchpins between inflammation and cancer.* Seminars in Immunology, 2014. **26**(1): p. 54-74.

87. Mauras, N., et al., *Sex Steroids, Growth Hormone, Insulin-Like Growth Factor-1: Neuroendocrine and Metabolic Regulation in Puberty.* Hormone Research in Paediatrics, 1996. **45**(1-2): p. 74-80.

88. LeRoith, D. and C.T. Roberts, *The insulin-like growth factor system and cancer.* Cancer Letters, 2003. **195**(2): p. 127-137.

89. Jones, J.I. and D.R. Clemmons, *Insulin-Like Growth Factors and Their Binding Proteins: Biological Actions*.* Endocrine Reviews, 1995. **16**(1): p. 3-34.

90. Khandwala, H.M., et al., *The effects of insulin-like growth factors on tumorigenesis and neoplastic growth.* Endocrine reviews, 2000. **21**(3): p. 215-244.

91. Hankinson, S.E., et al., *Circulating concentrations of insulin-like growth factor I and risk of breast cancer.* The Lancet, 1998. **351**(9113): p. 1393-1396.

92. Werner, H., et al., *Wild-type and mutant p53 differentially regulate transcription of the insulin-like growth factor I receptor gene.* Proceedings of the National Academy of Sciences, 1996. **93**(16): p. 8318-8323.

93. André, F., et al., *Integrins and E-cadherin cooperate with IGF-I to induce migration of epithelial colonic cells.* International journal of cancer, 1999. **83**(4): p. 497-505.

94. Pennisi, P.A., et al., *Reduced expression of insulin-like growth factor I receptors in MCF-7 breast cancer cells leads to a more metastatic phenotype.* Cancer research, 2002. **62**(22): p. 6529-6537.

95. Cauley, J.A., et al., *THE EPIDEMIOLOGY OF SERUM SEX HORMONES IN POSTMENOPAUSAL WOMEN.* American Journal of Epidemiology, 1989. **129**(6): p. 1120-1131.

96. Roberts, D.L., C. Dive, and A.G. Renehan, *Biological Mechanisms Linking Obesity and Cancer Risk: New Perspectives.* Annual Review of Medicine, 2010. **61**(1): p. 301-316.

97. Freedland, S.J. and E.A. Platz, *Obesity and prostate cancer: making sense out of apparently conflicting data.* Epidemiologic reviews, 2007. **29**(1): p. 88-97.

98. Roy, D. and J.G. Liehr, *Estrogen, DNA damage and mutations.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 1999. **424**(1): p. 107-115.

99. Hua, H., et al., *Mechanisms for estrogen receptor expression in human cancer.* Experimental Hematology & Oncology, 2018. **7**(1): p. 24.

100. Vona-Davis, L. and D.P. Rose, *Adipokines as endocrine, paracrine, and autocrine factors in breast cancer risk and progression.* Endocrine-related cancer, 2007. **14**(2): p. 189-206.

101. VanSaun, M.N., *Molecular pathways: adiponectin and leptin signaling in cancer*. Clinical Cancer Research, 2013: p. clincanres. 0930.2012.

102. Procaccini, C., et al., *Leptin signaling: A key pathway in immune responses*. Current signal transduction therapy, 2009. **4**(1): p. 22-30.

103. Fernández-Riejos, P., et al., *Role of leptin in the activation of immune cells.* Mediators of inflammation, 2010. **2010**: p. 568343-568343.

104. Luo, Y. and M. Liu, *Adiponectin: a versatile player of innate immunity*. Journal of molecular cell biology, 2016. **8**(2): p. 120-128.

105. Barb, D., et al., *Adiponectin in relation to malignancies: a review of existing basic research and clinical evidence.* The American Journal of Clinical Nutrition, 2007. **86**(3): p. 858S-866S.

106. Petridou, E., et al., *Leptin and Body Mass Index in Relation to Endometrial Cancer Risk.* Annals of Nutrition and Metabolism, 2002. **46**(3-4): p. 147-151.

107. Wu, M.H., et al., *Circulating levels of leptin, adiposity and breast cancer risk.* British Journal Of Cancer, 2009. **100**: p. 578.

