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# Roles of adipocytes in the resistance of breast cancer to trastuzumab-mediated antibody-dependent cellular cytotoxicity (ADCC)

Minh Ngoc Duong

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ROLES OF ADIPOCYTES IN THE RESISTANCE OF BREAST CANCER TO  
TRASTUZUMAB-MEDIATED ANTIBODY DEPENDENT CELLULAR CYTOTOXICITY  
(ADCC)

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*This dissertation is dedicated to the memory of  
Ms TRINH Chiem Huong, my biology teacher,  
who died from breast cancer 12 years ago...*



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# LIST OF ABBREVIATIONS

5-FU	5-FluoroUracil
AC	Adenylate Cyclase
ACS	Acyl-CoA Synthase
ADASs	Adipose-Derived Adult Stromal/adipose-derived adult Stem cells
ADCC	Antibody-Dependent Cellular Cytotoxicity
ADCP	Antibody-Dependent Cellular Phagocytosis
AdipoR	Adiponectin Receptors
ADM	ADrenoMedullin
ADP	Adenosine DiPhosphate
ALL	Acute Lymphoblastic Leukemia
AMP	Adenosine MonoPhosphate
AMPK	5' Adenosine MonoPhosphate-activated protein Kinase
Ang-1	Angiopoietin-1
ANGPTL	Angiopoietin-like protein
APC	Antigen-Presenting Cells
AR	Adrenergic Receptor
ASCs	Adipose tissue-derived Stem Cells
ATDCs	Adipose Tissue-Derived Cells
ATP	Adenosine TriPhosphate
ATSCs	Adipose Tissue-derived Stromal Cells
BAT	Brown Adipose Tissue
Bid	BH3 interacting-domain death agonist
BMI	Body Mass Index
BM-MSCs	Bone Marrow Mesenchymal Stem Cells
BMP	Bone Morphogenetic Protein
C/EBP $\alpha$	CCAAT/Enhancer Binding Protein $\alpha$
CCL	CC-chemokine Ligand
CD	Cluster of Differentiation
CDC	Complement-Dependent Cytotoxicity
CDRs	Complementarity-Determining Regions
ChemR23	Chemerin Receptor
CLL	Chronic Lymphocytic Leukemia
COX	CycloOXygenase
cSMAC	central SupraMolecular Activation Cluster
CTLA-4	Cytotoxic T Lymphocyte Antigen 4
CXC3R1	CXC chemokine Receptor 1
CXCL	CXC-chemokine Ligand
CXCR	chemokine (C-X-C motif) Receptor

DISC	Death Inducing Signaling Complex
EGFR	Epidermal Growth Factor Receptor
EGR1	Early Growth Response protein 1
EMT	Epithelial-to-Mechenchymal Transition
ERK	Extracellular-signal-Regulated Kinases
ER	Estrogen Receptor
Fab	Fragment antigen binding
FABP	Fatty Acid Binding Protein
FADD	Fas Associated Death Domain
FAP	Fibroblast Activation Protein
FasL	Fas Ligand
FASN	Fatty Acid SyNthase
FAT	Fatty Acid Translocase
FATP	Fatty Acid Transport Protein
Fc	Fragment crystallizable
FcR	Fc Receptors
FDA	Food and Drug Administration
FF	Phenylalanine-Phenylalanine
FFA	Free Fatty Acid
FGF-2	Fibroblast Growth Factor 2
FHL	Familial Haemophagocytic Lymphohistiocytosis
FITC	Fluorescein IsoThioCyanate
FSP1	Fibroblast-Specific Protein 1
GDF15	Growth Differentiation Factor 15
Glut	Glucose transporter
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HER2	Human Epidermal growth factor Receptor 2
HGF	Hepatocyte Growth Factor
HIF-1 $\alpha$	Hypoxia Induced Factor 1 $\alpha$
HLA	Human Leucocyte Antigen
HSL	Hormone-Sensitive Lipase
hMADS	human Multipotent Adipose-Derived Stem
HMGB-1	High-Mobility Group Box 1 protein
IARC	International Agency for Research on Cancer
ICAM-1	InterCellular Adhesion Molecule 1
IF	Invasive Front
IFN	InterFeroN
IGF-I	Insulin-like Growth Factor I
IGF-IR	Insulin-like Growth Factor I Receptor
IL	InterLeukin

IP-10	Interferon-gamma inducible Protein 10
IR	Insulin Receptor
ITAMs	Immunoreceptor Tyrosine-based Activating Motifs
ITIMs	Immunoreceptor Tyrosine-based Inhibitory Motifs
KIR	Killer cell Immunoglobulin-like Receptors
LPA	LysoPhosphatidic Acid
LPL	LipoProtein Lipase
mAbs	monoclonal Antibodies
MAC	Membrane Attack Complex
MAPK	Mitogen-Activated Protein Kinases
MCP-1	Macrophage and monocyte Chemoattractant Protein 1
MDSCs	Myeloid-Derived Suppressor Cells
MEF	Mouse Embryonic Fibroblasts
MEK	Mitogen Extracellular signal Kinase
MHC	Major Histocompatibility Complex
MICA/MICB	MHC class I Chain-related sequence A/B
MMP	Matrix MetalloProteinases
MSCs	Multipotent mesenchymal Stem Cells
MTOC	MicroTubule Organizing Center
mTOR	mammalian Target Of Rapamycin
MUC4	glycoprotein MUCin 4
NA	Not Approved
NAMPT	NicotinAMide PhosphoribosylTransferase
NCRs	Natural Cytotoxicity Receptors
NF- $\kappa$ B	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer
NKIS	NK cell Immunological Synapses
NKLAM	NK Lytic-Associated Molecule
NKT	Natural Killer T
NPI	Nottingham Prognostic Index
ObR	leptin Receptor
PAF	Platelet-Activating Factor
PAI-1	Plasminogen Activator Inhibitor 1
PCNA	Proliferating Cell Nuclear Antigen
PDGFR	Platelet Derived Growth Factor Receptor
PGE2	ProstaGlandin E2
PI3K	PhosphoInositide 3 Kinase
PKA	Protein Kinase A
PLAs	Processed LipoAspirate cells



PPAR $\gamma$	Peroxisome Proliferator-Activated Receptor $\gamma$
PR	Progesterone Receptor
PRDM16	PRD1-BF1-RIZ1 homologous DoMain containing 16
Pref-1	Preadipocyte factor 1
pSMAC	peripheral SupraMolecular Activation Cluster
PTEN	Phosphatase and TENsin homolog
RBP-4	Retinol Binding Protein 4
ROS	Reactive Oxygen Species
S1P	Spingosine-1-Phosphate
SERPINS	SERine Proteinase INhibitors
siRNA	small interfering RiboNucleic Acid
SMAC	SupraMolecular Activation Cluster
SMIC	SupraMolecular Inhibition Cluster
SNARE	Soluble N-éthylmaleimide-sensitive-factor Attachment protein REceptor
SOS	Son Of Sevenless
Syk	Spleen tyrosine kinase
TG	TriGlyceride
TGF	Transforming Growth Factor
TIMP-1	Tissue Inhibitor of MetalloProteinase-1
TNF- $\alpha$	Tumor Necrosis Factor alpha
TNM	Tumor Node Metastasis
TRAIL	TNF and TNF-related Apoptosis Inducing Ligand
Tregs	regulatory T cells
UCP1	UnCoupling Protein-1
ULBP	UL16-Binding Protein
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VF	Valine-Phenylalanine
VLDL	Very Low Density Lipoprotein
VV	Valine-Valine
WAT	White Adipose Tissue
WHO	World Health Organization
YAT	Yellow Adipose Tissue
$\alpha$ -SMA	alpha-Smooth Muscle Actin

# PUBLICATIONS AND COMMUNICATIONS

## Publications

- 1. Adipocytes inhibit trastuzumab-mediated ADCC via induction of GDF15.**  
Minh Ngoc Duong, Aurore Cleret, Eva-Laure Matera, Kamel Chettab, Doriane Poloni, Sandrine Valsesia-Wittmann, Béatrice Clémenceau, Charles Dumontet.  
(Manuscript in preparation)
- 2. Preclinical activity of the type II CD20 antibody GA101 (obinutuzumab) compared with rituximab and ofatumumab in vitro and in xenograft models.**  
Herter S, Herting F, Mundigl O, Waldhauer I, Weinzierl T, Fauti T, Muth G, Ziegler-Landesberger D, Van Puijenbroek E, Lang S, Duong MN, Reslan L, Gerdes CA, Friess T, Baer U, Burtscher H, Weidner M, Dumontet C, Umana P, Niederfellner G, Bacac M, Klein C.  
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## Communications

- 1. Adipocytes inhibit trastuzumab-mediated ADCC via induction of GDF15.**  
Minh Ngoc Duong, Aurore Cleret, Eva-Laure Matera, Kamel Chettab, Doriane Poloni, Sandrine Valsesia-Wittmann, Béatrice Clémenceau, Charles Dumontet (to be presented in the AACR annual meeting 2014, San Diego, California, USA. April 5-9, 2014).
- 2. Interference of fat tissue with NK cell-mediated antitumor immunity: towards a mechanism of tumor resistance**  
Minh Ngoc Duong, Aurore Cleret, Eva-Laure Peiller, Charles Dumontet. 1<sup>st</sup> International Symposium of Cancer Research Center of Lyon, France. February 13-15, 2013.
- 3. Interference of fat tissue with NK cell-mediated antitumor immunity: towards a mechanism of tumor resistance**  
MN.Duong, A.Cleret, EL.Peiller, C.Dumontet. 2<sup>nd</sup> Scientific Day of the Cancer Research Center of Lyon, France. October 3, 2012.
- 4. Interference of fat tissue with NK cell-mediated antitumor immunity: towards a mechanism of tumor resistance**  
MN.Duong, A.Cleret, C.Dumontet. 7<sup>th</sup> cancer scientific forum of the Cancéropole CLARA, Lyon, France. March 20-21, 2012.

# PREFACE

Breast cancer is the most frequent cancer in women and the leading cause of cancer-related mortality. The Human Epidermal growth factor Receptor 2 (HER2) is over-expressed in 15-20% of breast cancer and is a target for therapies. Trastuzumab is a humanized monoclonal antibody targeting HER2 and has been approved in the treatment of HER2-overexpressing breast cancer. The antitumor effects of trastuzumab are mainly mediated via the recruitment of immune effector cells which provoke the lysis of target tumor cells. Although trastuzumab has shown remarkable efficacy, resistance often occurs. Different mechanisms of resistance to trastuzumab have been proposed. However, they are mainly related to the alterations of the tumor cells and do not take into account the role of the tumor microenvironment.

On the other hand, adipocytes constitute the major component of the breast. Besides the obvious evidence linking obesity and cancer, raising data have indicated an important role of adipocytes in promoting tumor growth and tumor progression. Some studies also suggested that adipocytes may increase cancer resistance to chemotherapy and radiotherapy. Nevertheless, the involvement of adipocytes in cancer resistance to targeted therapy using monoclonal antibody is poorly known. Therefore, studies on the roles of adipocytes in cancer resistance to trastuzumab-mediated ADCC as well as the crosstalk between adipocytes and cancer cells would provide better understanding on the importance of adipocytes in the tumor microenvironment.

In the introduction of this thesis, I will present an overview of breast cancer and trastuzumab in the treatment of HER2-overexpressing breast cancer. The following parts will focus on the interplay between breast cancer cells, immune natural killer cells, and adipocytes. Obesity will be also evoked in relation with cancer risk and development.

In the section of results, I will present our work during my PhD study which consists of two parts. The first one demonstrates the roles of adipocytes in the inhibition of trastuzumab-mediated ADCC in cancer cells. In the second part, we will focus on the identification of the adipocyte-derived factors that could increase cancer resistance to ADCC.

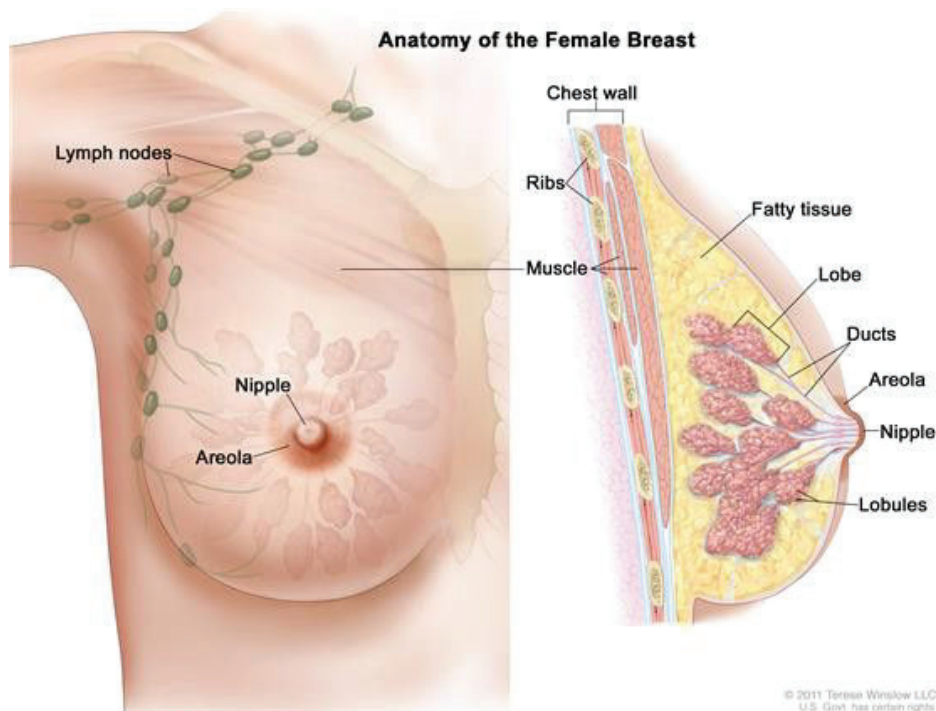
In the final section, discussion and perspectives, I will summarize our work and discuss in details about the objectives for future studies. Overall, our results contribute to the current knowledge about adipocytes and cancer, and suggest that adipocytes could be a good target in cancer treatment.

# **INTRODUCTION**

# 1. Breast cancer: an overview

## 1.1. Definition

Breast cancer is a type of cancer originating from the breast. Breasts are mainly made up of lobules (milk-producing glands), ducts (milk-draining tubes from lobules to the nipple), and stroma (adipose tissue, nerves, blood and lymph vessels) (Figure 1). Breast cancer starts most commonly in the ducts (ductal carcinoma) or the lobules (lobular carcinoma). The main sites of metastasis of breast cancer are bone, brain, liver and lung.



**Figure 1. Anatomy of the female breast**

The breast is composed of glandular structures (lobules and ducts) that are totally embedded in the stroma.

## 1.2. Incidence and mortality

While breast cancer is rare in men (approximately 1 out of every 150 breast cancers<sup>1</sup>), it is the most frequent cancer in women<sup>2</sup>. According to the data of the International Agency for Research on Cancer (IARC) in 2008, breast cancer accounts for 23% of newly diagnosed cancers in women in the world<sup>2</sup>. The incidence rates vary from less than 40 cases per 100,000 women in developing regions to greater than 80 cases per 100,000 women in developed regions.

The range of mortality of breast cancer is between 6 and 19 per 100,000 women<sup>2</sup>, depending on the country. Breast cancer accounts for 13.7% of cancer deaths in women worldwide. As a result, it remains the most frequent cause of cancer death in women.

### 1.3. Risk factors

Different factors can increase the risks developing breast cancer (Table I). It is worth noting that besides non-modifiable factors such as gender, age and genetic predisposition, many other factors are related to lifestyle, such as alcohol, weight/obesity, and lack of physical activity.

**Table I. Risk factors for breast cancer (Breast cancer Facts & Figures 2011-2012)**

Relative Risk	Factor
>4.0	<ul style="list-style-type: none"> <li>• Age (65+ vs. &lt;65 years, although risk increases across all ages until age 80)</li> <li>• Biopsy-confirmed atypical hyperplasia</li> <li>• Certain inherited genetic mutations for breast cancer (BRCA1 and/or BRCA2)</li> <li>• Mammographically dense breasts</li> <li>• Personal history of breast cancer</li> </ul>
2.1-4.0	<ul style="list-style-type: none"> <li>• High endogenous estrogen or testosterone levels</li> <li>• High bone density (postmenopausal)</li> <li>• High-dose radiation to chest</li> <li>• Two first-degree relatives with breast cancer</li> </ul>
1.1-2.0	<ul style="list-style-type: none"> <li>• Alcohol consumption</li> <li>• Ashkenazi Jewish heritage</li> <li>• Early menarche (&lt;12 years)</li> <li>• Height (tall)</li> <li>• High socioeconomic status</li> <li>• Late age at first full-term pregnancy (&gt;30 years)</li> <li>• Late menopause (&gt;55 years)</li> <li>• Never breastfed a child</li> <li>• No full-term pregnancies</li> <li>• Obesity (postmenopausal)/adult weight gain</li> <li>• One first-degree relative with breast cancer</li> <li>• Personal history of endometrium, ovary, or colon cancer</li> <li>• Recent and long-term use of menopausal hormone therapy containing estrogen and progestin</li> <li>• Recent oral contraceptive use</li> </ul>

\*Relative risks compare the risk of disease between people with or without exposure to a given factor. Relative risks higher 1.0 indicate higher risks in exposed people than non-exposed people.

## 1.4. Classification of breast cancer

Breast cancer is a group of heterogeneous diseases that show substantial variation in their molecular and clinical characteristics. As a consequence, a classification of breast cancer is required to provide prognostic information and to aid in the choice of adapted therapies.

Breast cancer can be classified into different stages according to the size of the tumor, lymph node involvement and metastasis status (the Tumor Node Metastasis, or TNM, staging system). It can be also divided into histological grades, based on tubule formation, nuclear pleomorphism and mitotic counts which reflect both the degree of differentiation and the proliferative activity of breast tumors<sup>3</sup>. Histological grade combines with the tumor size and the number of involved lymph nodes forming the Nottingham Prognostic Index (NPI), which provides complementary information for clinical outcome<sup>4-7</sup>. In 2003, the World Health Organization (WHO) classified breast cancer into 20 different histological subtypes<sup>8</sup>. However, the use of histological types has been limited, due to the lack of standardized criteria for their diagnosis and low inter-observer reproducibility<sup>9</sup>.

### 1.4.1. Molecular biomarkers of breast cancer

In addition to histopathological assessment, standard evaluation of breast cancer for clinical purposes involves immunohistochemistry characterization of molecular biomarkers, notably estrogen receptor alpha (ER $\alpha$ ), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2)<sup>10</sup>. This evaluation allows classification of breast cancers into three principal phenotypes: ER $\alpha$ -positive, ER $\alpha$ -negative/HER2-positive, and ER $\alpha$ -negative/HER2-negative (predominantly PR-negative, or triple-negative). These biomarkers are of particular importance for prognosis and adaption of systemic treatment.

#### 1.4.1.1. Estrogen receptor alpha (ER $\alpha$ )

Estrogen plays a crucial role not only in the morphogenesis of mammary glands but also in the promotion of growth in many hormone-dependent breast cancers<sup>11</sup>. It is synthesized mainly in the ovary of premenopausal women and in the adipose tissues of postmenopausal women<sup>12</sup>. Estrogen action is primarily mediated through two receptors: estrogen receptor alpha (ER $\alpha$ ) and estrogen receptor beta (ER $\beta$ )<sup>11</sup>. The oncogenic effect of estrogen is mediated by ER $\alpha$  via regulating expression of target genes involved in cell proliferation, cell survival and angiogenesis<sup>13</sup>. However, estrogen does not promote tumor metastasis in general, and ER $\alpha$ -positive breast cancers patients often have better prognosis than do ER $\alpha$ -negative breast



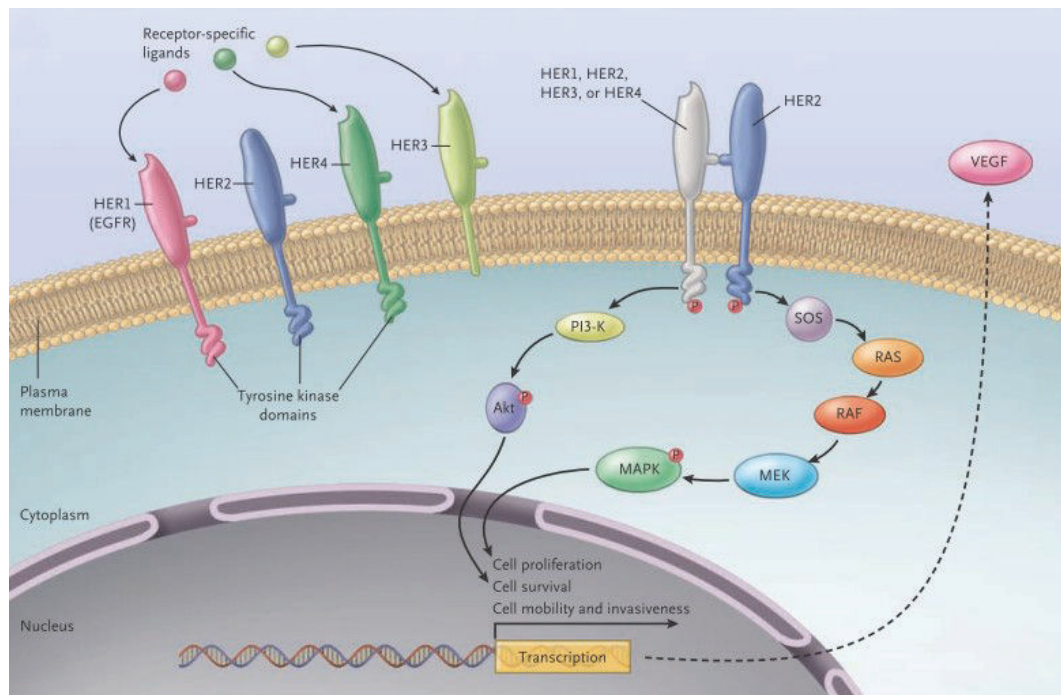
cancer patients<sup>14</sup>. ER $\alpha$  is expressed in about 75% of breast cancer patients, which underlines its importance as a biomarker in breast cancer.