108. Stattin, P., et al., *Plasma leptin and colorectal cancer risk: a prospective study in Northern Sweden.* Oncology reports, 2003. **10**(6): p. 2015-2021.

109. Hsing, A.W., et al., *Prostate cancer risk and serum levels of insulin and leptin: a population-based study*. Journal of the National Cancer Institute, 2001. **93**(10): p. 783-789.

110. Arditi, J., et al., *Antiproliferative effect of adiponectin on MCF7 breast cancer cells: a potential hormonal link between obesity and cancer*. Hormone and Metabolic Research, 2007. **39**(01): p. 9-13.

111. Kang, J.H., et al., *Adiponectin induces growth arrest and apoptosis of MDA-MB-231 breast cancer cell.* Archives of pharmacal research, 2005. **28**(11): p. 1263-1269.

112. Man, K., et al., *Suppression of liver tumor growth and metastasis by adiponectin in nude mice through inhibition of tumor angiogenesis and downregulation of Rho kinase/IFN-inducible protein 10/matrix metalloproteinase 9 signaling.* Clinical Cancer Research, 2010: p. 1078-0432. CCR-09-1487.

113. Ishikawa, M., J. Kitayama, and H. Nagawa, *Enhanced expression of leptin and leptin receptor (OB-R) in human breast cancer.* Clinical Cancer Research, 2004. **10**(13): p. 4325-4331.

114. Nieman, K.M., et al., *Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth.* Nat Med, 2011. **17**(11): p. 1498-1503.

115. Nieman, K.M., et al., *Adipose tissue and adipocytes support tumorigenesis and metastasis.* Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, 2013. **1831**(10): p. 1533-1541.

116. Okwan-Duodu, D., et al., *Obesity-driven inflammation and cancer risk: role of myeloid derived suppressor cells and alternately activated macrophages.* American journal of cancer research, 2013. **3**(1): p. 21.

117. Ostrand-Rosenberg, S., *Myeloid derived-suppressor cells: their role in cancer and obesity.* Current opinion in immunology, 2018. **51**: p. 68-75.

118. Budhwar, S., et al., *The Yin and Yang of myeloid derived suppressor cells.* Frontiers in Immunology, 2018. **9**.

119. Quail, D.F. and A.J. Dannenberg, *The obese adipose tissue microenvironment in cancer development and progression.* Nature Reviews Endocrinology, 2018: p. 1.

120. Cha, Y.J., E.-S. Kim, and J.S. Koo, *Tumor-associated macrophages and crown-like structures in adipose tissue in breast cancer.* Breast cancer research and treatment, 2018. **170**(1): p. 15-25.

121. Berger, N.A., *Crown-like structures in breast adipose tissue from normal weight women: important impact*. 2017, AACR.

122. Carter, J.M., et al., *Macrophagic "crown-like structures" are associated with an increased risk of breast cancer in benign*

breast disease. Cancer Prevention Research, 2018. **11**(2): p. 113-119.

123. Clements, V., et al., *High fat diet and leptin promote tumor progression by inducing myeloid-derived suppressor cells.* J. Leukoc. Biol, 2017.

124. Dirat, B., et al., *Cancer-Associated Adipocytes Exhibit an Activated Phenotype and Contribute to Breast Cancer Invasion.* Cancer Research, 2011. **71**(7): p. 2455.

125. Karagiannis, G.S., et al., *Cancer-Associated Fibroblasts Drive the Progression of Metastasis through both Paracrine and Mechanical Pressure on Cancer Tissue.* Molecular Cancer Research, 2012. **10**(11): p. 1403.

126. Seo, B.R., et al., *Obesity-dependent changes in interstitial ECM mechanics promote breast tumorigenesis.* Science translational medicine, 2015. **7**(301): p. 301ra130-301ra130.

127. Iyengar, P., et al., *Adipocyte-derived collagen VI affects early mammary tumor progression in vivo, demonstrating a critical interaction in the tumor/stroma microenvironment.* The Journal of clinical investigation, 2005. **115**(5): p. 1163-1176.

128. Cozzo, A.J., A.M. Fuller, and L. Makowski, *Contribution of adipose tissue to development of cancer.* Comprehensive Physiology, 2011. **8**(1): p. 237-282.