#### *1.4.1.2. Progesterone receptor (PR)*

Progesterone receptor (PR) is an estrogen-regulated gene and its expression is dependent on ER $\alpha$  activity<sup>15</sup>. Like ER, PR is a steroid nuclear receptor which regulates expression of genes related to cell adhesion, cell growth, apoptosis, steroid and fatty acid metabolism<sup>16</sup>. PR is expressed in more than 50% of ER $\alpha$ -positive tumors<sup>17</sup>. Tumors with PR positive and ER $\alpha$  negative are uncommon and represent 1-3% of all breast cancers<sup>17,18</sup>. In ER $\alpha$  positive patients, absence of PR is associated with more proliferative and aggressive tumors, poorer prognosis and recurrence<sup>17-20</sup>. Interestingly, PR status has been reported to be a predictive factor for benefit from adjuvant endocrine therapy independently of ER status<sup>21</sup>. However, in some studies, no association between PR expression and adjuvant therapy was observed<sup>22,23</sup>. Although the predictive value of PR has been recently questioned<sup>24</sup>, PR is still an interesting biomarker used in breast cancer<sup>25-27</sup>.

#### *1.4.1.3. Human epidermal growth factor receptor 2 (HER2)*

HER2, also named ERBB2, belongs to the epidermal growth factor receptor (EGFR) which comprises 4 members (HER1/EGFR, HER2, HER3 and HER4). HER2 is localized at the plasma membrane and has a tyrosine kinase activity. Although the ligand of HER2 remains unknown, HER2 activity is mediated through dimerization with other members of the EGFR family, phosphorylation and activation of downstream pathways such as mitogen-activated protein kinases (MAPK), phosphoinositide 3 kinase (PI3K)/Akt, resulting in cell proliferation, cell survival, invasion and angiogenesis<sup>28</sup> (figure 2). HER2 was found to be overexpressed in 15-20% of breast cancers<sup>29,30</sup>, and approximately 30% of HER2-positive cancers display ER $\alpha$  expression<sup>31</sup>. HER2 overexpression is correlated with poor prognosis in breast cancer patients<sup>32</sup>.



**Figure 2. HER2 signaling pathway (Hudis, 2007)**

HER2 possesses tyrosine kinase domains like HER1 and HER4. Upon activation, HER2 undergoes dimerization with other members of the EGFR family (HER1-4) and activates downstream pathways. The activated phosphoinositide 3-kinase (PI3-K) phosphorylates the enzyme Akt, leading to cell survival. In parallel, the mammalian homologue of the son of sevenless (SOS) subsequently activates, RAS, RAF, mitogen extracellular signal kinase (MEK), and the mitogen-activated protein kinase (MAPK). MAPK promotes cell proliferation and production of vascular endothelial growth factor (VEGF) supporting angiogenesis.

#### 1.4.1.4. Other biomarkers

Other biomarkers, even if they are not yet routinely evaluated, can be biologically informative and clinically useful. Ki67 is a biomarker of cell proliferation<sup>33</sup>. Given that cell proliferation is a hallmark of cancer<sup>34</sup> and proliferation influences the efficacy of therapy<sup>35</sup>, high levels of Ki67 are associated with poor prognosis in breast cancer<sup>36,37</sup>. However, the prognostic significance of Ki67 is modest<sup>37</sup>. Efforts have been made to standardize Ki67 measurement, Ki67 scoring and result interpretation<sup>38,39</sup>. Other emerging biomarkers, such as cyclin D1, cyclin E, ER $\beta$ , circulating tumor cells, mucin-like carcinoma associated antigen, and cancer antigens are potentially related to breast cancer prognosis<sup>10</sup>. However, due to the lack of evidence and reproducibility, further investigations are required.

### 1.4.2. Molecular classification of breast cancer

In 2000, using high throughput DNA microarray, Perou *et al.* characterized gene expression variation in human breast tumors. They identified at least four groups of breast cancer based on their relative unique molecular profiles: ER+/luminal-like, basal-like, HER2+ and normal breast-like tumors<sup>40</sup>. The luminal subtype was later divided by Sorlie *et al.* into two subgroups, luminal A and luminal B subtypes<sup>41,42</sup>. These studies also correlated each of these molecular breast cancer subtypes with distinct clinical outcome: worst for HER2+ and basal, best for luminal A, and intermediate for luminal B subtypes. This classification by Perou and Sorlie was further validated independently by Sotiriou's group<sup>43</sup>. Recent studies discovered a new molecular subtype, referred to as the "claudin-low" subtype<sup>44,45</sup>. This latter is characterized by the low expression of genes involved in tight junctions and cell-cell adhesion, including claudins 3, 4, 7, occludin, and E-cadherin. "Claudin-low" tumors are triple negative (ER-/PR-/HER2-) but enriched for epithelial-to-mechenchymal transition (EMT) markers and immune response genes. They also display cancer stem cell-like features. Clinically, "claudin-low" tumors present intermediate prognosis between basal and luminal subtypes. Thus, molecular classification of breast cancer provides high clinical relevance for prognosis. However, due to high cost and the lack of standardization, it is not fully applicable to clinical practice. Several studies using immunohistochemistry tissue microarray have reproduced a similar (not identical) molecular taxonomy of breast cancer<sup>46-49</sup>. Notably, classification of breast cancer using relevant IHC markers (ER $\alpha$ , PR, HER2 and Ki67) identified subgroups that are comparable to subtypes classified by molecular gene expression described by Perou and Sorlie<sup>39,50,51</sup> (Table II).

**Table II. Correspondence between classical IHC markers and molecular classification of breast tumors (from Spitale *et al.*, 2009)**

	Luminal A	Luminal B	HER2+	Basal-like/ triple negative
ER	+	+	-	-
PR	+/-	+/-	-	-
HER2	-	+	+	-
Ki67	low	intermediate	high	high

## **1.5. Breast cancer treatment**

Depending on the type of breast cancer and its extension, as well as the patient's preferences, breast cancer can be treated with different methods.

### **1.5.1. Surgery**

Most women with breast cancer undergo surgery to remove the tumor and to check/remove the axillary lymph nodes. There are two main types of surgery: breast-conserving surgery (only a part of the affected breast is removed) or mastectomy (the entire breast is removed, sometimes along with other nearby tissues).

### **1.5.2. Radiation therapy**

Radiation therapy is local treatment using high-energy X rays or particles that destroy cancer cells. It is often given after breast-conserving surgery or in patients with a cancer larger than 4 cm, or to sterilize adjacent lymph nodes. Radiation is also used to treat cancer that has spread to other areas, such as bone or brain. There are two types of radiation therapy: external beam radiation using a machine outside the body on the area affected by the cancer; and brachytherapy or internal radiation, using radioactive seeds or pellets placed into the breast tissue next to the cancer.

### **1.5.3. Chemotherapy**

Chemotherapy is systemic treatment using anti-cancer drugs that are given intravenously or orally. Chemotherapy is given in cycles, either after surgery (adjuvant chemotherapy) to kill undetected cancer cells, or before surgery (neoadjuvant chemotherapy) to shrink large cancers to facilitate the removal. Chemotherapy is also used for patients with advanced breast cancer that has spread outside the breast and nearby lymph nodes. Some of the most commonly used drugs are: cyclophosphamide, 5-fluorouracil, doxorubicin, paclitaxel and docetaxel. In most cases, combinations of more than one drug are used, alone or with targeted therapy.

### **1.5.4. Hormone therapy**

Estrogen promotes the growth of 70-80% of breast cancer. Hormone therapy is another form of systemic therapy, which targets estrogen synthesis or signaling in patients with ER+ and/or PR+ breast cancers. Hormone therapy is often given as adjuvant to reduce the risk of

cancer recurrence. There are different types of drugs; each one mediates its proper effect on the ER pathway. These include:

- Tamoxifen: this drug blocks the ER by preventing estrogen binding. Tamoxifen is used for both premenopausal and postmenopausal women.

- Aromatase inhibitors: these drugs, including letrozole, anastrozole, and exemestane, inhibit estrogen production by aromatases in various tissues in postmenopausal women. They cannot be used in premenopausal women whose estrogen is mainly produced by the ovaries.

- Fulvestrant: this drug reduces ER expression. It is often used in advanced breast cancer patients who no longer respond to tamoxifen treatment.

### **1.5.5. Targeted therapy**

Targeted therapies are agents, either monoclonal antibodies or small molecule tyrosine kinase inhibitors, which specifically inhibit targeted molecules that are expressed in tumor cells or required for tumor cell survival or proliferation. Targeted therapy is expected to be more effective than current treatments and have fewer side effects. The choice of drugs depends on the target molecules:

- HER2: HER2 is amplified in 15-20% of breast cancer and its overexpression is associated with bad prognosis. Monoclonal antibodies, such as trastuzumab or pertuzumab, target HER2 on tumor cells, blocking cell proliferation and leading to cell death. Monoclonal antibodies will be presented in greater detail in a later chapter. Lapatinib (Tyverb<sup>TM</sup>), a tyrosine kinase inhibitor, also targets HER2 protein. Lapatinib was approved by the U.S. Food and Drug Administration (FDA) in 2007 for treatment of HER2+ cancers with combination therapy<sup>52</sup>. Lapatinib inhibits tumor cell growth by reducing HER2 downstream signaling<sup>53</sup>.

- Mammalian target of rapamycin (mTOR): this protein normally promotes cell growth and division. Everolimus, recently approved in 2012 for advanced ER+/PR+/HER2- breast cancers in postmenopausal women, inhibits tumor cell growth by blocking mTOR.

## 2. Trastuzumab: a magic bullet for HER2-overexpressing breast cancers

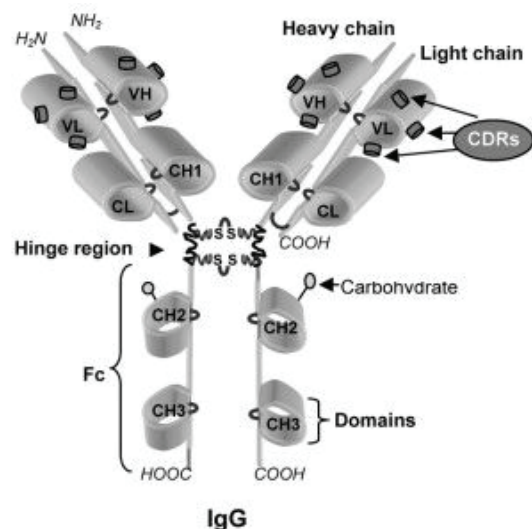
The concept of a “magic bullet” to selectively target tumors was first proposed by Paul Ehrlich at the beginning of the 20<sup>th</sup> century<sup>54</sup>. He postulated the existence of specific receptors that bind antigens and hypothesized that these receptors are either associated with cells or distributed more abundantly in the blood stream in response to antigen interaction. This concept constitutes the basis of the use of monoclonal antibodies (mAbs), which possess specific binding affinity to the targeted antigens. In 1975, using hybridomas (cells resulted from fusion between myeloma cells and murine B cells), Kohler and Milstein produced the first mAbs<sup>55</sup>. Since then, advances in antibody engineering technology have allowed the construction of various antibodies targeting different antigens, with diverse functions.

### 2.1. Structure of monoclonal antibodies

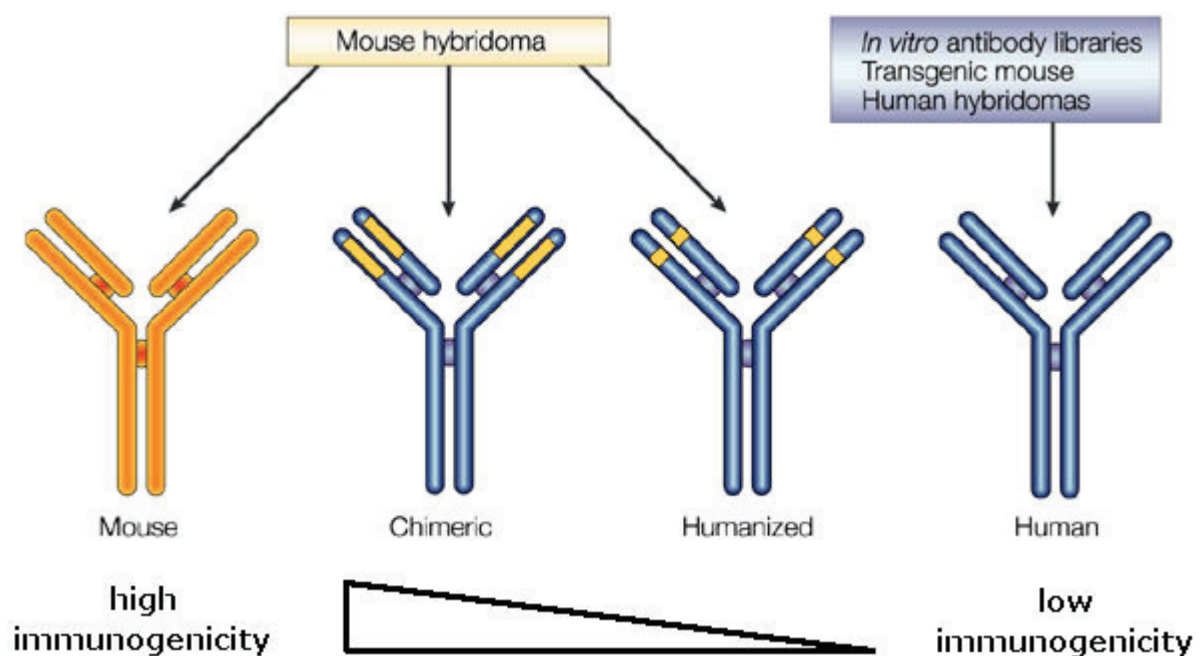
An antibody is a Y-shape protein belonging to the immunoglobulin superfamily, with a total mass about 150 kDa for most subtypes (figure 3). It contains two heavy chains and two light chains, linked to each other by disulfide bonds. Antibodies are grouped into five classes or isotypes based on their heavy chain regions: IgA, IgD, IgE, IgG, and IgM. Among these five classes, IgG is the most stable and abundant in human serum<sup>56</sup>, and the most frequently used in cancer immunotherapy<sup>57</sup>. Antibody binds to its target via the Fab (fragment antigen binding) region, which contains three complementarity-determining regions (CDRs). CDRs recognize a portion (epitope) of the antigen and are therefore responsible for the specificity of the antibody towards its antigen. Antibodies mediate their immune effector functions via the Fc (fragment crystallizable) region.

**Figure 3. Structure of an antibody (Sharkey and Goldenberg, 2006)**

A Y-shape antibody possesses two antigen binding (Fab) sites and a Fc region.



Monoclonal antibodies are antibodies that are specific for the same epitope. Originally, mAbs are made by identical immune cells (clones) that are derived from a unique parent cell. Early studies showed that murine mAbs were immunogenic in humans, had short serum half-life, and lacked abilities to induce human immune effector responses, thereby limiting their clinical applicability<sup>58</sup>. Later advances in genetic engineering have overcome these problems, giving rise to the development of chimeric, humanized, and fully human mAbs<sup>59</sup> (figure 4). Chimeric mAbs (suffix -ximab) are composed of mouse variable domains and human constant domains. Humanized mAbs (suffix -zumab) are constructed with only CDRs derived from mice and the remaining regions from humans. Fully human mAbs (suffix -mumab) contain no murine sequences.



**Figure 4. Evolution of monoclonal antibody structure (modified from Brekke and Sandlie, 2003)**

Mouse hybridoma technology generates mouse monoclonal antibodies. Cloning of mouse variable genes into human constant-region genes generates chimeric antibodies. Humanized antibodies are generated by the insertion of mouse complementarity-determining regions (CDRs) onto human constant and variable domain frameworks. Fully human antibodies can be generated by the selection of human antibody fragments from *in vitro* libraries, by transgenic mice, and through selection from human hybridomas. Immunogenicity of mAbs decreases from mouse, chimeric to humanized and fully human antibodies.

There are presently more than 30 mAbs approved for the treatments of various diseases, including cancer, autoimmune diseases, viral infections, and graft rejection. We will focus here on the use of mAbs for cancer treatment (table III).

**Table III. Therapeutic monoclonal antibodies approved in the treatment of cancer.**

Note: Information current as of 10 March, 2013

International non-proprietary name	Trade name	Type	Indication first approved	First EU (US) approval year
Rituximab	MabThera, Rituxan	Anti-CD20; Chimeric IgG1	Non-Hodgkin's lymphoma	1998 (1997)
Trastuzumab	Herceptin	Anti-HER2; Humanized IgG1	Breast cancer	2000 (1998)
Alemtuzumab	MabCampath, Campath-1H	Anti-CD52; Humanized IgG1	Chronic myeloid leukemia	2001 (2001)
Tositumomab-I131	Bexxar	Anti-CD20; Murine IgG2a	Non-Hodgkin lymphoma	NA (2003)
Cetuximab	Erbitux	Anti-EGFR; Chimeric IgG1	Colorectal cancer	2004 (2004)
Ibritumomab tiuxetan	Zevalin	Anti-CD20; Murine IgG1	Non-Hodgkin's lymphoma	2004 (2002)
Bevacizumab	Avastin	Anti-VEGF; Humanized IgG1	Colorectal cancer	2005 (2004)
Panitumumab	Vectibix	Anti-EGFR; Human IgG2	Colorectal cancer	2007 (2006)
Ofatumumab	Arzerra	Anti-CD20; Human IgG1	Chronic lymphocytic leukemia	2010 (2009)
Ipilimumab	Yervoy	Anti-CTLA-4; Human IgG1	Metastatic melanoma	2011 (2011)
Brentuximab vedotin	Adcetris	Anti-CD30; Chimeric IgG1; immunoconjugate	Hodgkin lymphoma	2012 (2011)
Pertuzumab	Perjeta	Anti-HER2; humanized IgG1	Breast Cancer	2013 (2012)
Trastuzumab emtansine	Kadcyla	Anti-HER2; humanized IgG1; immunoconjugate	Breast cancer	In review (2013)

**CD**, cluster of differentiation; **CTLA-4**, cytotoxic T lymphocyte antigen 4; **EGFR**, epidermal growth factor receptor; **HER2**, human epithelial growth factor receptor; **NA**, not approved; **VEGF**, vascular endothelial growth factor.

Source: Janice M. Reichert, Editor-in-Chief, mAbs.  
([http://www.antibodysociety.org/news/approved\\_mabs.php](http://www.antibodysociety.org/news/approved_mabs.php))



## **2.2. Mechanisms of action of mAbs**

Different mechanisms have been proposed to explain the observed anti-cancer effects of mAbs. These include direct actions of mAbs on tumor growth or tumor death and indirect effects via recruitment of effectors from the immune system. Are listed here the main mechanisms of tumor cell killing by mAbs: direct induction of programmed cell death (apoptosis), complement-dependent cytotoxicity, antibody-dependent cellular cytotoxicity, and antibody-dependent cellular phagocytosis.

### **2.2.1. Direct induction of apoptosis**

Many cancers overexpress growth factors and their receptors, which act in an autocrine/paracrine manner to promote tumor growth. In addition, many of these receptors, including epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), and HER2, are associated with activation of survival pathways, such as PI3K/Akt and MAPK pathways<sup>60-62</sup>. Thus, mAbs targeting these receptors can block their interactions with the ligands, and/or disrupt their signaling pathways, which results in tumor growth arrest and enhanced tumor susceptibility to conventional chemo- and radiotherapeutics<sup>63,64</sup>. In addition, mAbs can directly induce apoptotic signaling via cross-linking of the surface antigens or mimic ligands that mediate activation of death receptors<sup>65-67</sup>.

Direct apoptosis induction plays an important role in the mechanisms of action of certain mAbs. The most relevant examples are cetuximab and panitumumab, mAbs targeting EGFR and used in the treatment of colorectal cancer. Although EGFR protein is expressed in approximately 85% of metastatic colorectal cancer, only a subset of patients benefit from mAb treatment, either as monotherapy or in combination with chemotherapy. Several studies have pointed out that mutation of K-RAS, a downstream effector of the EGFR signaling pathway, was strongly associated with resistance to mAb treatment, as well as poor clinical outcome<sup>68-70</sup>.

### **2.2.2. Complement-dependent cytotoxicity**

MABs can also mediate cell death via recruitment of complement factors in the serum, a mechanism known as complement-dependent cytotoxicity (CDC). After recognizing targeted antigens on the tumor cell surface via the Fab fragments, mAbs bind C1q factors via the Fc fragments, which in turn recruits and activates other complement factors, resulting in

formation of the membrane attack complex (MAC)<sup>71</sup>. MAC creates pores on the membrane of target cells leading to cell death. It has been shown that CDC is important for the killing of CD20+ B cells by rituximab both *in vitro* and *in vivo*<sup>72,73</sup>.

### 2.2.3. Antibody-dependent cellular cytotoxicity

The Fc fragments of mAbs can be recognized by receptors expressed on immune cells, thus allowing recruitment of these effector cells to target tumor cells. Effector cells can directly induce target cell apoptosis via signal transduction of death receptors, or release mediators such as perforin and granzyme that cause target cell lysis<sup>74</sup>. Interestingly, this mechanism, named antibody-dependent cellular cytotoxicity (ADCC), is induced at Mab concentrations lower than those needed to activate CDC. Several *in vitro* and *in vivo* studies have suggested that ADCC is the main mode of action of many mAbs against cancer cells<sup>75</sup>.

The effect of mAbs via ADCC underlines the importance of effector immune cells bearing receptors for the Fc fragments (table IV). There are different classes of Fc receptors (FcR), each corresponding to an antibody isotype: Fc $\gamma$ R bind IgG, Fc $\alpha$ R bind IgA, Fc $\epsilon$ R bind IgE, Fc $\mu$ R bind IgM and Fc $\delta$ R bind IgD<sup>76</sup>. Since most anti-cancer mAbs belong to IgG class<sup>77</sup>, Fc $\gamma$ R is the most relevant in mAb action. Different subclasses of Fc $\gamma$ R have been identified in humans: Fc $\gamma$ RI (CD64), Fc $\gamma$ RII (CD32), and Fc $\gamma$ RIII (CD16)<sup>78</sup> (figure 5). Among them, Fc $\gamma$ RI has high affinity towards IgG Fc fragments, while other receptors (Fc $\gamma$ RIIA, Fc $\gamma$ RIIB, Fc $\gamma$ RIIC, Fc $\gamma$ RIIIA and Fc $\gamma$ RIIIB) possess low to medium affinity. Most of these receptors are activating, with the exception of Fc $\gamma$ RIIB, which is inhibitory. Fc $\gamma$ RI and Fc $\gamma$ RIIIA are associated with adaptor molecules containing the immunoreceptor tyrosine-based activating motifs (ITAMs). Binding of mAbs to these activating receptors leads to FcR cross-linking and activation of downstream pathways which results in ADCC response (figure 6). By contrast, Fc $\gamma$ RIIB (CD32b) is an inhibitory receptor with immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in its cytoplasmic domain, and its activation is associated with inhibition of effector cells and even apoptosis<sup>78</sup>. Engagement of both activating and inhibiting FcRs was shown to be essential for *in vivo* activity of both trastuzumab and rituximab against tumors in mice<sup>79</sup>.

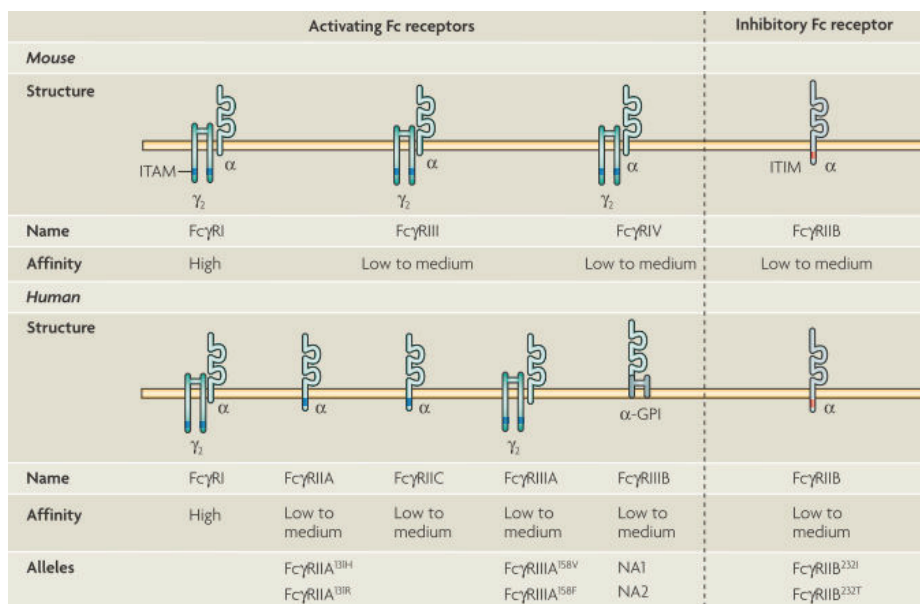
**Table IV. Human IgG receptor expression pattern (modified from Bruhns, 2012)**

	Fc $\gamma$ RI	Fc $\gamma$ RIIA	Fc $\gamma$ RIIB	Fc $\gamma$ RIIC*	Fc $\gamma$ RIIA	Fc $\gamma$ RIIB
B cells	-	-	+	-	-	-
T cells	-	-	-	-	-	-
NK cells	-	-	-†	+	+	-
Monocytes/macrophages	+	+	+/-	+	+	-
Neutrophils	(+)	+	+/-	+	-	+
DCs	+	+	+	-	-	-
Basophils	-	+	+	-	-	+/-
Mast cells	(+)	+	-	-	-	-
Eosinophils	-	+	-	-	-	-

+, expression; (+), inducible expression; +/-, very low percentages or rare subsets express the receptor; -, no expression; and NA, not analyzed.

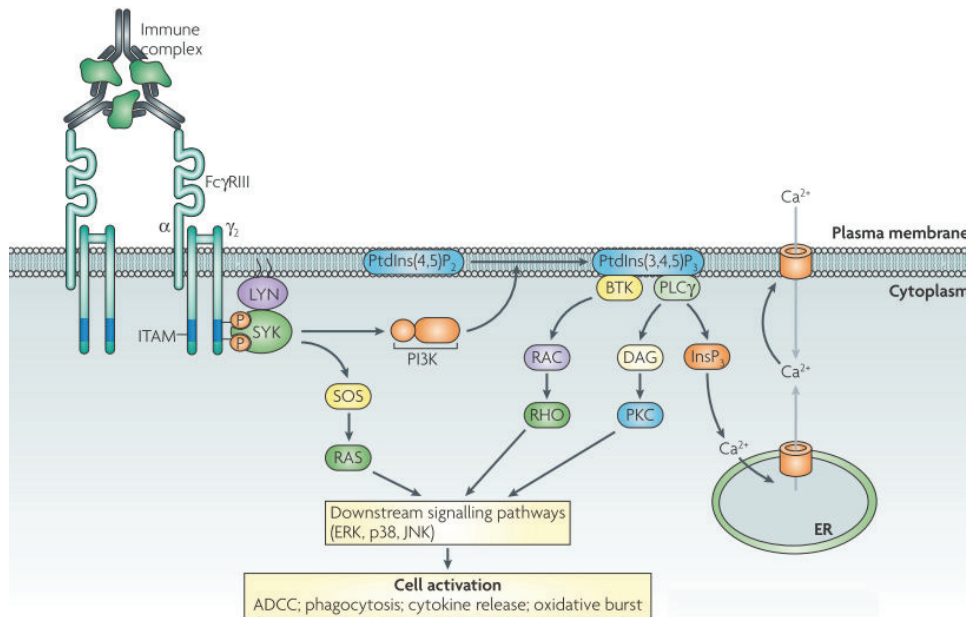
\*In Fcgr2c-open reading frame persons.

†Detectable and functional expression in nonconventional Fcgr2c-Stop persons.



**Figure 5. The family of FcR for IgG in mouse and human (Nimmerjahn and Ravetch, 2008)**

Mice and humans have one high-affinity receptor, Fc $\gamma$ RI; all other receptors have low to medium affinity. Most of the receptors are activating, and associated with adaptor molecules containing immunoreceptor tyrosine-based activating motif (ITAM). The Fc $\gamma$ RIIB is inhibitory and contains immunoreceptor tyrosine-based inhibitory motif (ITIM).



**Figure 6. Signaling pathways of activating FcR (Nimmerjahn and Ravetch, 2008)**

Crosslinking of activating FcR induces the phosphorylation of spleen tyrosine kinase (Syk) which in turn activates phosphoinositide 3-kinase (PI3K) and son of sevenless homologue (SOS). The final result is activation of effector cells, which mediate cytotoxic effects and secretion of cytokines.

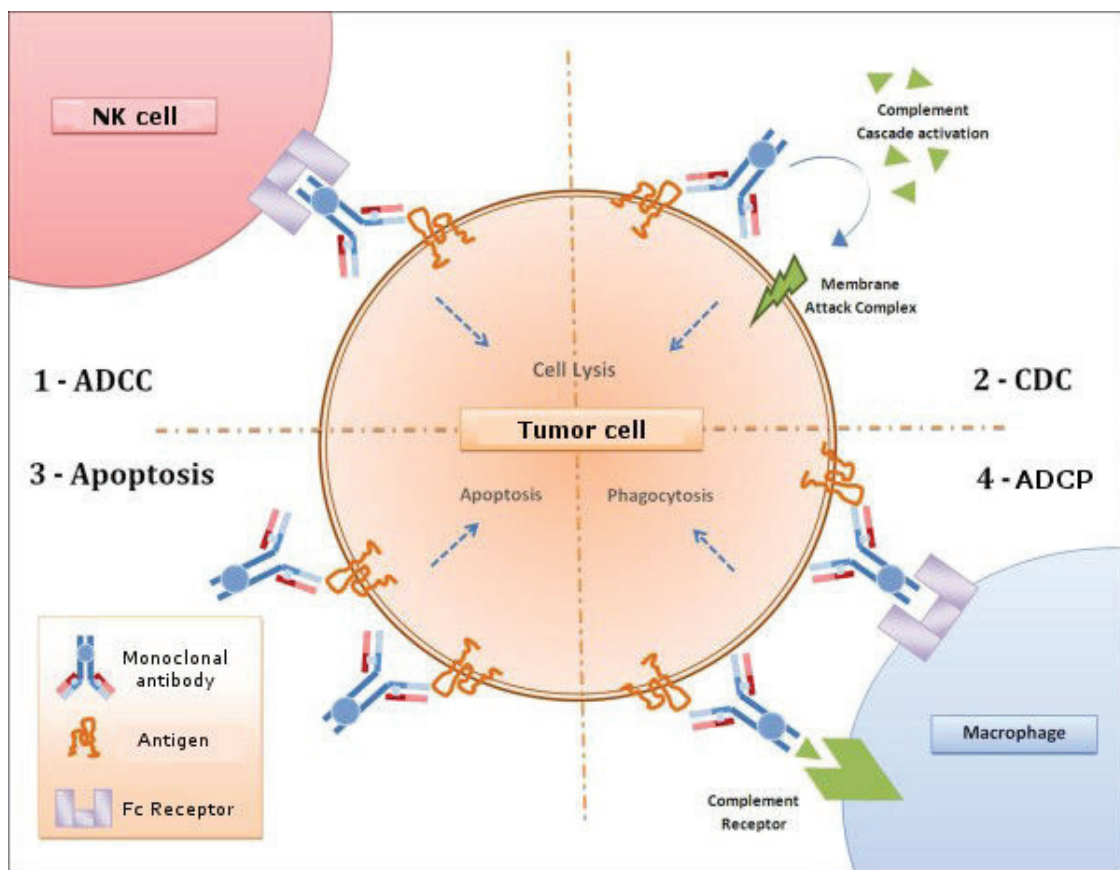
Although FcRs are expressed in all cells of the innate immune system<sup>78,80</sup>, ADCC is mainly mediated by natural killer (NK) cells (CD16+) and neutrophils (CD64+). *In vivo* studies have demonstrated that depletion of NK cells or neutrophils alone led to partial loss of antitumor effect of rituximab in mice<sup>81</sup>. Interestingly, NK cells express the activating receptors FcγRIIIA (CD16A), and to a lesser extent FcγRIIC (CD32C), but normally no inhibitory receptor FcγRIIB<sup>78,80,82</sup>. This data suggests that NK cells are by far the most important effector cells in ADCC. The importance of CD16 and NK cells in ADCC has been further demonstrated in humans, where the high-affinity [158(VF/VV)] but not the low-affinity [158(FF)] CD16 polymorphism was associated with better clinical outcome in patients having lymphoma or breast cancer and treated with rituximab or trastuzumab respectively<sup>83–85</sup>.

#### 2.2.4. Antibody-dependent cellular phagocytosis

Another mechanism of action of mAbs involves phagocytosis of target tumor cells by macrophages which express FcγRII (CD32). This mechanism, called antibody-dependent cellular phagocytosis (ADCP), has been demonstrated *in vitro*<sup>86,87</sup>. *In vivo*, macrophages were shown to be the important effector cells for B cell-depletion by anti-CD20 mAbs<sup>88</sup>.

Furthermore, clinical studies have shown that polymorphisms of CD32 can predict outcome in rituximab<sup>84</sup>, trastuzumab<sup>89</sup> and cetuximab<sup>90</sup> therapy.

Altogether, mAbs allow specific killing of target tumor cell, either directly or via recruitment of effectors from the immune system. Figure 7 summarizes the proposed mechanisms of action of mAbs. Although each mAb displays different preferential modes of action, the anti-tumor effect of mAbs *in vivo* is likely to result from all mechanisms. In clinical practice, mAbs are often combined with other approaches, such as chemotherapy, radiotherapy and immunomodulators<sup>57</sup>. Also, different strategies have been developed to optimize mAb structures and to enhance cytotoxicity, such as glycoengineering, bispecific antibodies and drug-conjugated antibodies<sup>91</sup>.



**Figure 7. Proposed mechanisms of action of mAbs (modified from Kasi *et al.*, 2012)**

There are four main proposed mechanisms of action of mAbs. First, mAbs directly inhibit cell growth and induce apoptosis. Second, mAbs recruit complement factors (C1q, C3a, C3b and C5a) in the serum to form a membrane attack complex that leads to cell lysis (complement-dependent cytotoxicity or CDC). Third, mAbs recruit immune effector cells that express activating FcR, notably natural killer (NK) cells (Fc $\gamma$ RIII+), leading to cell death by antibody-dependent cellular cytotoxicity (ADCC). Last but not least, mAbs facilitate antibody-dependent cellular phagocytosis (ADCP) by macrophages which express all types of Fc $\gamma$ R.

### **2.3. HER2, a target for mAbs**

HER2 was first shown in 1985 to be amplified in human mammary carcinoma<sup>92</sup>. Later, many other studies have shown that HER2/ERBB2 is overexpressed in 15-20% of breast cancers and its overexpression is associated with poor prognosis. Aberrant HER2 activation increased cell proliferation, resistance to apoptosis, angiogenesis and invasion<sup>28</sup>. It is interesting to note that increased HER2 signaling is always due to its overexpression in humans and not to mutations, contrary to its Neu homologue in rodents<sup>28</sup>. Moreover, it is also worth noting that HER2 overexpression was found in both primary tumors and metastatic sites of breast cancer<sup>93</sup>, while its expression is absent or present at very low levels in normal tissues, with the notable exception of heart tissue.

Importantly, several studies have demonstrated that HER2-overexpressing tumor cells are dependent on HER2 for survival and progression. Inhibition of HER2 expression in these cells by different approaches such as antisense oligonucleotides, HER2-targeted ribozymes, or siRNA, led to inhibition of cell growth and apoptosis *in vitro*<sup>94-97</sup> and *in vivo*<sup>98</sup>. In addition, HER2-induced transformed phenotype in fibroblasts could be reverted by expressing a kinase domain-mutated HER2 that competes with HER2<sup>99</sup>. Similarly, intracellular expression of antibodies targeting HER2 was shown to revert HER2-induced transformation or to induce apoptosis in HER2-overexpressing tumor cells<sup>100,101</sup>. Furthermore, using tetracycline-inducible systems, many groups have proved that HER2 expression induced tumorigenesis in mice, which could be reversed after HER2 withdrawal<sup>102-105</sup>.

Collectively, these data indicate that HER2 is specifically expressed in tumor cells, it drives tumorigenesis, and HER2-overexpressing tumor cells are addicted to HER2 for their growth and survival. Therefore, HER2 is a good target for therapeutic mAbs.

### **2.1. Trastuzumab, a mAb targeting HER2**

Trastuzumab is a humanized IgG1 antibody that targets the juxtamembrane portion of the extracellular domain of HER2<sup>93</sup>. In 1998, trastuzumab (Herceptin, Genetech) was approved by the FDA for the treatment of HER2-positive metastatic breast cancer. As single agent, trastuzumab displayed 11% to 26% overall response rates<sup>106-110</sup>. When combined with conventional agents, such as taxanes or vinorelbine, response rates ranged from 24% to

70%<sup>111–113</sup>. Importantly, combination of trastuzumab with chemotherapy provided greater clinical benefit than chemotherapy alone<sup>114–116</sup>.

### **2.1.1. Mechanisms of action of trastuzumab**

Although trastuzumab is now widely used in treatment of HER2 positive breast cancer, its mechanism of action is not fully understood.

#### *2.1.1.1. Direct apoptosis*

Trastuzumab is proposed to induce tumor cell apoptosis via blocking of HER2 dimerization and its signaling pathway, notably the survival PI3K/Akt downstream effectors. Studies in breast and ovarian cancer cell lines treated with trastuzumab showed decrease in Akt phosphorylation<sup>117,118</sup>. Moreover, Nagata *et al.* demonstrated that the phosphatase PTEN (phosphatase and tensin homolog) was rapidly recruited to the membrane and became activated in cells treated with trastuzumab. Trastuzumab-mediated activation of PTEN led to dephosphorylation and inactivation of HER2 downstream pathway, as well as inhibition of cell growth<sup>118</sup>.

#### *2.1.1.2. Antibody-dependent cellular cytotoxicity*

Several studies have underlined the role of ADCC as the important mechanism of action of trastuzumab. *In vitro* studies indicated that trastuzumab promoted NK-mediated killing of various HER2-overexpressing breast cancer cell lines<sup>119</sup>. *In vivo*, mice bearing BT474 tumor demonstrated inhibition of tumor growth when treated with trastuzumab, and this anti-cancer effect was significantly altered in mice lacking FcRs<sup>79</sup>. In humans, the therapeutic efficacy of trastuzumab was shown to be correlated with CD16 polymorphism<sup>85</sup>, indicating the importance of NK cells in trastuzumab action.

Intriguingly, in a recent study, using 7.16.4, an antibody against neu (homolog of HER2 in rodents) which competed with 4D5, the precursor of trastuzumab, Park *et al.* demonstrated the involvement of adaptive immune cells in the action of anti-HER2/neu antibody<sup>120</sup>. That anti-HER2/neu antibody mediated inhibition of tumor growth in wild-type control mice, but not in mice depleted for CD8+ T cells. These authors also showed that anti-HER2/neu antibody treatment induced release of danger signals, such as HMGB1. They postulated that HMGB1, as well as Fc-mediated tumor phagocytosis, might enhance cross-priming of antigen-presenting cells (APC), which in turn induced CD8+ T cell cytotoxicity against tumor

cells. However, the role of ADCC in the initiation of adaptive immune response remains unclear and requires further investigations.

#### *2.1.1.3. Inhibition of HER2 shedding*

Overexpressed HER2 can undergo proteolytic cleavage by matrix metalloproteinases which releases the extracellular domain into the circulation and a p95-membrane bound fragment<sup>121</sup>. Increase of p95HER2 was associated with nodal metastasis of breast cancer<sup>121,122</sup>. Trastuzumab treatment was shown to block HER2 shedding, which results in the decrease of p95 fragments in breast cancer specimens<sup>123</sup> in parallel with the decline of serum HER2 extracellular domain<sup>124,125</sup>.

#### *2.1.1.4. Inhibition of angiogenesis*

HER2 overexpression plays an important role in promoting angiogenesis<sup>126</sup>. Trastuzumab treatment was shown to inhibit secretion of angiogenic factors, such as VEGF (vascular-endothelial growth factor), TGF- $\alpha$  (transforming growth factor- $\alpha$ ), Ang-1 (angiopoietin-1) and PAI-1 (plasminogen-activator inhibitor-1) *in vitro* as well as reduced tumor blood vessels *in vivo*<sup>127</sup>. Interestingly, this anti-angiogenic effect of trastuzumab was more enhanced when combined with paclitaxel<sup>128</sup>.

### **2.1.2. Resistance to trastuzumab**

Resistance is observed in most patients treated with trastuzumab. Resistance may exist in cancer cells before exposure to trastuzumab (*de novo* resistance), or arise after exposure (acquired resistance). Several hypotheses have been proposed to explain resistance to trastuzumab.