129. DiGirolamo, M., F.D. Newby, and J. Lovejoy, *Lactate production in adipose tissue: a regulated function with extra-adipose implications.* FASEB J, 1992. **6**(7): p. 2405-12.

130. Warburg O, P.K., Negelein E, *Über den Stoffwechsel der Tumoren.* Biochem Z, 1924. **152**: p. 319-344.

131. Warburg, O., *On the Origin of Cancer Cells.* Science, 1956. **123**(3191): p. 309.

132. O'Neill, L.A., R.J. Kishton, and J. Rathmell, *A guide to immunometabolism for immunologists.* Nature Reviews Immunology, 2016. **16**(9): p. 553.

133. Kuo, C.-Y. and D.K. Ann, *When fats commit crimes: fatty acid metabolism, cancer stemness and therapeutic resistance.* Cancer Communications, 2018. **38**(1): p. 47.

134. Monaco, M.E., *Fatty acid metabolism in breast cancer subtypes.* Oncotarget, 2017. **8**(17): p. 29487.

135. Cluntun, A.A., et al., *Glutamine metabolism in cancer: understanding the heterogeneity.* Trends in cancer, 2017. **3**(3): p. 169-180.

136. Currie, E., et al., *Cellular Fatty Acid Metabolism and Cancer.* Cell Metabolism, 2013. **18**(2): p. 153-161.

137. Zhao, Y., E.B. Butler, and M. Tan, *Targeting cellular metabolism to improve cancer therapeutics.* Cell death & disease, 2013. **4**(3): p. e532.

138. Qu, Q., et al., *Fatty acid oxidation and carnitine palmitoyltransferase I: emerging therapeutic targets in cancer.* Cell death & disease, 2016. **7**(5): p. e2226.

139. Lyssiotis, C.A. and A.C. Kimmelman, *Metabolic Interactions in the Tumor Microenvironment.* Trends in Cell Biology, 2017. **27**(11): p. 863-875.

140. Draoui, N. and O. Feron, *Lactate shuttles at a glance: from physiological paradigms to anti-cancer treatments.* Disease Models & Mechanisms, 2011. **4**(6): p. 727.

141. Sonveaux, P., et al., *Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice.* The Journal of clinical investigation, 2008. **118**(12): p. 3930-3942.

142. Pavlides, S., et al., *The reverse Warburg effect: Aerobic glycolysis in cancer associated fibroblasts and the tumor stroma.* Cell Cycle, 2009. **8**(23): p. 3984-4001.

143. Mantovani, A., et al., *The origin and function of tumor-associated macrophages.* Immunology today, 1992. **13**(7): p. 265-270.

144. Warburg, O., K. Gawehn, and A. Geissler, *Metabolism of leukocytes.* Zeitschrift fur Naturforschung. Teil B, Chemie,

Biochemie, Biophysik, Biologie und verwandte Gebiete, 1958. **13**(8): p. 515.

145. O'Neill, L.A.J. and E.J. Pearce, *Immunometabolism governs dendritic cell and macrophage function.* The Journal of Experimental Medicine, 2016. **213**(1): p. 15.

146. Papandreou, I., et al., *HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption.* Cell metabolism, 2006. **3**(3): p. 187-197.

147. Kim, J.-w., et al., *HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia.* Cell metabolism, 2006. **3**(3): p. 177-185.

148. Tannahill, G., et al., *Succinate is an inflammatory signal that induces IL-1β through HIF-1α.* Nature, 2013. **496**(7444): p. 238.

149. Lambeth, J.D., *Nox/Duox family of nicotinamide adenine dinucleotide (phosphate) oxidases.* Current opinion in hematology, 2002. **9**(1): p. 11-17.

150. Forman, H.J. and M. Torres, *Reactive oxygen species and cell signaling: respiratory burst in macrophage signaling.* American journal of respiratory and critical care medicine, 2002. **166**(supplement_1): p. S4-S8.

151. Everts, B., et al., *TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKε supports the anabolic demands of dendritic cell activation.* Nature immunology, 2014. **15**(4): p. 323.

152. Haschemi, A., et al., *The sedoheptulose kinase CARKL directs macrophage polarization through control of glucose metabolism.* Cell metabolism, 2012. **15**(6): p. 813-826.

153. Diskin, C. and E.M. Pålsson-McDermott, *Metabolic modulation in macrophage effector function.* Frontiers in Immunology, 2018. **9**: p. 270.

154. Rath, M., et al., *Metabolism via Arginase or Nitric Oxide Synthase: Two Competing Arginine Pathways in Macrophages.* Frontiers in immunology, 2014. **5**: p. 532-532.

155. Mori, M., *Regulation of Nitric Oxide Synthesis and Apoptosis by Arginase and Arginine Recycling.* The Journal of Nutrition, 2007. **137**(6): p. 1616S-1620S.

156. El Mjiyad, N., et al., *Sugar-free approaches to cancer cell killing.* Oncogene, 2011. **30**(3): p. 253.

157. Yoo, B.C., et al., *Decreased pyruvate kinase M2 activity linked to cisplatin resistance in human gastric carcinoma cell lines.* International journal of cancer, 2004. **108**(4): p. 532-539.

158. Guo, W., et al., *Efficacy of RNAi targeting of pyruvate kinase M2 combined with cisplatin in a lung cancer model.* Journal of cancer research and clinical oncology, 2011. **137**(1): p. 65-72.

159. Le, A., et al., *Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression.* Proceedings of the National Academy of Sciences, 2010. **107**(5): p. 2037-2042.

160. Gandhi, N. and G.M. Das, *Metabolic Reprogramming in Breast Cancer and Its Therapeutic Implications.* Cells, 2019. **8**(2): p. 89.

161. O'Mahony, F., et al., *Estrogen modulates metabolic pathway adaptation to available glucose in breast cancer cells.* Molecular endocrinology, 2012. **26**(12): p. 2058-2070.

162. Yang, J., et al., *Estrogen receptor-α directly regulates the hypoxia-inducible factor 1 pathway associated with antiestrogen response in breast cancer.* Proceedings of the National Academy of Sciences, 2015. **112**(49): p. 15172-15177.

163. Kim, S., Y. Lee, and J.S. Koo, *Differential expression of lipid metabolism-related proteins in different breast cancer subtypes.* PloS one, 2015. **10**(3): p. e0119473.

164. Bettencourt, I.A. and J.D. Powell, *Targeting metabolism as a novel therapeutic approach to autoimmunity, inflammation, and transplantation.* The Journal of Immunology, 2017. **198**(3): p. 999-1005.

165. O'Sullivan, D. and E.L. Pearce, *Targeting T cell metabolism for therapy.* Trends in Immunology, 2015. **36**(2): p. 71-80.

166. Crompton, J.G., et al., *Akt inhibition enhances expansion of potent tumor-specific lymphocytes with memory cell characteristics.* Cancer research, 2015. **75**(2): p. 296-305.

167. Macintyre, A.N. and J.C. Rathmell, *Activated lymphocytes as a metabolic model for carcinogenesis.* Cancer & metabolism, 2013. **1**(1): p. 5.

168. Ciuleanu, T.E., et al., *A randomised Phase III trial of glufosfamide compared with best supportive care in metastatic pancreatic adenocarcinoma previously treated with gemcitabine.* European journal of cancer, 2009. **45**(9): p. 1589-1596.

169. De Palma, M. and C.E. Lewis, *Macrophage regulation of tumor responses to anticancer therapies.* Cancer cell, 2013. **23**(3): p. 277-286.

170. Bonapace, L., et al., *Cessation of CCL2 inhibition accelerates breast cancer metastasis by promoting angiogenesis.* Nature, 2014. **515**(7525): p. 130.

171. Colegio, O.R., et al., *Functional polarization of tumour-associated macrophages by tumour-derived lactic acid.* Nature, 2014. **513**(7519): p. 559.

172. Cheng, W.Y., et al., *Macrophage PPARγ inhibits Gpr132 to mediate the anti-tumor effects of rosiglitazone.* Elife, 2016. **5**: p. e18501.