#### *2.1.2.1. PTEN deficiency*

As mentioned above, PTEN is recruited to the cell membrane upon trastuzumab exposure and it induces Akt inactivation. When PTEN is inactive or deficient, Akt remains active and trastuzumab treatment becomes ineffective. Nagata *et al.* demonstrated that reducing PTEN by antisense oligonucleotides conferred trastuzumab resistance both *in vitro* and *in vivo*<sup>118</sup>. Additionally, they showed that patients with PTEN-deficiency displayed poorer responses to trastuzumab-based therapy than those with normal PTEN<sup>118</sup>.



#### 2.1.2.2. Activation of alternative pathways

Insulin-like growth factor I receptor (IGF-IR) is a transmembrane tyrosine kinase receptor whose signaling pathways are associated with cell proliferation and cell survival<sup>129,130</sup>. Breast cancer cells frequently express high levels of IGF-IR, and circulating levels of IGF-I have been associated with increased breast cancer risk in women<sup>131,132</sup>. Interestingly, several studies suggested that IGF-I was not produced by cancer cells but rather by cells in the breast stroma<sup>133–135</sup>. Lu *et al.* demonstrated that IGF-I decreased trastuzumab-mediated growth inhibition in breast cancer cells<sup>136</sup>. This result was further confirmed by Nahta *et al.* who showed that IGF-IR formed heterodimers with HER2 in trastuzumab-resistant cell lines<sup>137</sup>. Additionally, inhibition of IGF-IR tyrosine kinase activity increased sensitivity of resistant cells to trastuzumab<sup>137</sup>. These results indicate the involvement of IGF-IR signaling pathway in the resistance of cancer cells to trastuzumab-induced apoptosis.

#### 2.1.2.3. HER2 downregulation

Whether trastuzumab treatment induces HER2 endocytosis and HER2 destruction in cancer cells is not clear. While Valabrega *et al.* showed that trastuzumab down-regulated HER2 in breast cancer cell lines *in vitro*<sup>138</sup>, Austin *et al.* indicated that trastuzumab action did not involve surface HER2 downregulation<sup>139</sup>. In line with this finding, Gennari *et al.* showed no down-modulation of HER2 in tumors of patients treated with neoadjuvant trastuzumab<sup>140</sup>. However, in this study, the tumor samples were removed and analyzed 7 days after the last trastuzumab treatment, which did not exclude re-expression of HER2 by tumor cells. Recently, Valabrega *et al.* observed HER2 down-regulation in trastuzumab-resistant cell lines after chronic exposure to trastuzumab<sup>141</sup>, suggesting that HER2 down-modulation may be involved in trastuzumab resistance rather than trastuzumab action.

#### 2.1.2.4. HER2 shedding and HER2 masking

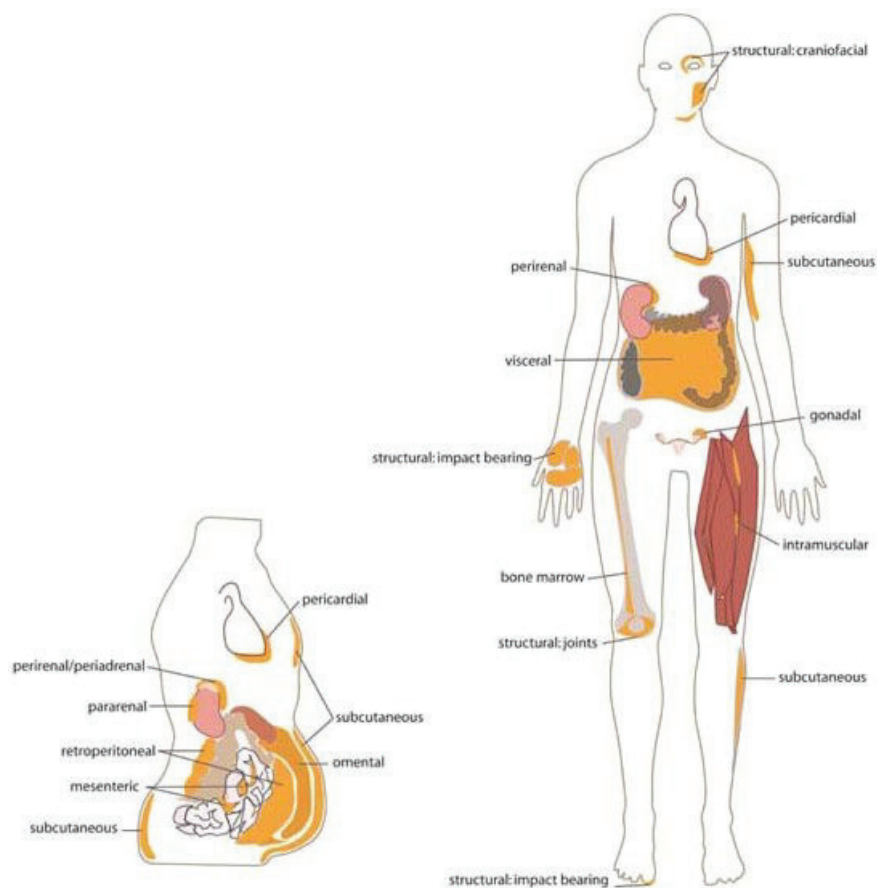
HER2 shedding by breast cancer was shown to neutralize the binding and effect of anti-HER2 antibody<sup>142</sup>. Moreover, an increase in serum levels of HER2 has been correlated with resistance to trastuzumab and disease progression<sup>143</sup>. On the other hand, several studies showed that the binding epitope of HER-2 for trastuzumab can be masked by the expression of glycoprotein mucin-4 (MUC4) in breast cancer cells<sup>144,145</sup>. Over-expression of MUC4 decreased the binding of trastuzumab to its target, while knockdown of MUC4 increased trastuzumab binding. Recently, upregulation of MUC4 was reported in breast cancer xenografts with acquired resistance to trastuzumab and lapatinib<sup>146</sup>.

Collectively, many mechanisms can be involved in the resistance of breast cancer cells to trastuzumab. However, these proposed mechanisms only implicated modifications of the cancer cells themselves, but not the tumor microenvironment. The surrounding stromal cells in the breast tumor microenvironment are permanently in interactions with cancer cells, which may participate in the sensitivity or resistance of cancer cells to trastuzumab treatment.

# 3. Adipose tissue: more than a fat storage

## 3.1. Types and distribution

Adipose tissue is one of the main components of the human body. Imaging methods, including computed tomography, magnetic resonance imaging and ultrasound, have allowed the quantification of adipose tissue<sup>147</sup>. The latter represents 18-25% of the body mass in a reference man, 25-31% in a reference woman, and higher percentage in overweight people<sup>148</sup>. Adipose tissue is distributed throughout the human body (figure 8) and its distribution pattern is influenced by many factors such as sex, age, genotype, diet, physical activity level, hormones and drugs<sup>149</sup>.



**Figure 8. Distribution of adipose tissue in humans (Cook and Cowan, 2009)**

Adipose tissue is found in different depots throughout the human body. White adipose tissue is located in subcutaneous regions (under the skin and abundantly in female breasts), and visceral regions (omentum, mesenteric, pericardial, peritoneal, perirenal, and intrapelvic depots). Brown adipose tissue is found in paracervical and supraclavicular regions after cold exposure. Yellow adipose tissue occupies the bone marrow cavities.

Based on its biological functions, the adipose tissue can be classified into three main types: white adipose tissue, brown adipose tissue and yellow adipose tissue.

### 3.1.1. White adipose tissue

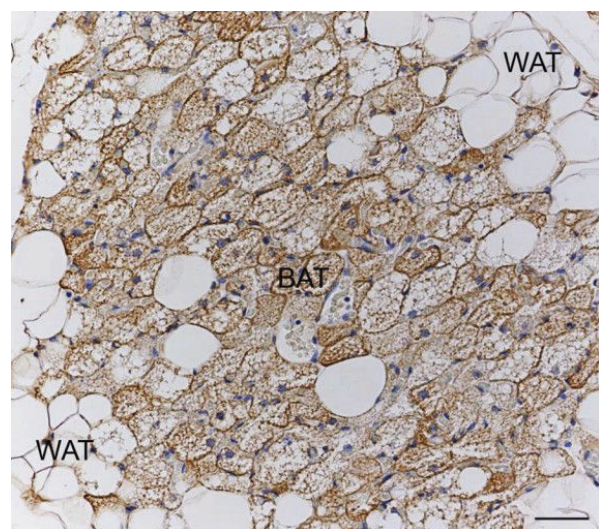
White adipose tissue (WAT) is the main type of adipose tissue found in adult humans. It is localized in subcutaneous regions (layer found under the dermis) and surrounding visceral organs (omental, mesenteric, pericardial, peritoneal and intrapelvic depots)<sup>149</sup>. WAT contains white adipocytes that are characterized by a large unilocular lipid droplet that occupies more than 90% of the cytoplasm. WAT is both an energy storage depot and a paracrine/endocrine source.

### 3.1.2. Brown adipose tissue

In contrast to WAT, brown adipose tissue (BAT) is composed of multiloculated adipocytes enriched in mitochondria that confer the brown coloring. Particularly, brown adipocytes are characterized by the expression of uncoupling protein-1 (UCP1, thermogenin) that is absent in WAT (figure 9)<sup>150</sup>. UCP1 is a trans-membrane protein found in the mitochondrial inner membrane. It allows the passage of protons from the inter-membrane space to the mitochondrial matrix, leading to the decrease in proton gradient generated from oxidative phosphorylation. Due to this uncoupling function of UCP1, BAT plays important roles in thermogenesis<sup>151</sup>. BAT is found around the major organs (heart, kidney, aorta, gonads)<sup>152</sup>. Recent studies have detailed the distribution of BAT in adults, with localization in paracervical and supraclavicular regions<sup>153–155</sup>.

**Figure 9. White and brown adipocytes from the neck of a 24-year-old woman (Cinti, 2009)**

White adipose tissue (WAT) contains large unilocular adipocytes while brown adipose tissue (BAT) contains multilobular adipocytes. BAT, but not WAT, is stained with uncoupling protein 1 (UCP1) in brown.



Thus, WAT and BAT are two morphologically and functionally different types of adipose tissue. Interestingly, based on the co-localization of WAT and BAT in many depots as well as *in vitro* data, Cinti has suggested the trans-differentiation between the two types of adipose tissue<sup>156,157</sup>. Indeed, it was observed that with cold acclimatization, there was a decrease in the WAT mass in mice in parallel with an increase in BAT. Inversely, BAT could be converted into white-like adipose tissue in warm-acclimatized animals<sup>158</sup>. In humans, BAT was found to be increased in patients with pheochromocytoma where adrenergic activity was high<sup>159</sup>, or in outdoor workers in northern Finland where they were exposed to prolonged cold<sup>160</sup>. In keeping with these data, several studies showed that ectopic expression in WAT of PRDM16 (PRD1-BF1-RIZ1 homologous domain containing 16), a brown adipose determination factor, or of COX-2 (cyclooxygenase), a downstream effector of  $\beta$ -adrenergic signaling, or of Rb (retinoblastoma) induced the development of brown-like adipocytes in WAT<sup>161-165</sup>. Recently, Wu *et al.* isolated “beige cells” from murine white adipose tissue<sup>166</sup>. These beige cells, or “brite” cells, have intermediate characteristics between white and brown adipocytes, in particular low expression of UCP1 which can be upregulated after stimulation by cyclic AMP<sup>166-168</sup>.

### 3.1.3. Yellow adipose tissue

In addition to WAT and BAT, a third type of adipose tissue, yellow adipose tissue (YAT), is found in the bone marrow. YAT accumulates in the bone marrow cavity and its adiposity varies with age<sup>169</sup>. YAT plays important roles in the bone marrow micro-environment, including bone metabolism, bone modulation, and hematopoiesis<sup>170,171</sup>. Until now, YAT has acquired little attention from clinicians and researchers, and is often considered by most investigators as WAT<sup>172,173</sup>. However, some recent studies have characterized YAT with distinct phenotype and gene expression profile from WAT and BAT<sup>174,175</sup>. Additionally, adipocytes derived from bone marrow progenitor cells display multilocular phenotypes, moderate number of mitochondria, with low expression of leptin and UCP1, and elevated inflammatory cytokine production<sup>176,177</sup>.

Overall, there are different types of adipose tissue localized in different sites of depot throughout the body; each type is characterized with distinct phenotypes and functions. Importantly, these adipose tissues display high plasticity, which implicates their susceptibility to be modified by the (micro)environment.

## **3.2. Components of the adipose tissue**

Adipose tissue is a heterogeneous tissue composed mainly of adipocytes but also preadipocytes, fibroblasts, pericytes, endothelial cells, and leukocytes<sup>152</sup> (figure 10).

### **3.2.1. Adipocytes and preadipocytes**

Adipocytes are the most abundant cells in the adipose tissue. When isolated from adipose tissue, adipocytes correspond to the floating fraction after digestion of the tissue with collagenase and centrifugation<sup>152</sup>. Morphologically, they have spherical shape with lipid droplets in the cytoplasm surrounded by a specific protein, perilipin<sup>178</sup>.

Preadipocytes are adipocyte-precursor cells with fibroblast-like morphology and high proliferative ability. They are characterized by the presence of the preadipocyte factor 1 (Pref-1)<sup>179</sup>. Pref-1 is a transmembrane protein that is cleaved to generate a soluble form which inhibits adipocyte differentiation<sup>180</sup>.

### **3.2.2. Fibroblasts**

Fibroblasts are elongated mesenchymal cells found in the connective tissue. They are characterized by the expression of fibroblast-specific protein 1 (FSP1)<sup>181</sup>, and by other non exclusive markers such as vimentin<sup>182</sup>,  $\alpha$ -smooth-muscle-actin<sup>183</sup>, desmin<sup>184</sup>, or fibroblast activation protein (FAP)<sup>185</sup>. Fibroblasts secrete components rich in collagen and fibronectin that constitute the extracellular matrix<sup>186,187</sup>. Besides, fibroblasts participate in the extracellular matrix modeling via secretion of matrix metalloproteinases<sup>188,189</sup>.

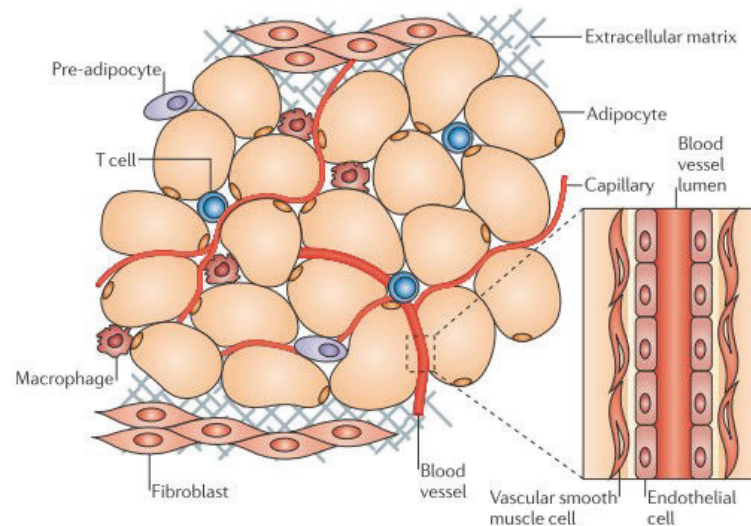
### **3.2.3. Endothelial cells and pericytes**

Endothelial cells with pericytes (vascular smooth muscle cells) form blood vessels that ensure delivery of nutrients, oxygen, growth factors, and hormones, as well as transportation of waste and secreted substances by resident cells. Besides, blood vessels allow recruitment of circulating cells from peripheral blood into the adipose tissue, including immune cells and progenitor cells. During development and tumorigenesis, blood vessels can be formed from existing vasculature in a process called angiogenesis. Angiogenesis is regulated by various factors, such as VEGF, TGF- $\beta$ , TNF- $\alpha$ , FGF-2, or angiogenin<sup>190</sup>.

### **3.2.4. Leukocytes**

Leukocytes are cells of the immune system involved in elimination of foreign material. All types of immune cells can be detected in adipose tissue-infiltrating leukocytes:

macrophages, neutrophils, B and T lymphocytes, Natural Killer (NK) cells, Natural Killer T (NKT) cells, and regulatory T cells<sup>191</sup>. Interestingly, many cancers are associated with chronic inflammation and increased infiltration of immune cells<sup>192</sup>. These cells have a double role: they participate in elimination of tumor cells, and paradoxically promote cell survival and tumorigenesis<sup>193</sup>.



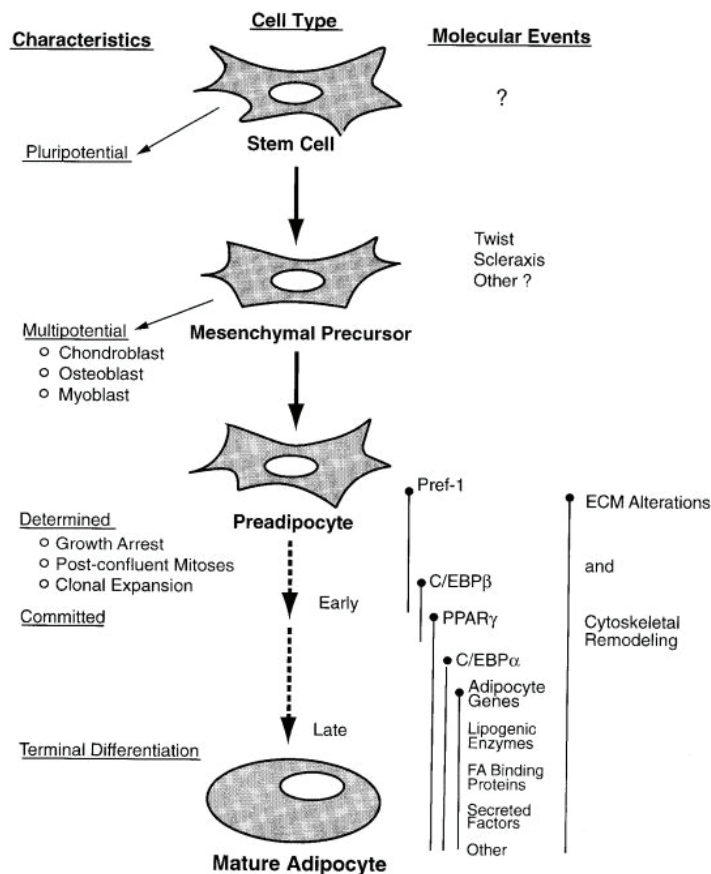
**Figure 10. Components of adipose tissue (Ouchi *et al.*, 2011)**

Adipose tissue is mainly composed of adipocytes with spherical shape and high volume. Other cell types present in the adipose tissue are preadipocytes, fibroblasts, endothelial cells, pericytes (vascular smooth muscle cells), and immune cells.

### 3.3. Adipocyte differentiation

Adipocytes derive from multipotent mesenchymal stem cells (MSCs) through a differentiation process called adipogenesis<sup>194</sup>. Although molecular markers for intermediate stages are not well characterized, for practical purposes, most workers in the field described two phase of adipogenesis. The first phase, known as determination, corresponds to the conversion of MSCs into preadipocytes that are committed to the adipocyte lineage. During the second phase, called terminal differentiation, preadipocytes acquired fully characteristics of mature adipocytes with the machinery to transport and store lipids, as well as secretion of adipocyte-specific molecules. The second phase involves four steps: growth arrest, clonal expansion, early differentiation and terminal differentiation<sup>195</sup> (figure 11). Different factors, such as insulin, glucocorticoids, triiodothyronine, retinoic acid, growth hormone and IGF-I, are required and control the adipogenesis<sup>195</sup>. These extracellular signals lead to activation of

transcriptional factors, in particularly peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) and CCAAT/enhancer binding protein  $\alpha$  (C/EBP $\alpha$ ) that play key roles in the differentiation process<sup>196</sup>. Interestingly, Seale *et al.* have demonstrated that white and brown adipocytes arise from different precursors; brown but not white adipocytes share the same precursors as muscle cells<sup>167</sup>. Transcriptional factor PRDM16 and bone morphogenetic protein 7 (BMP7) trigger the brown adipocyte lineage<sup>167,197</sup>.



**Figure 11. Overview of stages in adipocyte differentiation (Gregoire *et al.*, 1998)**

Adipocytes derive from mesenchymal precursors that are multipotent. It is proposed that during adipogenesis, these mesenchymal precursors undergo first a commitment into preadipocytes (determination). When exposed to adipogenic conditions, these preadipocytes give rise to mature adipocytes (terminal differentiation). The differentiation process is tightly controlled by peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) and CCAAT/enhancer binding protein  $\alpha$  (C/EBP $\alpha$ ).

Interestingly, it has been shown that mature adipocytes can also dedifferentiate to preadipocytes *in vitro*<sup>198,199</sup>. However, whether this dedifferentiation from adipocytes to preadipocytes occurs *in vivo* remains unknown. In humans, the number of adipocytes seems to be determined during childhood and adolescence, and remains constant throughout adulthood independently of the lean or obese status of individuals<sup>200</sup>. Nonetheless, the adipose tissue



displays a dynamic turnover with adipocyte death, preadipocyte replication, and regeneration of adipocytes from differentiated preadipocytes<sup>200–202</sup>. The balance between adipocytes and preadipocytes depends on several factors, including the stage of development<sup>203</sup>, weight changes<sup>173,204</sup>, and aging<sup>205</sup>.