173. Chen, P., et al., *Gpr132 sensing of lactate mediates tumor–macrophage interplay to promote breast cancer metastasis.* Proceedings of the National Academy of Sciences, 2017. **114**(3): p. 580-585.

174. Penny, H.L., et al., *Warburg metabolism in tumor-conditioned macrophages promotes metastasis in human pancreatic ductal adenocarcinoma.* Oncoimmunology, 2016. **5**(8): p. e1191731.

175. Chen, W., et al., *Macrophage-induced tumor angiogenesis is regulated by the TSC2–mTOR pathway.* Cancer research, 2012. **72**(6): p. 1363-1372.

176. Wenes, M., et al., *Macrophage metabolism controls tumor blood vessel morphogenesis and metastasis.* Cell metabolism, 2016. **24**(5): p. 701-715.

177. El-Abd, E., et al., *15 Animal Models of Breast Cancer.* Omics Approaches in Breast Cancer: Towards Next-Generation Diagnosis, Prognosis and Therapy, 2014: p. 297.

178. Holen, I., et al., *In vivo models in breast cancer research: progress, challenges and future directions.* Disease models & mechanisms, 2017. **10**(4): p. 359-371.

179. Hennighausen, L., *Mouse models for breast cancer.* Breast Cancer Research, 1999. **2**(1): p. 2.

180. Fantozzi, A. and G. Christofori, *Mouse models of breast cancer metastasis.* Breast Cancer Research, 2006. **8**(4): p. 212.

181. Yang, Y., et al., *Immunocompetent mouse allograft models for development of therapies to target breast cancer metastasis.* Oncotarget, 2017. **8**(19): p. 30621-30643.

182. Hu, X., et al., *Genetic alterations and oncogenic pathways associated with breast cancer subtypes.* Molecular Cancer Research, 2009. **7**(4): p. 511-522.

183. Horii, R., et al., *Assessment of hormone receptor status in breast cancer.* Pathology international, 2007. **57**(12): p. 784-790.

184. Casey, A.E., W.R. Laster, Jr., and G.L. Ross, *Sustained enhanced growth of carcinoma EO771 in C57 black mice.* Proc Soc Exp Biol Med, 1951. **77**(2): p. 358-62.

185. Ewens, A., E. Mihich, and M.J. Ehrke, *Distant metastasis from subcutaneously grown E0771 medullary breast adenocarcinoma.* Anticancer Research, 2005. **25**(6b): p. 3905-3915.

186. Urs, S., *E0771 Syngeneic Breast Cancer Model* in *Model Spotlight*. 2018: MI Bioresearch.

187. García-Teijido, P., et al., *Tumor-infiltrating lymphocytes in triple negative breast cancer: the future of immune targeting.* Clinical Medicine Insights: Oncology, 2016.

188. Emens, L.A., *Breast cancer immunobiology driving immunotherapy: vaccines and immune checkpoint blockade.* Expert Review of Anticancer Therapy, 2012. **12**(12): p. 1597-1611.

189. Fridman, W.H., et al., *The immune contexture in human tumours: impact on clinical outcome.* Nat Rev Cancer, 2012. **12**(4): p. 298-306.

190. Johnstone, C.N., et al., *Functional and molecular characterisation of EO771. LMB tumours, a new C57BL/6-mouse-derived model of spontaneously metastatic mammary cancer.* Disease models & mechanisms, 2015: p. dmm. 017830.

191. Foulkes, W.D., I.E. Smith, and J.S. Reis-Filho, *Triple-negative breast cancer.* New England journal of medicine, 2010. **363**(20): p. 1938-1948.

192. Davies, L.C., et al., *Tissue-resident macrophages.* Nature immunology, 2013. **14**(10): p. 986.

193. Hashimoto, D., et al., *Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes.* Immunity, 2013. **38**(4): p. 792-804.

194. Landsman, L., C. Varol, and S. Jung, *Distinct differentiation potential of blood monocyte subsets in the lung.* The Journal of Immunology, 2007. **178**(4): p. 2000-2007.

195. Wang, C., et al., *Characterization of murine macrophages from bone marrow, spleen and peritoneum.* BMC immunology, 2013. **14**(1): p. 6.

196. Berghaus, L.J., et al., *Innate immune responses of primary murine macrophage-lineage cells and RAW 264.7 cells to ligands of Toll-like receptors 2, 3, and 4.* Comparative immunology, microbiology and infectious diseases, 2010. **33**(5): p. 443-454.

197. Chamberlain, L.M., et al., *Phenotypic non-equivalence of murine (monocyte-) macrophage cells in biomaterial and inflammatory models.* Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials, 2009. **88**(4): p. 858-871.

198. Spiller, K.L., et al., *Differential gene expression in human, murine, and cell line-derived macrophages upon polarization.* Experimental cell research, 2016. **347**(1): p. 1-13.

199. Galván-Peña, S. and L.A. O'Neill, *Metabolic reprograming in macrophage polarization.* Frontiers in immunology, 2014. **5**: p. 420.

200. Mestas, J. and C.C.W. Hughes, *Of Mice and Not Men: Differences between Mouse and Human Immunology*. The Journal of Immunology, 2004. **172**(5): p. 2731.

201. Hassnain Waqas, S.F., et al., *Adipose tissue macrophages develop from bone marrow–independent progenitors in Xenopus laevis and mouse.* Journal of leukocyte biology, 2017. **102**(3): p. 845-855.

202. Marathe, C., et al., *Preserved glucose tolerance in high-fat-fed C57BL/6 mice transplanted with PPARγ−/−, PPARδ−/−, PPARγδ−/−, or LXRαβ−/− bone marrow.* Journal of lipid research, 2009. **50**(2): p. 214-224.

203. Waqas, S.F.H., et al., *Neuropeptide FF increases M2 activation and self-renewal of adipose tissue macrophages.* The Journal of clinical investigation, 2017. **127**(7): p. 2842-2854.

204. Röszer, T., *Understanding the biology of self-renewing macrophages.* Cells, 2018. **7**(8): p. 103.

205. Chance, B. and G.R. Williams, *Respiratory enzymes in oxidative phosphorylation. III. The steady state.* J Biol Chem, 1955. **217**(1): p. 409-27.

206. Brand, M.D. and D.G. Nicholls, *Assessing mitochondrial dysfunction in cells.* Biochemical Journal, 2011. **435**(2): p. 297-312.

207. Perry, C.G., et al., *Methods for assessing mitochondrial function in diabetes.* Diabetes, 2013. **62**(4): p. 1041-1053.

208. Rousset, S., et al., *The biology of mitochondrial uncoupling proteins.* Diabetes, 2004. **53**(suppl 1): p. S130-S135.

209. Bedard, K. and K.-H. Krause, *The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology.* Physiological reviews, 2007. **87**(1): p. 245-313.

210. Brown, G.C., *Nitric oxide and mitochondrial respiration.* Biochimica et Biophysica Acta (BBA) - Bioenergetics, 1999. **1411**(2): p. 351-369.

211. Umpleby, A.M., *HORMONE MEASUREMENT GUIDELINES: Tracing lipid metabolism: the value of stable isotopes.* J Endocrinol, 2015. **226**(3): p. G1-10.

212. Tumanov, S., V. Bulusu, and J.J. Kamphorst, *Analysis of fatty acid metabolism using stable isotope tracers and mass spectrometry*, in *Methods in enzymology*. 2015, Elsevier. p. 197-217.

213. Dranka, B.P., et al., *Assessing bioenergetic function in response to oxidative stress by metabolic profiling.* Free Radical Biology and Medicine, 2011. **51**(9): p. 1621-1635.

214. Folmes, C.D., et al., *Metabolic plasticity in stem cell homeostasis and differentiation.* Cell stem cell, 2012. **11**(5): p. 596-606.

215. Chen, C.-T., S.-H. Hsu, and Y.-H. Wei, *Mitochondrial bioenergetic function and metabolic plasticity in stem cell differentiation and cellular reprogramming.* Biochimica et Biophysica Acta (BBA)-General Subjects, 2012. **1820**(5): p. 571-576.

216. Yepez, V.A., et al., *OCR-Stats: Robust estimation and statistical testing of mitochondrial respiration activities using Seahorse XF Analyzer.* PloS one, 2018. **13**(7): p. e0199938.