### **3.4. Functions of adipocytes**

Adipocytes were considered for a long time as simple energy storage depots (figure 12). However, since the discovery of leptin in 1994, accumulating evidence has brought to light the roles of adipocytes as sources of abundant paracrine/endocrine factors.

#### **3.4.1. Lipogenesis: adipocytes store energy in triglycerides**

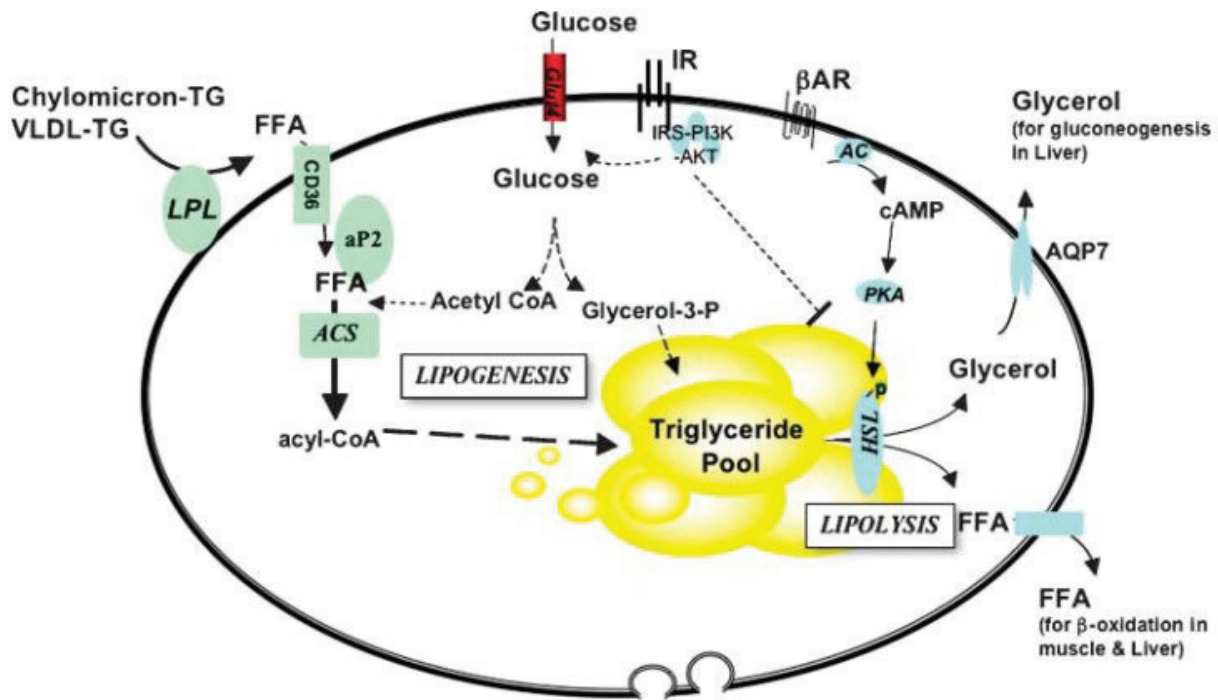
The first role of adipocytes is to store energy under the form of triglycerides. More than 95% of triglycerides in the body are found in the adipose tissue, while a small fraction is stored in other tissues such as liver and muscle<sup>206</sup>. Triglyceride synthesis requires fatty acids and glycerol<sup>207</sup>. On the one hand, fatty acids are either produced in the cells from non lipid substrates (mainly carbohydrates) through a process called *de novo* lipogenesis, or taken up from the plasma by different membrane transporters, including fatty acid translocase (FAT/CD36), fatty acid transport protein (FATP) and fatty acid binding protein (FABP). Glycerol is produced from glucose through glycolysis or from glycerogenesis, or provided by the plasma through the membrane glucose transporter 1 and 4 (Glut1 and Glut4). Triglycerides are synthesized after successive esterification steps, probably in the endoplasmic reticulum, which gives rise to lipid droplet(s)<sup>208</sup>. The whole process is tightly controlled by insulin which induces fatty acid uptake and triglyceride synthesis, in parallel with an inhibition of lipolysis.

#### **3.4.2. Lipolysis: adipocytes mobilizes energy**

A storage role of adipocytes implicates the mobilization of energy when necessary. During fasting, energy is mobilized through a process called lipolysis. Triglycerides are broken down to diacylglycerol and monoacylglycerol, with the release of free fatty acids. The latter is transported through the plasma membrane via FABP and associated with carrier proteins, for example albumin, to be mobilized in the circulation<sup>207</sup>. The key enzyme in lipolysis is the hormone-sensitive lipase (HSL) which is tightly controlled by reversible phosphorylation. Perilipin, which is also phosphorylated like HSL during lipolysis, may

facilitate the docking of the lipase to the lipid droplets. The whole process is under the control of catecholamines which stimulate lipolysis<sup>207</sup>.

Thus, lipogenesis and lipolysis are opposite processes tightly controlled by hormonal factors. It is interesting to note that lipogenic and lipolytic activities are different between adipose depots. For example, visceral adipocytes exhibited higher lipolytic activity and produced more interleukin-6 than subcutaneous adipocytes<sup>209,210</sup>.



**Figure 12. Lipid metabolism in adipocytes (Sethi and Vidal-Puig, 2007)**

Adipocytes are equipped with the machinery to store and mobilize lipids. This includes membrane transporters, lipogenic and lipolytic enzymes. AC, adenylate cyclase; ACS, acyl-CoA synthase; AR, adrenergic receptor; FFA, free fatty acid; HSL, hormone sensitive lipase; IR, insulin receptor; LPL, lipoprotein lipase; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; TG: triglyceride; VLDL, very low density lipoprotein.

### 3.4.3. Secretion of adipocytokines: adipocytes are a paracrine and endocrine source

Besides the role of energy storage and mobilization, adipocytes are a paracrine and endocrine source. Adipocytes, but also preadipocytes, secrete various factors, including growth factors, cytokines and adipokines<sup>211,212</sup> (table IV). It is worth noting that the secretion profiles are different between adipocytes and preadipocytes, as well as between specific sites of adipose depots<sup>147,213</sup>.

**Table V. Adipocytokines secreted by white adipose tissue (Fonseca-Alaniz et al., 2007)**

Substance	Biological effect
Leptin	Signals to the CNS about the body's energy stocks
Adiponectin	Increases sensitivity to insulin, is antiinflammatory and attenuates the progression of atherosclerosis
Resistin	Increases insulin resistance
TNF- $\alpha$	Lipolytic, increases energy consumption and reduces sensitivity to insulin
Interleukin-6	Proinflammatory, lipolytic, reduces sensitivity to insulin
Adipsin	Activates the alternative complement pathway
ASP	Stimulates triacylglycerol synthesis in WAT
Angiotensinogen	Precursor of angiotensin II, involved in regulating arterial blood pressure
PAI-1	Inhibits plasminogen activation, blocking fibrinolysis
Tissue factor	Initiates the coagulation cascade
VEGF	Stimulates vascular proliferation (angiogenesis) in WAT
Visfatin	Insulinomimetic predominantly produced by visceral fat
Monobutyryn*	Vasodilator and inducer of vascular neoformation
TGF- $\beta$	Regulates a series of processes in WAT, including proliferation of preadipocytes and differentiation, development and apoptosis of adipocytes
IGF-1	Stimulates proliferation and differentiation of adipocytes
HGF	Stimulates differentiation and development of adipocytes
MIF	Immunoregulator with paracrine action in WAT
LLP <sup>†</sup>	Hydrolysis stimulating enzyme in the TAG of lipoproteins (chylomicron and VLDL)
CETP <sup>†</sup>	Transfers cholesterol esters between lipoproteins
Apo-E <sup>†</sup>	Protein component of lipoproteins, especially VLDL
Prostaglandins*	Regulators of many cellular processes, active during inflammation, blood coagulation, ovulation and secretion of gastric acid
Estrogens*	Produced by the action of aromatase, this is the principal source of estrogen in men and postmenopausal women
Glucocorticoids*	Generated by the action of 11-hydroxysteroid dehydrogenase, type II, which transforms cortisone into cortisol in WAT
Apelin	Its biological actions are not very clear yet, but are related to control of the body's energy stores

ASP = acylation stimulating protein; CETP = cholesterol ester transfer protein; CNS = central nervous system; HGF = hepatocyte growth factor; IGF1 = insulin-like growth factor-1; LLP = lipoprotein lipase; MIF = macrophage migration inhibitory factor; PAI-1 = plasminogen activation inhibitor -1; TAG = triacylglycerols; TGF- $\beta$  = transforming growth factor- $\beta$ ; TNF- $\alpha$  = tumor necrosis factor- $\alpha$ ; VEGF = vascular endothelial growth factor; VLDL = very low density lipoprotein; WAT = white adipose tissue.

\* Non-protein substances.

<sup>†</sup> Proteins without hormonal action.

### 3.4.3.1. Leptin

Leptin, a 16kDa polypeptide, is the first adipocyte-secreted factor to have been described<sup>214</sup>. Its secretion was observed mainly in adipocytes, but also in other cell types including gastric mucosa cells<sup>215</sup>, mammary epithelial cells<sup>216</sup>, myocytes<sup>217</sup> and the placenta<sup>218</sup>. Preadipocytes were also shown to secrete leptin under hypoxic conditions<sup>219</sup>. Subcutaneous adipose tissue secreted higher levels of leptin than visceral adipose tissue<sup>220</sup>. Bone marrow-differentiated adipocytes also expressed and secreted high levels of leptin<sup>221</sup>.

Leptin acted as an endocrine factor on the central nervous system to regulate body weight and energy balance by inhibiting food intake and stimulating energy expenditure<sup>222</sup>.

#### 3.4.3.2. *Adiponectin*

Adiponectin, a 30 kDa polypeptide, is mainly produced by white adipocytes, and brown adipocytes at lower concentrations<sup>223</sup>. It was also reported to be expressed in other tissues but in much lower amounts<sup>224</sup>. Like leptin, adiponectin was detected in human plasma where it exerts its endocrine function. Adiponectin was shown to decrease insulin resistance by reducing triglyceride content in muscle and liver by different mechanisms: while adiponectin enhanced fatty acid transport and oxidation in muscle, it inhibited fatty acid influx into the liver<sup>225</sup>.

#### 3.4.3.3. *Estrogen*

Adipose tissue is a major source of estrogen production in men and postmenopausal women<sup>12</sup>. Aromatase, a key enzyme in the estrogen production, was shown to be highly expressed in breast preadipocytes but not adipocytes<sup>226</sup>. Estrogen exerts a paracrine effect on adjacent cells in the adipose tissue, and is also released into the circulation where it mediates systemic effects.

#### 3.4.3.4. *Tumor necrosis factor alpha*

Tumor necrosis factor alpha (TNF- $\alpha$ ) is a pro-inflammatory cytokine and its production was demonstrated in both adipocytes and preadipocytes in the adipose tissue<sup>213</sup>. Locally, TNF- $\alpha$  inhibited lipogenesis, adipogenesis, and it induced insulin resistance in adipocytes. Moreover, action of TNF- $\alpha$  on adipose tissue altered the production of many adipokines that participate in insulin sensitivity and whole body energy homeostasis<sup>227</sup>.

#### 3.4.3.5. *Interleukin 6*

Interleukin 6 (IL-6) is another inflammatory cytokine secreted by both adipocytes and preadipocytes<sup>213</sup>. IL-6 was produced by visceral adipose tissue at higher quantity than by subcutaneous adipose tissue<sup>210</sup>. It was also shown to be highly expressed in bone marrow-derived mesenchymal stem cells and inhibited adipogenesis<sup>228</sup>. Contrary to TNF- $\alpha$ , IL-6 circulated at high levels in the bloodstream and one third of circulating IL-6 was estimated to originate from adipose tissue<sup>229</sup>. IL-6 played important roles in the induction of insulin resistance in humans<sup>230</sup>.

#### 3.4.3.6. *Transforming growth factor beta*

Contrary to TNF- $\alpha$  and IL-6, transforming growth factor beta (TGF- $\beta$ ) is an anti-inflammatory cytokine. Its production was observed in both preadipocytes and adipocytes<sup>231</sup>. TGF- $\beta$  plays important roles in both tumor development and regulation of the immune system<sup>232,233</sup>.

#### 3.4.3.7. *Other factors*

Adipose tissue was shown to secrete various other factors, such as hepatocyte growth factor (HGF), insulin-like growth factor I (IGF-I), prostaglandin E2 (PGE2), resistin, adipon, chemerin, visfatin, omentin, vaspin, angiopoietin, angiotensin, plasminogen activator inhibitor 1 (PAI-1), macrophage and monocyte chemoattractant protein 1 (MCP-1), retinol binding protein 4 (RBP-4), interleukin 18 (IL-18), interferon-gamma inducible protein 10 (IP-10)<sup>211,234</sup>. Presently, more than 50 adipocytokines have been described, and this number is still increasing. These factors may act locally in the adipose tissue or enter the circulation where they mediate systemic effects. Altogether, these data demonstrate the role of adipose tissue as a paracrine/endocrine source. This may impact on different processes, notably in tumorigenesis and immunosurveillance as detailed in following parts.

#### **3.4.4. Mammary adipose tissue: participation in breast development**

Anatomic quantification from mastectomy specimens and by ultrasound showed that adipose tissue occupied up to 56% of breast volume in non-lactating breast<sup>235</sup>, while this volume ranged around 35% in lactating breasts<sup>236</sup>. Although mammary adipose tissue is considered by most investigators as a portion of subcutaneous adipose compartment<sup>149</sup>, it is distinct from other subcutaneous regions in several aspects. Mammary adipose tissue, also called mammary fat pad, continually changes in structure throughout the lifetime of reproductively active females and permanently interacts with epithelial cells<sup>237</sup>. During breast development, epithelial cells invade the mammary fat pad, forming branching ducts and terminal end buds. Thus these glandular structures are totally embedded in the mammary fat pad, which is composed primarily of adipocytes in rodents and fibroblast-enriched stroma in humans<sup>238</sup>. During pregnancy and lactation, terminal mammary epithelium expands into secretory, milk-producing alveoli, in parallel with a reduction of mammary fat pad volume. After the breastfeeding period, the gland volume is reduced and the fat pad remodels back to its previous state. Thus, breast development and lactation involve different processes, including proliferation of epithelial cells, differentiation and dedifferentiation of adipocytes,

extracellular matrix remodeling, angiogenesis, and most importantly bidirectional interactions between stromal cells and epithelial cells. Curiously, these processes are similarly observed during breast cancer tumorigenesis, which strongly suggests the direct implication of mammary adipose tissue in breast cancer<sup>203</sup>.

### 3.5. Adipose tissue and obesity

Obesity is a pathological condition accompanied by an excessive fat deposition and is often estimated through the measure of body mass index (BMI) (weight in kg / height in m<sup>2</sup>). The WHO classification of BMI is described in the table below.

**Table VI. WHO classification of overweight and obesity (Calle and Kaaks, 2004)**

BMI (kg/m <sup>2</sup> )	WHO classification	Popular description
< 18.5	Underweight	Thin
18.5–24.9	Normal range	'Healthy', 'normal' or 'acceptable' weight
25.0–29.9	Grade 1 overweight	Overweight
30.0–39.9	Grade 2 overweight	Obesity
≥ 40.0	Grade 3 overweight	Morbid obesity

According to WHO data in 2008, obesity has become a global epidemic with more than 1.4 billion overweight adults, including 500 million obese (<http://www.who.int/mediacentre/factsheets/fs311/en/index.html>). The prevalence of adult obesity is high in Eastern Europe, the eastern Mediterranean, North America (especially the United States), Central and South America, and some countries of Western Europe (Finland, Germany, Spain and the United Kingdom). The trend of obesity is increasing worldwide, and obesity has been recently declared by the American Medical Association as a disease<sup>239</sup>.

Obesity is due to an imbalance between dietary energy intake and energy output<sup>240</sup>. The excessive energy intake in obese people is stored mainly in adipose tissue that increases in mass. The expansion of adipose tissue is essentially due to an increase in adipocyte volume (hypertrophy) and to a lesser extent to an increase in adipocyte cell number (hyperplasia)<sup>196,241</sup>. Importantly, obesity is not only related to an increase of adipose quantity, but also an alteration of adipose quality. Obesity has been characterized as a chronic inflammation state, with remodeling of local adipose tissue and dysregulation of secreted adipocytokines<sup>241,242</sup>. Moreover, obesity has been shown to be associated with increased risk for insulin resistance and type 2 diabetes, as well as for cardiovascular disease<sup>243–245</sup>.

Furthermore, several studies have pointed out the implication of obesity in the occurrence of different types of cancer<sup>246,247</sup>.

### **3.6. Models to study adipocytes**

A variety of models have been used so far to study the differentiation and functions of adipocytes<sup>248</sup>. These include preadipocyte cell lines, multipotent stem-cell lines, isolated adipocyte-precursors, and freshly isolated adipocytes.

#### **3.6.1. Preadipocyte cell lines**

Preadipocyte cell lines are already committed to the adipocyte lineage and can only differentiate into adipocytes when exposed to appropriate hormonal and pharmacological agents. The murine preadipocyte cell lines widely used are 3T3-L1 and 3T3-F442A. Both cell lines derived from clones of Swiss 3T3 mouse fibroblasts that can convert to adipocytes in culture<sup>249–251</sup>. However, 3T3-F442A is considered to be more committed to adipocyte differentiation than 3T3-L1. When induced by hormonal agents (insulin), these preadipocytes rapidly expressed PPAR $\gamma$  and C/EBP $\alpha$  that drove the differentiation process. Differentiated adipocytes accumulated triglycerides in multiple lipid droplets that could be stained with Oil Red O. They also developed responses to lipolytic factors. Moreover, subcutaneous injection of 3T3-F442 preadipocytes, but not 3T3-L1 cells, into athymic mice at anatomical site devoid of adipose tissue gave rise to fat pads that were not histologically and biologically different from normal fat pads<sup>252,253</sup>. In particular, *in vivo* differentiated 3T3-F442 adipocytes expressed leptin, which could not be observed in cells differentiated *in vitro*<sup>253</sup>.

#### **3.6.2. Multipotent stem-cell lines**

Multipotent stem-cell lines are not committed, and under specific conditions, can differentiate into other lineages than adipocytes, such as myoblasts, osteoblasts and chondrocytes. A human multipotent adipose-derived stem cell population, named hMADS, was isolated from the white adipose tissue of young donors and established in culture<sup>254</sup>. hMADS cells displayed normal karyotype, had active telomerase, and maintained their characteristics with long-term passaging. They were characterized by flow cytometry as CD44+, CD49b+, CD105+, CD90+, CD13+, Stro-1-, CD34-, CD15-, CD117-, Fl-1-, glyA-, CD133-, HLA-DR-, and HLA-I low<sup>255</sup>. hMADS cells exhibited gene expression patterns similar to that of primary preadipocytes. In serum-free chemically defined conditions, they

differentiated into adipocytes with adipose specific molecular markers and lipolytic response. Importantly, differentiated hMADS were shown to secrete leptin and adiponectin, which were not observed with *in vitro* differentiated 3T3-L1 and 3T3-F442 cells. It is noteworthy that the capacities to differentiate into adipocytes of all cell lines declined with the number of passages.

### 3.6.3. Isolated adipocyte-progenitors

Adipocyte-progenitors, or adipose tissue-derived stem cells (ASCs), can be isolated from bone marrow or peripheral adipose tissue. They were found in the stromal vasculature fraction (SVF) after digestion of adipose tissue with collagenase followed by centrifugation. When isolated from bone marrow, adipocyte-precursors are often referred to as bone marrow mesenchymal stem cells (BM-MSCs) or mesenchymal stem cells (MSCs). When isolated from peripheral adipose tissue, they are called by different names: processed lipoaspirate cells (PLAs), adipose tissue-derived stromal cells (ATSCs), adipose-derived stromal/stem cells (ASCs), adipose-derived adult stromal cells (ADASs), adipose-derived adult stem cells (ADASs), adipose tissue-derived cells (ATDCs), or simply MSCs<sup>256</sup>. Peripheral and bone marrow-derived ASCs can all differentiate into adipocytes under adipogenic conditions, but also into myocytes, osteoblasts and chondrocytes<sup>256</sup>. Furthermore, they share many molecular markers such as CD31- CD45- CD73+ CD90+ CD105+<sup>256,257</sup>. In contrast, some molecular markers allow distinction between BM-MSC and peripheral ASCs, such as CD104 and CD106 on BM-MSCs, CD34 and CD49d on peripheral ASCs<sup>256,258</sup>. Moreover, microarray analysis has revealed distinct gene expression profiles between these populations<sup>259,260</sup>. It is interesting to note that when *in vitro* studies on cell lines distinguished committed preadipocytes from multipotent stem cells, the difference between the two cell types was much less marked in isolated adipocyte-progenitors<sup>256</sup>. Since no direct conversion from multipotent mesenchymal stem cells into committed preadipocytes has been observed *in vivo*, it is possible that preadipocytes are the same population as ASCs, and that preadipocytes are only observed in experimental contexts.

### 3.6.4. Freshly isolated adipocytes

In parallel with adipocyte progenitors, mature adipocytes can be isolated and correspond to the floating fraction after collagenase digestion of adipose tissue. Unlike adipocytes obtained from *in vitro* differentiation of preadipocytes that are unable to acquire hypertrophy, isolated (white) adipocytes have a single lipid droplet, which may reflect better adipocyte



biology and metabolism. However, these isolated mature adipocytes were unstable in culture conditions; after 48 h of culture their survival and functions were severely altered<sup>248,261</sup>. This difficulty limited the use of isolated adipocytes in long-term experiments.

Together, these different models provide useful and complementary tools to study the biology and functions of adipocytes, both *in vitro* and *in vivo*.

## 4. Cancer cells, adipocytes and Natural Killer cells: a “ménage à trois”

Tumor cells, adipocytes, and natural killer (NK) cells are all components of the tumor microenvironment in many cancers, such as breast cancer, ovarian cancer, and lymphoma. They are permanently in interaction with each other, thus participating in a “ménage à trois”, resulting in modification of cell characteristics, and promotion or inhibition of tumor progression.

### 4.1. Cancer cells and NK cells: the bad and the killers

#### 4.1.1. Evidence of the antitumor role of NK cells

NK cells are cytotoxic lymphocytes that belong to the innate immune system. Their name as “natural killer cells” was attributed after the discovery of their capacity to kill tumor cells without immunization<sup>262–266</sup>. *In vitro* mouse and human NK cells can kill a broad range of tumor cells of haematopoietic and non-haematopoietic origins. *In vivo* evidence of the antitumor role of NK cells has been shown using NK-depleting antibodies<sup>267–269</sup> or using genetically NK-deficient mice<sup>270</sup>. Mice lacking NK cells showed impaired rejection of tumors. In humans, selective NK deficiencies are rare<sup>271</sup>. However, low NK cytotoxicity has been correlated with high incidence of cancer<sup>272</sup>, suggesting the importance of NK cells in tumor surveillance.

#### 4.1.2. Recruitment of NK cells to tumor sites

To exert their cytotoxicity, NK cells need to extravasate through blood vessels, migrate into stromal tissues and eventually penetrate the basement membrane, before getting in contact with tumor cells. NK cells were shown to eradicate large solid tumors in mice<sup>273</sup>. In humans, infiltration of NK cells has been observed in various types of solid tumors, including colon<sup>274</sup>, stomach<sup>275</sup>, lung<sup>276</sup>, and breast<sup>277</sup>. In the majority of cases, high number of tumor infiltrating NK cells is associated with good prognosis.

The mobilization of NK from the peripheral blood to tumor sites implicates chemokines and chemokine receptors. NK cells express various chemokine receptors<sup>278</sup>, among which

CXCR3 and CXCR3R1 were shown to play major roles in tumor infiltration. Over-expression of their ligands in tumor cells led to increased accumulation of NK cells in tumor sites<sup>279,280</sup>. Moreover, CXCR3<sup>-/-</sup> and CXCR3R1<sup>-/-</sup> mice display reduced infiltrating NK cells in the tumor sites<sup>280,281</sup>. Interestingly, inflammatory cytokines such as TNF- $\alpha$  and IFN- $\gamma$  can induce the expression of CXCR3 ligands in tumor cells<sup>282</sup>, enabling amplification of NK infiltration.

In humans, NK cells can be divided into two subtypes, based on the expression levels of CD56: CD56<sup>dim</sup> and CD56<sup>bright</sup><sup>283</sup>. The CD56 antigen is a neural cell adhesion molecule and its function in NK cells remains unknown<sup>284</sup>. CD56<sup>dim</sup> NK cells constitute approximately 90% of the NK population in the peripheral blood. They express high levels of CD16, mediate strong cytolytic activity and low cytokine production. By contrast, CD56<sup>bright</sup> NK cells, less abundant in the blood, express low levels of CD16 and mediate low cytolytic activity but high cytokine production<sup>285</sup>. In the tumor sites, the majority of tumor-infiltrating NK cells belongs to the CD56<sup>dim</sup>/CD16<sup>high</sup> subtype<sup>286,287</sup>. However, the ratio CD56<sup>dim</sup> : CD56<sup>bright</sup> was found to be lower in tumor sites than in the peripheral blood<sup>277</sup>. This can be explained by a preferential recruitment of CD56<sup>bright</sup> NK cells to tumor sites, since CD56<sup>dim</sup> cells express less frequently CXCR3 than CD56<sup>bright</sup> cells<sup>288</sup>. Another possible explanation is the alteration of NK phenotypes by the tumor microenvironment<sup>289</sup>.

Tumor infiltrating NK cells are often located in the surrounding stroma and only few NK cells are in direct contact with tumor cells<sup>290,291</sup>. Moreover, the basement membrane with matrix proteins such as laminin and collagen type IV constitutes a barrier for NK cells<sup>290</sup>. However, NK cells were shown to express metalloproteinases which facilitate their migration and disruption of the basement membrane<sup>292,293</sup>. Besides, invasive tumors with disrupted basement membrane can come into direct interaction with NK cells in the stroma at the invasive front. The contact between NK cells and target cells is estimated to be short, between 5-10 min, leading to the lysis of MHC-mismatched target cells in lymph nodes<sup>294</sup> or of NKG2D ligand-overexpressing tumors in subcutaneous sites<sup>295</sup>. Tumor-targeting antibody was shown to increase number of infiltrating NK cells as well as contact stability and tumor lysis<sup>296,297</sup>.

#### **4.1.3. Recognition of tumor cells**

NK cells possess a number of receptors that allow the recognition of ligands expressed on potential target cells<sup>298</sup> (figure 13). These receptors can be classified as either inhibitors or activators, and the integration of signals from both types of receptors determines whether NK

cells kill or spare target cells. Tumor cells display different patterns of ligand expression from those of normal cells, allowing their specific recognition by NK cells.

#### *4.1.3.1. The missing-self*

NK cells express inhibitory receptors for the major histocompatibility complex (MHC) class I molecules<sup>298,299</sup>. These inhibitory receptors, including LY49 in mice, the killer cell immunoglobulin-like receptors (KIR) in humans and CD94/NKG2A in both species, contain the ITIMs in their cytoplasmic domain. Engagement of the inhibitory receptors counteracts NK activation and cytolytic function. Non-tumor cells with normal levels of MHC class I – self peptides activate NK inhibitory receptors, thus prevent lysis by NK cells. In contrast, certain tumor cells have been shown to lose or downregulate MHC class I antigens<sup>300,301</sup>, thus becoming susceptible to NK lysis<sup>302,303</sup>. It has been suggested that T cell immunoselection of tumor variants can be the cause of the loss of MHC class I in tumor cells<sup>304,305</sup>.

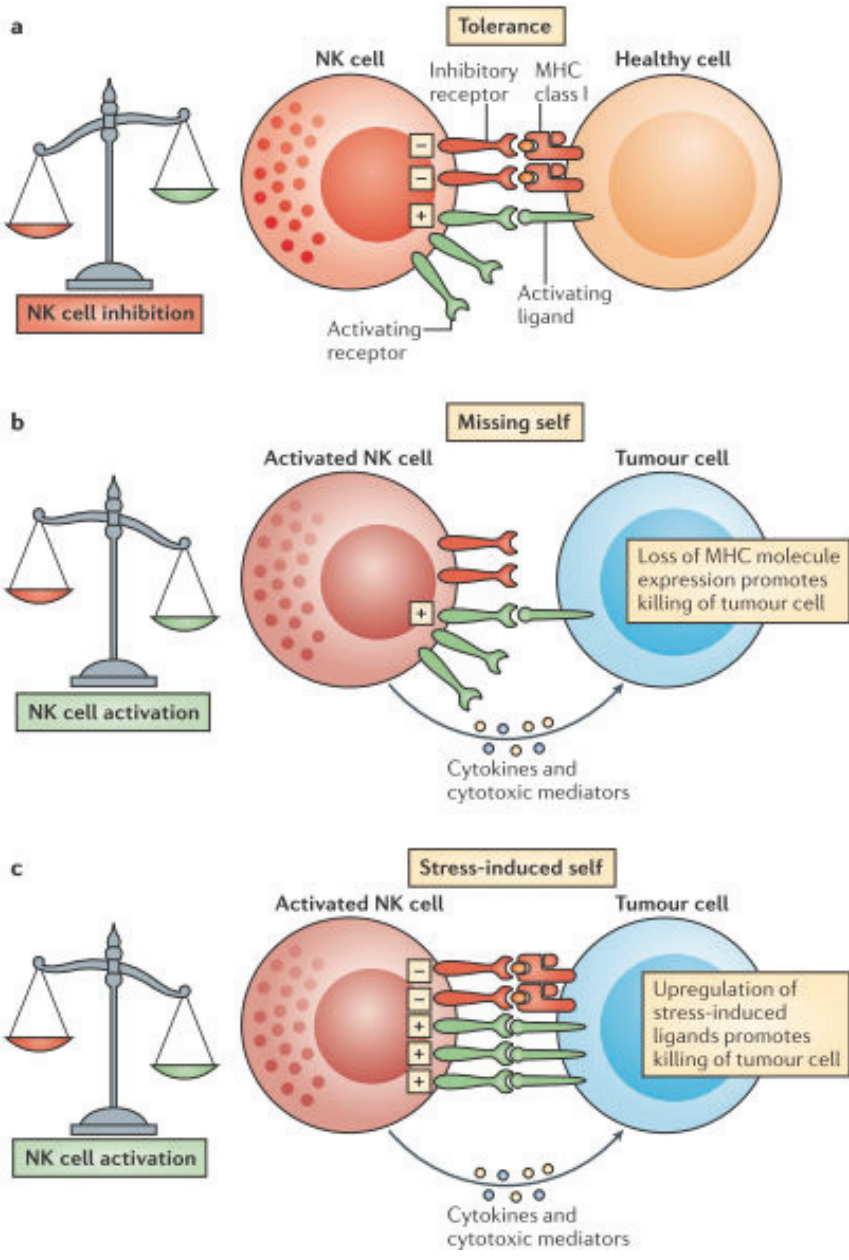
#### *4.1.3.2. Danger/stress signal*

In addition to inhibitory receptors, NK cells express several activating receptors<sup>306</sup>. These receptors can detect ligands that are barely present in normal cells, but whose expression increases in transformed cells in stress conditions. NKG2D is the most documented activating receptor. It recognizes a broad range of stress-induced ligands<sup>307</sup>, such as MICA<sup>308</sup>, MICB<sup>308</sup> and ULBP<sup>309</sup> in humans, RAE1<sup>310</sup>, H60<sup>310</sup> and MULT1<sup>311</sup> in mice. Expression of these ligands sensitizes target cells to NK lysis. Besides NKG2D, NK cells express natural cytotoxicity receptors (NCRs) that include NKp46<sup>312</sup>, NKp44<sup>313</sup>, and NKp30<sup>314</sup>. These receptors are associated with adaptor proteins, such as DAP12, FcR $\gamma$  and CD3 $\zeta$  containing ITAMs. Masking of one or many NCRs by antibodies was shown to reduce NK cytotoxicity against various tumors. Until now, ligands of NKp30, B7-H6<sup>315</sup> and BAG6/BAT3<sup>316</sup> have been identified in tumor cells. These ligands were not detected in normal cells but up-regulated in response to stress, illustrating the stress-induced signal for NK cell lysis.

#### *4.1.3.3. Antibody-dependent cellular cytotoxicity*

Besides direct recognition, NK cells can bind to and lyse tumor cells through the involvement of antibodies that recognize tumor-overexpressed antigens. NK cells express high levels of activating receptor Fc $\gamma$ RIII (CD16) but no inhibitory receptor Fc $\gamma$ RIIB<sup>78,317</sup>. Binding of CD16 to antibody-coated tumor cells leads to NK cells activation and tumor lysis.

As mentioned above, this ADCC mechanism is the main mode of action of many monoclonal antibodies used in therapy, particularly trastuzumab.



**Figure 13. Recognition of tumor cells by NK cells (Vivier *et al.*, 2012)**

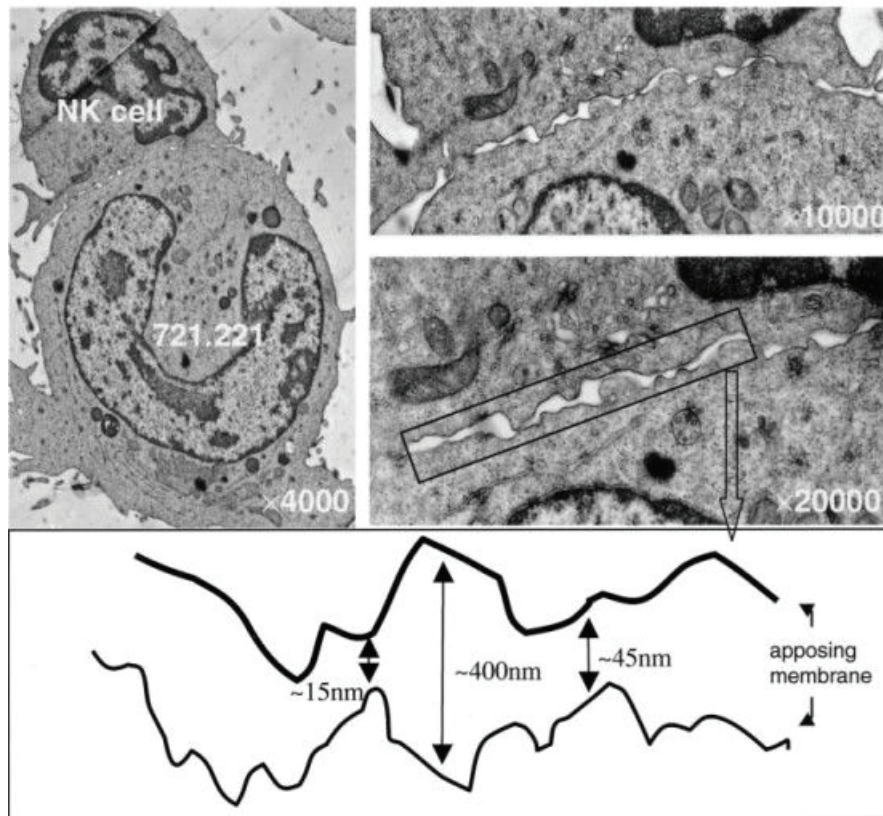
a) NK cells are tolerant towards healthy cells, as the result of the balance between activating and inhibitory signals. b) Missing self: tumor cells lose expression of MHC class I molecules, resulting in decrease in inhibitory signals and NK cell activation. c) Stress-induced self: tumor cells overexpress activating ligands, which increases activating signals and NK cell activation. The ADCC mechanism was already shown in figure 7.

#### 4.1.4. Formation of an immunological synapse

After recognition, NK cells bind to the target cells through the action of adhesion molecules, in particular CD2, CD11a (LFA-1) and CD11b (Mac-1). The importance of these adhesion molecules in NK function has been shown. Absence of LFA-1 expression or blocking of LFA-1 or CD2 by monoclonal antibodies inhibited NK cytotoxicity<sup>318,319</sup>. Binding of NK cells to target cells leads to formation of NK cell immunological synapses (NKIS) (figure 14) whose patterns can be either activating or inhibiting, depending on cytolytic or non-cytolytic interactions. In the activating NKIS, adhesion molecules and NK receptors form a supramolecular activation cluster (SMAC), in which adhesion molecules are organized in the peripheral SMAC (pSMAC) while perforin in the central SMAC (cSMAC)<sup>320,321</sup>. By contrast, in the supramolecular inhibition cluster (SMIC) of the non-cytolytic NKIS, CD11a and its ligand ICAM-1 cluster at the center of the synapse, while KIR/HLA-C complex forms a ring<sup>322</sup>.

The formation of activating NKIS between NK cells and the target cells leads to re-arrangement of the actin cytoskeleton in NK cells. Actin and the actin-binding protein talin accumulate in the pSMAC and interact with LFA-1, stabilizing the cell-cell contact<sup>323</sup>. Subsequent to actin remodeling, the microtubule organizing center (MTOC) is reorientated to the contact sites, allowing the polarization of lytic granules to the proximity of the synapses. Absence of MTOC reorientation prevents polarization of lytic granules and defect in cytotoxicity<sup>324</sup>. In contrast, in non-cytolytic NKIS, no large-scale remodeling of actin or MTOC polarization was observed<sup>323</sup>.

Lytic granules pass through a hypodense of actin region at the center of the synapse before fusing with plasma membranes, leading to the release of granule contents<sup>325</sup>. During the formation of NKIS, the distances between the membranes of NK cells and target cells are small, between 15-400 nm<sup>326</sup>. This small space allows a concentration of lytic factors and their specificity towards target cells. NK cells do not release all their lytic granules at once, only a subset of granules are released upon activation, which allows serial killing of multiple target cells<sup>327</sup>.



**Figure 14. Visualization of NK immunological synapse (NKIS) (Vyas *et al.*, 2002)**

Transmission electron microscopy was used to visualize the immunological synapse between NK cells and target sensitive 721.221 cells. The interaction surface is characterized by multiple membrane invaginations and projections. Distances between the two apposing membranes in the IS are short, which allows efficiency and specificity of cytotoxic factors released by NK cells.

#### **4.1.5. Mechanisms of killing**

Upon recognition of target cells and formation of immunological synapse, NK cells trigger target-killing through various mechanisms: release of perforin and granzymes, expression of tumor necrosis factor (TNF) family factors, and secretion of cytokines (figure 15).

##### *4.1.5.1. Exocytosis of granules containing perforin and granzymes*

NK cells are characterized by the constitutive presence of granules in their cytoplasm<sup>328</sup>. These granules contain essentially perforin and granzymes, which constitute the main effectors of tumor killing by NK cells<sup>329</sup>. Perforin is a membrane-disrupting protein, which allows formation of pores and the delivery of granzymes into the cytosol of target cells<sup>330</sup>. Granzymes belong to the serine protease family. Eleven granzymes have been described, including ten in mice (granzymes A-K, M and N) and five in humans (granzymes

A, B, H, K and M)<sup>331</sup>. They induce target cell necrosis or apoptosis through cleavage of multiples substrates, including caspases and BH3 interacting-domain death agonist (Bid), leading to DNA fragmentation, mitochondria depolarization and cytochrome c release<sup>332</sup>.

The importance of perforin in the anti-tumor activity has been shown *in vivo* by genetic knock-down of the perforin gene. Perforin-deficient mice failed to eliminate various types of tumors<sup>333,334</sup>. Deficiency of perforin, but not of Fas ligand, was shown to abrogate IL-12-induced NK cytotoxicity and anti-metastasis effects in mice<sup>335</sup>. In humans, mutations of perforin were found in patients with familial hemophagocytic lymphohistiocytosis (FHL) and were associated with lymphocyte-defective cytotoxicity<sup>336</sup>. Besides, patients with temperature-sensitive mutations in their perforin (*Prfl*) genes displayed loss of cytotoxicity and a predisposition to hematological malignancy<sup>337</sup>. By contrast, the roles of granzymes seem to be unclear in tumor rejection. Pardo *et al.* showed that mice deficient for either granzyme A or B were more susceptible to tumor growth<sup>338</sup>. Using the same models, Davis and Smyth showed that granzyme A and B-deficient mice retain potency to mediate anti-tumor activity similar to that of wild-type mice<sup>339,340</sup>, implying the involvement of other factors in anti-tumor activity.

Granulysin is another lytic molecule expressed in NK cells<sup>341,342</sup>. Granulysin is a member of the saposin-like protein family<sup>343</sup>. It is localized in the cytolytic granules and released upon activation<sup>344</sup>. Purified granulysin was shown to induce apoptosis of target tumor cells, through release of cathepsin B from lysosomes, cleavage of Bid, disruption of mitochondrial membrane and release of cytochrome c into the cytosol<sup>345-347</sup>. Expression of granulysin in mouse adenocarcinoma cells led to apoptosis<sup>348</sup>. Kishi *et al.* correlated the decreased levels of granulysin expression with cancer progression, while perforin expression levels did not change<sup>349</sup>. *In vivo* evidence of the role of granulysin is limited, because of the absence of granulysin homologue in mice. However, using transgenic mice expressing granulysin, Huang *et al.* have shown a protective effect of granulysin against C6VL tumors, but not RMA-S tumor challenge<sup>350</sup>.