217. Hughes, P., et al., *The costs of using unauthenticated, over-passaged cell lines: how much more data do we need?* Biotechniques, 2007. **43**(5): p. 575-586.

218. Vara-Perez, M., B. Felipe-Abrio, and P. Agostinis, *Mitophagy in Cancer: A Tale of Adaptation.* Cells, 2019. **8**(5): p. 493.

219. Wang, C., et al., *Characterization of murine macrophages from bone marrow, spleen and peritoneum.* BMC Immunol, 2013. **14**(1): p. 6.

220. Van den Bossche, J., et al., *Mitochondrial dysfunction prevents repolarization of inflammatory macrophages.* Cell reports, 2016. **17**(3): p. 684-696.

221. Van den Bossche, J., J. Baardman, and M.P. de Winther, *Metabolic characterization of polarized M1 and M2 bone marrow-derived macrophages using real-time extracellular flux analysis.* JoVE (Journal of Visualized Experiments), 2015(105): p. e53424.

222. Le Bourgeois, T., et al., *Targeting T cell metabolism for improvement of cancer immunotherapy.* Frontiers in oncology, 2018. **8**.

223. Dmitriev, R.I. and D.B. Papkovsky, *Optical probes and techniques for O 2 measurement in live cells and tissue.* Cellular and molecular life sciences, 2012. **69**(12): p. 2025-2039.

224. Reina-Campos, M., J. Moscat, and M. Diaz-Meco, *Metabolism shapes the tumor microenvironment.* Current opinion in cell biology, 2017. **48**: p. 47-53.

225. Sundaram, S., A.R. Johnson, and L. Makowski, *Obesity, metabolism and the microenvironment: Links to cancer.* Journal of carcinogenesis, 2013. **12**.

226. Carmichael, A.R. and T. Bates, *Obesity and breast cancer: a review of the literature.* The Breast, 2004. **13**(2): p. 85-92.

227. Wernstedt Asterholm, I., et al., *Adipocyte inflammation is essential for healthy adipose tissue expansion and remodeling.* Cell Metab, 2014. **20**(1): p. 103-18.

228. Dunn, G.P., L.J. Old, and R.D. Schreiber, *The three Es of cancer immunoediting.* Annu. Rev. Immunol., 2004. **22**: p. 329-360.

229. Weinberg, R.A., *The Biology of Cancer: Second International Student Edition*. 2013: WW Norton & Company.

230. van Dalen, F., et al., *Molecular repolarisation of tumour-associated macrophages.* Molecules, 2019. **24**(1): p. 9.

231. Bazan, J.F., et al., *A new class of membrane-bound chemokine with a CX3C motif.* Nature, 1997. **385**(6617): p. 640.

232. Sierra-Filardi, E., et al., *CCL2 shapes macrophage polarization by GM-CSF and M-CSF: identification of CCL2/CCR2-dependent gene expression profile.* The Journal of Immunology, 2014. **192**(8): p. 3858-3867.

233. Roca, H., et al., *CCL2 and interleukin-6 promote survival of human CD11b+ peripheral blood mononuclear cells and induce M2-type macrophage polarization.* Journal of biological chemistry, 2009. **284**(49): p. 34342-34354.

234. Kim, J., et al., *Silencing CCR2 in macrophages alleviates adipose tissue inflammation and the associated metabolic syndrome in dietary obese mice.* Molecular Therapy-Nucleic Acids, 2016. **5**: p. e280.

235. Kitade, H., et al., *CCR5 plays a critical role in obesity-induced adipose tissue inflammation and insulin resistance by regulating both macrophage recruitment and M1/M2 status.* Diabetes, 2012. **61**(7): p. 1680-1690.

236. Lumeng, C.N., et al., *Phenotypic switching of adipose tissue macrophages with obesity is generated by spatiotemporal differences in macrophage subtypes.* Diabetes, 2008. **57**(12): p. 3239-3246.

237. Medrikova, D., et al., *Sex differences during the course of diet-induced obesity in mice: adipose tissue expandability and glycemic control.* International journal of obesity, 2012. **36**(2): p. 262.