Recently, a new lytic factor named NK lytic-associated molecule (NKLAM) has been identified in humans and mice<sup>351,352</sup>. NKLAM is an E3 ubiquitin ligase and colocalizes with granzyme B to the cytoplasmic granules of NK cells<sup>351</sup>. Its role in anti-tumor activity was shown both *in vitro* and *in vivo*. NKLAM antisense oligonucleotide treatment decreased NK



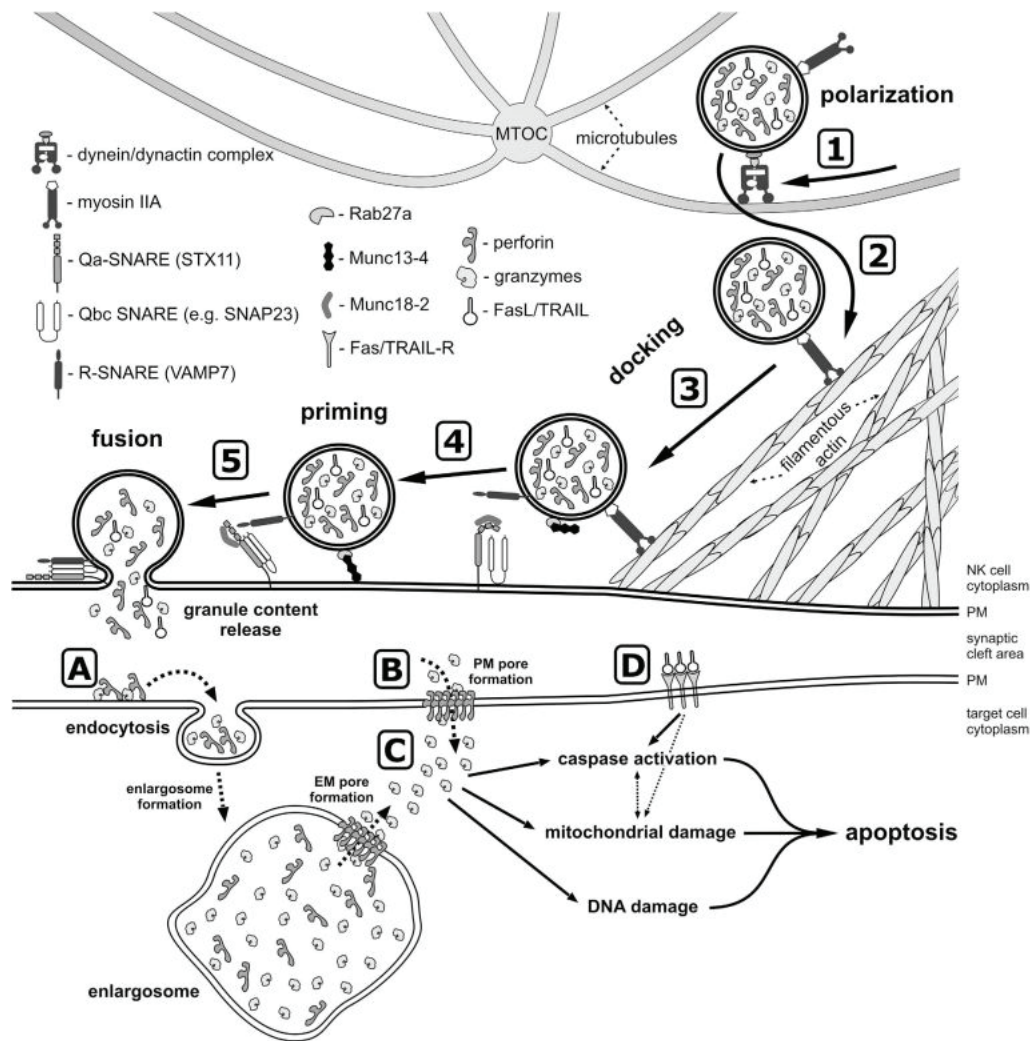
cytolytic activity<sup>351</sup>. NK/LAM-deficient mice displayed less NK cytotoxicity and weaker control of tumor dissemination<sup>353,354</sup>.

#### 4.1.5.2. Death-receptor-mediated apoptosis

Besides lytic granules, NK cells constitutively express a number of the TNF family ligands for death receptors on tumor cells. These ligands include Fas ligand (FasL), TNF and TNF-related apoptosis inducing ligand (TRAIL)<sup>355</sup>. They are expressed as membrane-bound proteins, but their extracellular regions can be also secreted after cleavage by matrix metalloproteinases. It was also shown that Fas ligand could be stored in the same lytic granules as perforin and granzymes, and be delivered to the cell membrane by exocytosis of the lytic granules<sup>356,357</sup>. Binding of the TNF family ligands on their receptors expressed on tumor cells leads to trimerization of the receptors, recruitment of an adaptor protein called Fas associated death domain (FADD), formation of the death inducing signaling complex (DISC), activation of caspases which results in apoptosis<sup>358</sup>. NK cells producing FasL can kill Fas-expressing tumor cells independently of perforin<sup>359</sup>, while they fail to kill tumor cells from Fas-mutated *lpr* mice<sup>360</sup>. Interestingly, NK cells can mediate upregulation of Fas in tumors that normally do not express Fas, and thereafter kill the tumor in a Fas-dependent manner<sup>361</sup>. In parallel, TRAIL can participate in lysis of TRAIL-sensitive tumor cells along with perforin and FasL<sup>362,363</sup>. Mice deficient for TRAIL showed increased susceptibility to tumor initiation and metastasis<sup>364,365</sup>.

#### 4.1.5.3. Secretion of cytokines

After activation, NK cells secrete various cytokines such as interferon  $\gamma$  (IFN- $\gamma$ ), tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 3 (IL-3) and others<sup>366</sup>. IFN- $\gamma$  is the most documented and plays an important role in anti-tumor activity. IFN- $\gamma$  was demonstrated to inhibit tumor growth *in vitro*<sup>367</sup>. IFN- $\gamma$ -insensitive mice showed increased susceptibility to spontaneous and chemically induced tumors<sup>368</sup>. IFN- $\gamma$  induces TRAIL expression<sup>363</sup> and is essential in the anti-metastatic effect of NK cells, independently of perforin<sup>369</sup>. Additionally, IFN- $\gamma$  can activate antigen presenting cells (APC) by upregulation of MHC class I and II<sup>370</sup>, and by inducing IL-12 production in dendritic cells<sup>371</sup>. Subsequent priming and activation of cytotoxic T lymphocytes participate in the anti-tumor response together with NK cells.



**Figure 15. Tumor cell killing by cytotoxic granules and death receptors (Krzewski and Coligan, 2012)**

Upon activation, cytolytic granules are polarized towards the microtubule organizing center (MOTC) at the proximity of the immunological synapse via the action of dynein/dynactin complex (1). They are next transported along filamentous actin network by myosin to the close proximity of the plasma membrane (PM) (2). Their docking (3) and priming (4) processes are ensured by Rab27a, Munc13-4 and Munc18-2 proteins. By means of SNARE proteins, these lytic granules are fused with the plasma membrane and their contents, including granzymes, perforin, and FasL are released into the synapse (5). Perforin and granzymes enter target cells via endocytosis (A) or formation of pores in the plasma membrane (B). After being released in the target cell cytosol, granzymes cleave various substrates, leading to caspase activation, mitochondrial or DNA damage, which results in cell apoptosis. In parallel, FasL and TRAIL bind to their receptors on the target cell surface (D) and initiate apoptosis. The large number of pores formed by perforin in the plasma membrane may lead to disruption of plasma membrane integrity and subsequently cell necrosis.

#### 4.1.6. Resistance of tumor cells

Despite immunosurveillance, tumor cells can evade NK lysis by developing various strategies: escaping recognition by NK cells, increasing resistance to lysis/apoptosis, altering NK functions and remodeling the tumor microenvironment.

##### 4.1.6.1. Escaping recognition by NK cells

While high or low expression levels of MHC class I on tumor cells are both subjects for recognition by either cytotoxic T cells or NK cells, downregulation of NKG2D ligands may constitute a means for tumor evasion. In human glioma cells, the NKG2D ligand MICA was shown to be upregulated in early grades of tumorigenesis, but decreased in higher grades of malignancy<sup>372</sup>. This downregulation of MICA was mediated by TGF- $\beta$ , and silencing TGF- $\beta$  by siRNA restored MICA levels as well as sensitivity to NK lysis<sup>372,373</sup>. Besides, cellular microRNAs which are overexpressed in various tumors could also downregulate MICA and MICB, leading to tumor evasion<sup>374</sup>.

Ligand shedding can also be used by tumor cells to avoid NK recognition. Soluble MICA (sMICA) has thus been detected in sera of patients with gastrointestinal malignancies<sup>375</sup>, colon adenocarcinoma<sup>376</sup>, neuroblastoma<sup>377</sup>, or prostate cancer<sup>378</sup>. Moreover, elevated levels of sMICA have been correlated with cancer stage and metastasis<sup>379</sup>. sMICA is released from tumor cells after proteolytic cleavage by metalloproteinases. On the one hand, release of sMICA leads to decreased expression levels of this ligand on the tumor surface. On the other hand, sMICA serves as decoy to subvert NK targeting. Inhibition of metalloproteinases has been shown to block MIC shedding and to increase MIC expression on the cell surface<sup>375</sup>, which restored sensitivity to NK cytotoxicity<sup>372</sup>.

Recently, a novel strategy for NK cell evasion has been observed in bladder cancer cells expressing the carbohydrate group core2 O-glycans on MICA<sup>380</sup>. Upregulation of the core2 O-glycan branching enzyme C2GNT in bladder cancer correlated with progression of the disease. Core2 O-glycans recruits galectin-3 to the NKG2D-binding site on MICA, which reduces affinity of MICA to NKG2D, impairs NK cell recognition and killing.

In anti-cancer targeted therapy using monoclonal antibodies, evasion may occur despite the fact that the antigens chosen to be targeted are supposed to be stably expressed in cancer cells. In B cell lymphoma, downregulation of CD20 after rituximab treatment was observed in some patients<sup>381</sup> and in resistant cell lines<sup>382</sup>. In breast cancer, shedding of the antigen HER2

neutralized the effect of anti-HER2 antibody and correlated with disease progression<sup>143</sup>. In addition, the binding epitope of HER2 for trastuzumab could be masked by the expression of glycoprotein mucin-4 (MUC-4) in breast cancer cells<sup>144,145</sup>.

#### 4.1.6.2. Increasing resistance to lysis/apoptosis

Since perforin is essential in NK cytotoxicity, failure of its delivery into tumor cells can confer resistance to lysis. Lehmann *et al.* correlated the absence of perforin binding to the surface of human leukemia cells and their resistance to NK lysis<sup>383</sup>. Besides, perforin was suggested to bind to the platelet-activating factor (PAF) secreted by NK cells upon activation<sup>384</sup>. The PAF/perforin complex interacts with the PAF receptor expressed on sensitive cells leading to lysis. Absence of PAF receptor expression on tumor cells could induce resistance to the perforin lytic pathway<sup>384</sup>.

Besides inhibition of perforin, tumor cells can resist to NK cytotoxicity by inhibiting granzymes, through the action of the proteinase inhibitor PI-9. PI-9 belongs to the family of serine proteinase inhibitors (serpins) that counts over 1000 members<sup>385</sup>. PI-9 specifically binds granzyme B and inhibits granzyme B-mediated apoptosis<sup>386</sup>. PI-9 has been found to be expressed in many cancers, such as lymphoma<sup>387</sup>, melanoma<sup>388</sup>, lung<sup>389</sup> and breast<sup>390</sup>. The association between PI-9 levels and unfavorable clinical outcome<sup>388</sup> suggests the involvement of PI-9 in tumor resistance and immune evasion. Another serpin, SerpinA3, has been described to bind to purified granzyme B and inhibited its enzymatic activity<sup>391</sup>. SerpinA3 is secreted by the immunoprotective Sertoli cells<sup>391</sup> and is also expressed in human adenocarcinomas<sup>392</sup>. However, the role of SerpinA3 in cancer resistance remains to be determined.

Alternatively, cancer cells can become resistant to death receptor-induced apoptosis by expressing mutated forms of these receptors, such as inactive mutations of TRAIL receptors in metastatic breast cancer<sup>393</sup> or deletion of Fas death domain in diffuse large B-cell lymphoma<sup>394</sup>.

#### 4.1.6.3. Altering NK functions

NK cell functions have been shown to be impaired in many tumors, such as prostate<sup>378</sup>, breast<sup>286</sup>, stomach<sup>395</sup>, colon<sup>376</sup>, leukemia<sup>396</sup> and glioma<sup>373</sup>. Alteration of NK cell functions, in particular NK cytotoxicity, has been correlated with disease progression. Interestingly, alteration of NK cytotoxicity is more prominent in tumor sites than in the peripheral blood.

The activating receptor NKG2D is a well documented target for impairment. NKG2D was shown to be downregulated after sustained exposure to NKG2D ligands present on tumor cells<sup>397,398</sup> or to soluble ligand (sMIC) shed from tumor cells<sup>376,378</sup>. Tumor-derived exosomes expressing NKG2D ligands and TGF- $\beta$ 1 could also contribute to NKG2D downregulation<sup>399,400</sup>. Although these effects are reversible, close contact of NK cells with tumor cells and high serum levels of sMIC may lead to a permanent dysfunction of NK cells in cancer patients.

Besides NKG2D, NCRs have been shown to be downregulated in acute myeloid leukemia<sup>396</sup> and chronic lymphocytic leukemia (CLL)<sup>401</sup>. Recently, Reiners *et al.* demonstrated different roles of the NKp30 ligand BAG6/BAT3 in evasion of CLL cells from NK cell anti-tumor activity<sup>402</sup>. While exosomal BAG6 secreted by tumor cells triggered NK cytotoxicity, soluble BAG6 inhibited NK cytotoxicity. Elevated concentrations of soluble BAG6 are present in patients with advanced disease, suggesting a mechanism for immune escape. Additionally, Rosental *et al.* showed an impairment of NK cell function by the proliferating cell nuclear antigen (PCNA), a ligand for NKp44<sup>403</sup>. PCNA is overexpressed in cancer cells and associated with malignancy. Unlike other NCR ligands, PCNA binds to NKp44 and activates the NKp44 cytoplasmic tail containing ITIM, leading to the inhibition of NK cell function<sup>403</sup>.

In addition, CD16, the receptor involved in ADCC, is also subject to down-regulation during cancer progression<sup>277</sup>. CD16 expression levels were reported to be lower in tumor-infiltrating NK cells in comparison with peripheral NK cells<sup>277</sup>. Recent studies suggested a down-modulation of CD16 in activated NK cells after cleavage of CD16 by metalloproteinases<sup>404,405</sup>.

Furthermore, tumor cells can directly inhibit NK cytotoxicity by expressing non classical HLA (human leucocyte antigen) class I molecules, HLA-G, HLA-E, HLA-F<sup>406</sup>. HLA-G, the most documented molecule, is expressed in different types of cancers, such as breast carcinoma<sup>407</sup>, melanoma<sup>408</sup>, and ovarian carcinoma<sup>409</sup>. It can be membrane-bound, or secreted, or incorporated into tumor-derived exosomes<sup>410</sup>. Interactions between HLA-G and KIR2DL4 on NK cells<sup>411</sup> led to inhibition of NK cytotoxicity and favored immune evasion<sup>412-</sup>  
414.

#### 4.1.6.4. Remodeling the tumor microenvironment

Tumor cells can generate an immunosuppressive microenvironment that participates in NK dysfunction and tumor resistance. Hypoxia is an important feature that characterizes the tumor sites, where the availability of oxygen is less than 2%<sup>415</sup>. Hypoxia has been associated with malignancy, resistance to radiotherapy and chemotherapy<sup>415</sup>. In addition, tumor cells in hypoxic conditions showed resistance to NK lysis<sup>416,417</sup>. This could be due to a hypoxia-induced downregulation of surface MICA<sup>416</sup> or a hypoxia-induced shedding of MICA through the alteration of nitric oxide signaling<sup>417</sup>. In parallel, hypoxia reduced NK cell killing by downregulating the levels of NKG2D, perforin and granzyme B<sup>418</sup>. Hypoxia also induced the expression of HLA-G on tumor cells<sup>419</sup>. Furthermore, combination of hypoxia with other physical parameters of the tumor microenvironment such as acidic pH and low glucose concentrations strongly decreased NK cytotoxicity<sup>420</sup>.

The over-production of immunosuppressive cytokines, such as TGF- $\beta$ , contributes to immune evasion and tumor progression. High levels of TGF- $\beta$  have been observed in many cancers, including breast cancer<sup>421</sup>, lung cancer, and colorectal cancer<sup>422</sup>. TGF- $\beta$  is secreted by tumor cells and various cells of the tumor stroma<sup>423</sup>. On the one hand, TGF- $\beta$  promoted tumor growth and tumor invasion<sup>423</sup>. On the other hand, TGF- $\beta$  inhibited cytolytic activity of NK cells by downregulating expression of NKp30 and NKG2D receptors<sup>422,424,425</sup>. Moreover, extended exposure of NK cells to TGF- $\beta$  inhibited ADCC, with repression of granzymes A and B<sup>426</sup>.

Besides TGF- $\beta$ , adenosine is an immunosuppressor which is upregulated in the tumor microenvironment<sup>427</sup>. Adenosine is a purine nucleoside and it is generated from the catabolism of ATP into AMP then into adenosine, through the activity of the ectoenzymes CD39 and CD73, respectively. Adenosine has been found to inhibit NK cytolytic activity<sup>428,429</sup> by interfering with both perforin-mediated and Fas ligand-mediated killing pathways<sup>430</sup>. In parallel, adenosine stimulated the proliferation of cancer cells<sup>431</sup>. Interestingly, different factors of the tumor microenvironment, such as hypoxia<sup>432</sup>, TGF- $\beta$ <sup>433</sup>, IL- $\beta$ <sup>434</sup>, TNF- $\alpha$ <sup>434</sup> and IFN- $\alpha$ <sup>435</sup>, were shown to drive adenosine generation by upregulating CD73 expression.

Furthermore, the alteration of NK functions by the tumor microenvironment can be mediated through the recruitment of immunosuppressive cells, such as myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs). The accumulation of these

immunosuppressive cells has been observed in many cancers and correlated with increased tumor burden. MDSCs were shown to inhibit NK functions by downregulating NKp30, NKG2D, perforin, and IFN- $\gamma$  production<sup>436-438</sup>. This inhibition depended on cell-cell contact and the membrane-bound TGF- $\beta$  expressed on MDSCs<sup>438</sup>. In parallel, Tregs inhibited NK functions through direct interactions, including by expressing membrane-bound TGF- $\beta$ <sup>439,440</sup>. Additionally, Tregs in the tumor microenvironment were shown to express granzyme B and kill NK cells, thus promoting tumor evasion<sup>441-444</sup>. Interestingly, MDSCs were shown to promote the expansion and activation of Tregs, thus strengthening the immunosuppressive properties of the tumor microenvironment<sup>445</sup>.

Collectively, NK cells play an anti-tumor role in the immune system. They infiltrate the tumor sites and kill tumor cells via different mechanisms. However, tumor cells develop various strategies to evade the immunosurveillance exerted by NK cells, either by increasing their resistance or altering NK cell recognition and cytotoxicity. Other cell types, such as MDSCs and Tregs, are recruited to the tumor sites and mediate an immunosuppression. What about the role of resident cells, in particular adipocytes?

## 4.2. Adipocytes and cancer cells: the fat and the bad

### 4.2.1. Links between adipose tissue and cancer

Emerging roles of adipose tissue in cancer have been raised based on two key observations. On the one hand, epidemiological studies have shown that being overweight (BMI between 25 and 30 kg/m<sup>2</sup>) and obesity (BMI greater than 30 kg/m<sup>2</sup>) are important risk factors for many cancers such as post-menopausal breast cancer, endometrial cancer, kidney cancer, adenocarcinoma of the oesophagus, gallbladder cancer, ovarian cancer, gastric cancer, pancreatic cancer, liver cancer, leukemia and non-Hodgkin lymphoma<sup>247,446,447</sup>. In addition, increased body mass index is associated with higher mortality of cancer patients<sup>448</sup> (table VI). Interestingly, weight loss after gastric bypass surgery was shown to reduce cancer incidence and mortality<sup>449</sup>. On the other hand, adipose tissue plays the role of a niche in which cancer cells develop (in breast), metastasize (in stomach, colon, ovary), and resist to therapy (in bone marrow). Adipocytes constitute major components of the adipose tissue and are in close proximity of cancer cells. Thus, they can interact and modulate cancer properties, in particularly tumor growth, invasion and resistance<sup>450</sup>. Inversely, cancer cells can modify their microenvironment, notably by transforming cancer-associated adipocytes into fibroblast-like cells that favor cancer development<sup>451</sup>.