238. Boutens, L., et al., *Unique metabolic activation of adipose tissue macrophages in obesity promotes inflammatory responses.* Diabetologia, 2018. **61**(4): p. 942-953.

239. Sun, S., et al., *Mechanisms of inflammatory responses in obese adipose tissue.* Annual review of nutrition, 2012. **32**: p. 261-286.

240. Russo, L. and C.N. Lumeng, *Properties and functions of adipose tissue macrophages in obesity.* Immunology, 2018. **155**(4): p. 407-417.

241. Galvan, G.C., et al., *Effects of obesity on the regulation of macrophage population in the prostate tumor microenvironment.* Nutrition and cancer, 2017. **69**(7): p. 996-1002.

242. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* cell, 2011. **144**(5): p. 646-674.

243. Chih, C.-P. and E.L. Roberts Jr, *Energy substrates for neurons during neural activity: a critical review of the astrocyte-neuron*

lactate shuttle hypothesis. Journal of Cerebral Blood Flow & Metabolism, 2003. **23**(11): p. 1263-1281.

244. Abdel-Haleem, A.M., et al., *The emerging facets of non-cancerous warburg effect.* Frontiers in endocrinology, 2017. **8**: p. 279.

245. Zaidi, N., et al., *Lipogenesis and lipolysis: The pathways exploited by the cancer cells to acquire fatty acids.* Progress in Lipid Research, 2013. **52**(4): p. 585-589.

246. Prajapati, P. and D.W. Lambert, *Cancer-associated fibroblasts - Not-so-innocent bystanders in metastasis to bone?* Journal of bone oncology, 2016. **5**(3): p. 128-131.

247. Santi, A., et al., *Cancer associated fibroblasts transfer lipids and proteins to cancer cells through cargo vesicles supporting tumor growth.* Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 2015. **1853**(12): p. 3211-3223.

248. Tisdale, M.J., *Mechanisms of Cancer Cachexia.* Physiological Reviews, 2009. **89**(2): p. 381.

249. Yanagihara, K., et al., *Development and characterization of a cancer cachexia model employing a rare human duodenal neuroendocrine carcinoma-originating cell line.* Oncotarget, 2019. **10**(25): p. 2435.

250. Lottes, R.G., et al., *Lactate as substrate for mitochondrial respiration in alveolar epithelial type II cells.* American Journal of Physiology-Lung Cellular and Molecular Physiology, 2015. **308**(9): p. L953-L961.

251. Fischer, K., et al., *Inhibitory effect of tumor cell–derived lactic acid on human T cells.* Blood, 2007. **109**(9): p. 3812-3819.

252. Katz, J. and P. Wals, *Lipogenesis from lactate in rat adipose tissue.* Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism, 1974. **348**(3): p. 344-356.

253. Prior, R.L., S.B. Smith, and J. Jacobson, *Metabolic pathways involved in lipogenesis from lactate and acetate in bovine adipose tissue: Effects of metabolic inhibitors.* Archives of biochemistry and biophysics, 1981. **211**(1): p. 202-210.

254. San-Millán, I. and G.A. Brooks, *Reexamining cancer metabolism: lactate production for carcinogenesis could be the purpose and explanation of the Warburg Effect.* Carcinogenesis, 2017. **38**(2): p. 119-133.

255. Antoniewicz, M.R., *A guide to 13 C metabolic flux analysis for the cancer biologist.* Experimental & molecular medicine, 2018. **50**(4): p. 19.

256. Mounier, C., L. Bouraoui, and E. Rassart, *Lipogenesis in cancer progression.* International journal of oncology, 2014. **45**(2): p. 485-492.

257. Luo, X., et al., *Emerging roles of lipid metabolism in cancer metastasis.* Molecular cancer, 2017. **16**(1): p. 76.

258. Nakajima, E.C. and B. Van Houten, *Metabolic symbiosis in cancer: refocusing the Warburg lens.* Molecular carcinogenesis, 2013. **52**(5): p. 329-337.

259. Morrot, A., et al., *Metabolic symbiosis and immunomodulation: how tumor cell-derived lactate may disturb innate and adaptive immune responses.* Frontiers in oncology, 2018. **8**: p. 81.