**Table VII. Relative risk of cancer incidence and mortality in association with obesity (Park *et al.*, 2011)**

Type of cancer	Incidence (risk ratio, per 5 kg/m <sup>2</sup> increase <sup>a</sup> )		Type of cancer	Mortality (risk ratio, normal vs. obese <sup>b</sup> )	
	Men	Women		Men	Women
Liver	1.24	1.07	Liver (BMI ≥35)	4.52	1.68
Pancreas	1.07	1.12	Pancreas (BMI ≥35)	2.61	2.76
Esophagus (adenocarcinoma)	1.52	1.51	Esophagus (BMI ≥30)	1.91	2.64
Colorectal	1.24	1.09	Colon and rectum (BMI ≥35)	1.84	1.46
Gallbladder	1.09	1.59	Gallbladder (BMI ≥30)	1.76	2.13
Kidney	1.24	1.34	Kidney (BMI ≥35)	1.70	4.75
Prostate	1.03		Prostate (BMI ≥35)	1.34	
Breast (postmenopausal)		1.12	Breast (BMI ≥40)		2.12
Endometrium		1.59	Uterus (BMI ≥40)		6.25
			Cervix (BMI ≥35)		3.20
			All other cancers (BMI ≥35)	1.68	2.51
			All cancers (BMI ≥40)	1.52	1.88

<sup>a</sup>Risk ratio represents increase of incidence rate per 5 kg/m<sup>2</sup> increase in BMI

<sup>b</sup>Risk ratio represents relative mortality rate of high BMI (35-39.9 kg/m<sup>2</sup>) patients compared to normal BMI (18.5-24.9 kg/m<sup>2</sup>)



#### 4.2.2. Adipocytes support tumor growth

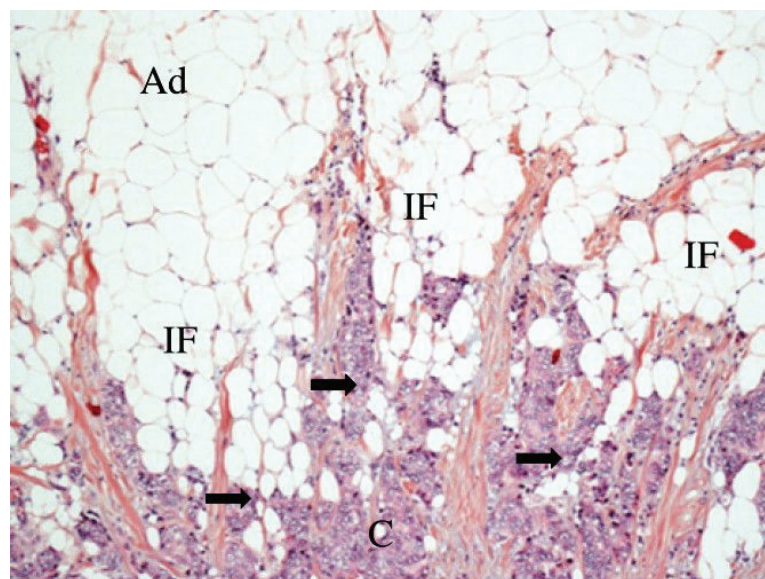
Several studies have shown the role of adipose tissue in support and promotion of tumor growth. Elliott *et al.* showed that the murine mammary carcinoma SPI cells grew better when injected in the mesenteric and ovarian fat pad or the mammary gland than in subcutis or peritoneal cavity of mice<sup>452</sup>. Moreover, co-transplantation of SPI cells with mammary or ovarian fat fragments into the subcutis increased tumor growth<sup>452</sup>. Manabe *et al.* showed that mature rat adipocytes, but not rat preadipocytes, increased tumor growth of breast carcinoma cell lines<sup>453</sup>. However, Zimmerlin *et al.* clearly demonstrated that human preadipocytes could also promote the *in vitro* growth and *in vivo* tumorigenesis of metastatic breast cancer cells from clinical isolates<sup>454</sup>. In addition, coculture of human adipocytes with ovarian cancer cells promoted cancer cell growth both *in vitro* and *in vivo*<sup>455</sup>. Different mechanisms have been proposed to explain the promotion of tumor growth by adipocytes.

##### 4.2.2.1. Adipocytes provide energy to fuel tumor growth

The “Warburg effect”, in which oxidative phosphorylation is shifted to glycolysis to provide energy for rapid tumor growth, even under normal oxygenic conditions, has been described in cancer cells several decades ago<sup>456</sup>. In parallel, cancer cells are capable of using alternative sources of energy, such as amino acids and lactate from the microenvironment<sup>457,458</sup>. In 2009, Lisanti *et al.* proposed the “reverse Warburg effect”: cancer cells induce glycolysis in cancer-associated fibroblasts which in turn produce lactate and pyruvate for cancer cell metabolism and proliferation<sup>459,460</sup>. This concept can be extended to other cells of the tumor stroma, notably the adipocytes. These cells were shown to release lactate through the monocarboxylate transporters, in particularly under hypoxic conditions<sup>461,462</sup>.

Other important sources of energy for tumor cells are fatty acids, since when metabolized, they yield large quantity of ATP. Using radioactive palmitate injected either intraperitoneally or intravenously into mice, Mermier and Baker suggested that most of the free fatty acids in tumor cells are directly transferred from host stroma tissues but not from the plasma<sup>463</sup>. Since adipocytes are specialized in lipid storage, release of fatty acids by adipocytes can be useful to fuel tumor growth. Indeed, co-cultivation of adipocytes with breast cancer cells led to adipocyte delipidation and decreased expression of the adipocyte marker adiponectin<sup>451</sup>. Likewise, ovarian cancer cells cocultured with adipocytes activated lipolysis in adipocytes<sup>455</sup>. In line with these *in vitro* data, Dirat *et al.* showed that cancer-

associated adipocytes located at the invasive front of breast cancer displayed smaller sizes and less lipid than adipocytes far from the tumors<sup>451</sup> (figure 16). In parallel, fatty acids released from adipocyte lipolysis could be taken by ovarian cancer cells which accumulated lipids in their cytoplasm and upregulated their rate of  $\beta$ -oxydation<sup>455</sup>. The fatty acid-binding protein 4 (FABP4) was identified as key role in the transfer of fatty acids. Mutation of FABP4 reduced significantly tumor growth<sup>455</sup>. However, the factors that induce the energy crosstalk between adipocytes and cancer cells remain unknown. It is worth noting that induced lipolysis of adipose tissue contributed to cancer-associated cachexia<sup>464</sup>, a syndrome characterized by the loss of adipose and muscle mass, and frequently observed in untreated cancer patients<sup>465</sup>. Interestingly, many tumor cells express high levels of fatty acid synthase (FASN) which plays a key role in the biogenesis of fatty acids<sup>466-471</sup>, suggesting *de novo* synthesis of fatty acids by cancer cells. Inhibition of FASN decreased cell proliferation and tumor growth *in vivo*<sup>472,473</sup>. Together, these data underline that fatty acids, either from *de novo* synthesis or transferred from adipocytes, are pivotal to fuel tumor growth.



**Figure 16. Cancer-associated adipocytes at the invasive front of breast tumors (Wang *et al.*, 2012)**

Immunohistochemistry of the invasive front (IF) of breast cancer reveals the existence of cancer-associated adipocytes with reduced sizes compared to adipocytes far from the tumors. Ad, adipose tissue; C, tumor center.

#### 4.2.2.2. Adipocytes secrete adipocytokines

As mentioned above, both adipocytes (and also preadipocytes) secrete various factors that act either locally or systemically. In parallel, many tumor cells express receptors for these adipocytokines which can affect tumor growth. These factors include leptin, adiponectin, estrogen, IGF-I and HGF.

##### 4.2.2.2.1. Leptin

The role of leptin in tumorigenesis was suggested by the high expression of the leptin receptor (ObR) in several cancer cells, such as breast<sup>474</sup>, lung<sup>475</sup>, stomach<sup>476</sup>, colon<sup>477</sup>, ovary<sup>478</sup> and leukemia<sup>479</sup>. *In vitro* studies using recombinant leptin showed an increase in cell proliferation by leptin in different cancer cell types, via the activation of the ERK1/2 pathway<sup>480</sup>. Particularly, leptin could augment proliferation of breast cancer cells via transactivation of HER-2<sup>481,482</sup>. Amemori *et al.* showed that in a three dimensional collagen gel culture system, adipocytes and preadipocytes from wild-type mice increased the proliferation of colon cancer cells<sup>483</sup>. This effect was abolished in adipocytes, but not in preadipocytes, from *ob/ob* mice deficient for leptin<sup>483</sup>. This result suggests that leptin may participate, at least in adipocytes, in the promotion of tumor growth. However, the *in vivo* role of leptin in tumorigenesis is controversial. While *ob/ob* mice were shown to display decreased growth of colorectal tumors<sup>484</sup> and mammary tumors<sup>485</sup>, increased tumor growth was observed in prostate tumors<sup>486</sup>. Additionally, Aparicio *et al.* showed that leptin did induce growth of colon cancer cells *in vitro* but not in nude mice and *Apc*<sup>Min/+</sup> mice that were susceptible to spontaneous intestinal adenoma formation<sup>487</sup>. This result may be partly explained by the local and not systemic effect of leptin on adjacent tumor cells. Interestingly, many cancer cells were shown to express leptin<sup>474,476,488</sup>, suggesting leptin may affect tumor growth in an autocrine and paracrine manner.

##### 4.2.2.2.2. Adiponectin

A number of human cancers were observed to express high levels of the adiponectin receptors (AdipoR1 and AdipoR2)<sup>489-493</sup>. *In vitro* exposition of cancer cells to adiponectin inhibited proliferation and induced apoptosis in different cancer cell lines, such as breast<sup>489,494,495</sup>, liver<sup>496</sup>, colon<sup>496</sup>, stomach<sup>497</sup> and endometrium<sup>498</sup>. *In vivo*, adiponectin reduced tumorigenesis of many cancer cells<sup>496,497,499,500</sup>, and adiponectin deficiency promoted tumor growth<sup>501,502</sup>. The growth inhibition of adiponectin in cancer cells was shown to be mediated through activation of AMPK, inhibition of PI3K/Akt and ERK1/2 pathways, down-regulation

of leptin-induced STAT3 phosphorylation, inhibition of NF- $\kappa$ B and Wnt/ $\beta$ -catenin pathways, and decrease of ROS production<sup>224,503</sup>.

Thus, adiponectin mediates opposite effects on tumor growth compared to leptin. Since both adipokines are secreted by adipocytes and present in the blood, the ratio leptin: adiponectin may be a major factor regarding tumor growth. Interestingly, there is an inverse correlation of the serum levels of these two adipokines during fat mass changes<sup>448</sup>. High BMI was associated with increased leptin and decreased adiponectin levels in the serum. While *in vitro* studies showed divergent responses of different breast cancer cell lines to various leptin : adiponectin ratios<sup>504</sup>, clinical studies indicated a positive correlation between high leptin: adiponectin ratios and increased risk of postmenopausal breast<sup>505,506</sup>, colorectal<sup>507</sup> and endometrial cancer<sup>508</sup>.

#### 4.2.2.2.3. Estrogen

ERs are expressed by many cancer cells, in particularly breast cancer (70-80%). Thus, the production of estrogen by adjacent mammary adipose tissue has a strong positive impact on tumor growth in estrogen-dependent breast cancer<sup>509</sup>. Preadipocytes, but not adipocytes, were shown to express aromatase, the key enzyme in the estrogen production<sup>226</sup>. Interestingly, the aromatase expression in preadipocytes was demonstrated to be induced by tumor cells via secretion of PGE2<sup>510</sup>. This data underlines the cross-talk between tumor cells and cancer-associated preadipocytes in favor of tumorigenesis.

#### 4.2.2.2.4. Insulin-like growth factor-I (IGF-I)

Epidemiological studies have revealed a correlation between high IGF-I circulating levels and increased risk of cancers, such as premenopausal breast<sup>131</sup>, prostate<sup>511</sup>, lung<sup>512</sup> and colorectal<sup>513-515</sup> cancers. Tumor cells were shown to express IGF-IR, and binding of IGF-IR to its ligand promoted tumor growth through activation of PI3K/Akt and MAPK pathways<sup>132</sup>. IGF-I was shown to be secreted by human adipocytes, and by preadipocytes in lower amounts<sup>516</sup>. Inhibition of IGF-IR prevents the growth-promoting effect of adipocytes in breast cancer cells<sup>516</sup>.

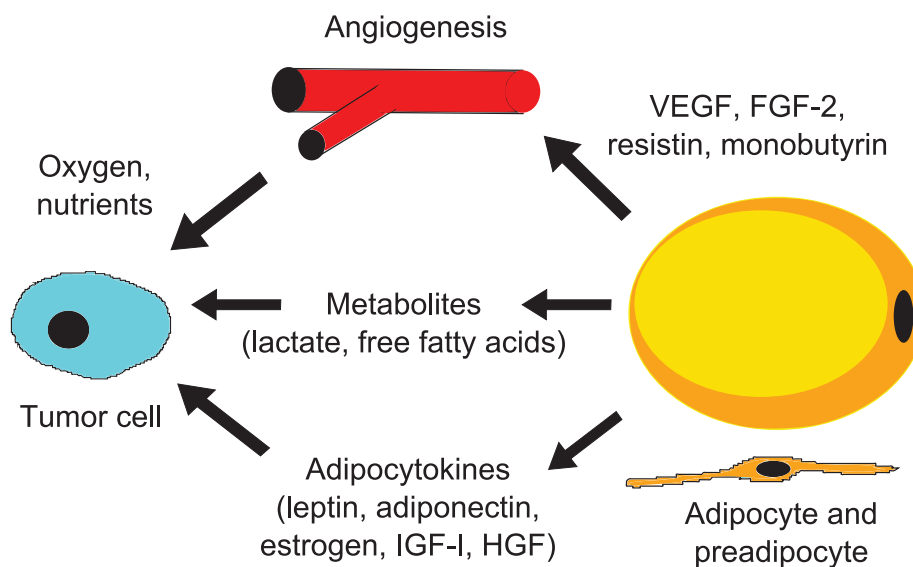
#### 4.2.2.2.5. Hepatocyte growth factor (HGF)

HGF was shown to be secreted by both adipocytes and preadipocytes<sup>517,518</sup>. In parallel, the HGF receptor (c-Met) was found to express in breast<sup>519</sup>, ovarian<sup>520</sup>, and lung<sup>521</sup> cancers. Interestingly, it was observed that in breast cancer, c-Met expression was increased at the adipocyte-tumor interface<sup>522</sup>. This expression pattern suggests a paracrine interaction between

stromal adipocytes, preadipocytes and tumor cells. Rahimi *et al.* showed that HGF promoted proliferation of murine breast carcinoma SP1 cells. Moreover, neutralization of HGF in the conditioned medium from murine 3T3-L1 adipocytes suppressed adipocyte-induced proliferation of SP1 cells<sup>523</sup>. However, a direct role of HGF in growth of human cancer cells has not been demonstrated. *In vivo*, overexpressing HGF in different tissues in mice resulted in tumorigenesis of those tissues<sup>524</sup>.

#### 4.2.2.2.6. Adipocytes promote angiogenesis

Angiogenesis, the process of new blood vessel formation, plays a critical role in tumor expansion<sup>34</sup>. Blood vessels supply oxygen, nutrients, and growth factors from the plasma to tumor cells. This process is tightly regulated by tumor cells and stromal cells in the tumor microenvironment. It was shown that adipocytes and preadipocytes actively participated in the angiogenic modulation by secretion of adipokines<sup>525</sup>. Angiogenic factors, such as vascular endothelial growth factor A (VEGFA) and fibroblast growth factor 2 (FGF-2), are produced by adipocytes and preadipocytes, respectively<sup>526,527</sup>. Other adipokines, such as leptin, adiponectin, resistin, and HGF, promoted angiogenesis *in vitro* and *in vivo*<sup>528-532</sup>. Certain adipocyte-derived lipids such as monobutyryl also induced angiogenesis<sup>533</sup>. Interestingly, it was shown that adipose-derived stem cells (ASCs) could differentiate into various cell types including endothelial and perivascular cells<sup>534</sup>. These ASCs, which were presumably considered to only differentiate into a limited number of lineages, were shown to express pericyte markers  $\alpha$ -SMA (alpha-smooth muscle actin), PDGFR- $\beta$  (platelet derived growth factor receptor beta) and NG2 (chondroitin sulfate proteoglycan), and localize around the adipose vasculature<sup>535</sup>. In line with this data, a recent study showed that circulating preadipocytes from endogenous WAT could be recruited into tumor sites, where they were incorporated into blood vessels to promote tumor vascularization<sup>536</sup>.



**Figure 17. Adipocytes and preadipocytes promote tumor growth**

Adipocytes and preadipocytes secrete metabolites, growth factors and angiogenic factors that promote tumor growth. FGF-2, fibroblast growth factor 2; HGF, hepatocyte growth factor; IGF-I, insulin-like growth factor I; VEGF, vascular endothelial growth factor.

#### 4.2.3. Adipocytes promote tumor invasion and metastasis

The invasion-metastasis of carcinomas consists of a cascade of events: (1) invade locally through surrounding extracellular matrix and stromal cell layers, (2) intravasate into the lumina of blood vessels, (3) survive the transport through the vasculature, (4) arrest at distant organ sites, (5) extravasate into the parenchyma of distant tissues, (6) initially survive in these foreign microenvironments, and (7) reinitiate their proliferative program at metastatic sites<sup>537</sup>. During these processes, adipocytes and preadipocytes play important roles, either by increasing tumor invasion, remodeling extracellular matrix, or promoting tumor homing.

##### 4.2.3.1. Adipocytes increase tumor invasion

Several studies indicate the role of adipocytes in promoting tumor invasion. It was observed that in ductal breast carcinoma, the scattered invasion into fat and the length of the invasive patterns of cancer cells were correlated with tumor aggressiveness and poor prognosis<sup>538-540</sup>. *In vitro*, coculture of adipocytes with breast and ovarian cancer cells increased tumor invasion<sup>451,455</sup>. Visceral adipocytes were shown to be more efficient to promote tumor invasion of ovarian cancer cells than subcutaneous adipocytes<sup>455</sup>. *In vivo* studies demonstrated that breast cancer cells previously cocultured with adipocytes displayed

more aggressive phenotype and stronger invasive capacity than cancer cells alone<sup>451</sup>. The promoting effect of tumor invasion by adipocytes is likely mediated by soluble factors secreted by these cells. Interestingly, Dirat *et al.* showed that the conditioned medium from cancer-associated adipocytes, but not adipocytes which had never been cocultivated with tumor cells, could promote tumor invasion<sup>451</sup>. This data illustrates the tumor-adipocyte crosstalk and the ability of tumor cells to modulate stroma cells in favor of tumor progression. As for preadipocytes, their roles in tumor invasion are unclear<sup>451,541</sup>.

IL-6 has been identified as the adipose-derived factor that promotes tumor migration and invasion. Depletion of IL-6 by neutralizing antibody abrogated the stimulation of tumor invasion by adipocytes or preadipocytes<sup>451,541</sup>. Another adipocyte/preadipocyte-secreted factor, HGF, was shown to promote tumor invasion via its receptor MET<sup>542</sup>. HGF triggered downregulation of E-cadherin, destabilization of adherent junctions, and dissociation of cell-cell contact<sup>543,544</sup>. Interestingly, MET expression is altered in many cancers, either by overexpression, mutation or HGF induction, leading to cancer aggressivity and metastatic dissemination<sup>545</sup>.

#### 4.2.3.2. Adipocytes remodel the extracellular matrix

Adipocytes secrete various constituents of the extracellular matrix, such as different types of collagen<sup>546</sup>. During tumorigenesis, cancer cells upregulated the secretion of collagen VI from adipocytes<sup>547</sup>. Collagen VI was shown to promote tumor growth and survival via signaling through the NG2/chondroitin sulfate proteoglycan receptor expressed on tumor cells<sup>548</sup>. In the background of the mouse mammary tumor virus/polyoma virus middle T oncogene, mice lacking collagen VI exhibited reduced rates of hyperplasia and primary tumor growth<sup>548</sup>. Furthermore, endotrophin, a cleaved fragment of the collagen VI  $\alpha 3$  chain, promoted the epithelial-mesenchymal transition (EMT) process and metastasis of mammary epithelial cancer cells<sup>549</sup>. Like collagen VI in adipocytes, secretion of fibronectin by preadipocytes was also enhanced by cancer cells<sup>550</sup>. Fibronectin activated the STAT3 signaling pathway which promoted EMT of breast cancer cells<sup>551</sup>.

Adipocytes secrete a number of matrix metalloproteinases (MMP)<sup>552</sup>, which allows the degradation of extracellular matrix and the promotion of tumor invasion<sup>553</sup>. Cancer cells were shown to induce the expression of MMP-11 in adipocytes as tumor invaded the surrounding adipose tissue<sup>554</sup>. Although the substrates of MMP-11 remain unknown<sup>555</sup>, some suggested that MMP-11 can cleave collagen VI<sup>556</sup>. *In vivo*, MMP-11 was shown to promote cancer

progression by remodeling the extracellular matrix<sup>557</sup>. Downregulation of MMP-11 by siRNA attenuated cancer metastasis<sup>558,559</sup>. Additionally, high levels of MMP-11 expression were correlated with increased invasion and bad prognosis in breast carcinoma<sup>560</sup>, pancreatic cancer<sup>561</sup> and colon cancer<sup>562</sup>. In parallel, MMP-11 can directly act on adipocytes and preadipocytes. MMP-11 negatively regulated adipogenesis by inhibiting preadipocyte differentiation and enhancing adipocyte dedifferentiation, leading to accumulation of fibroblast-like cells which favor tumorigenesis<sup>563</sup>. Moreover, adipocytes can indirectly promote tumor invasion via leptin and HGF, which in turn induce the secretion of various MMP by cancer cells<sup>564–566</sup>.

#### 4.2.3.3. Adipocytes promote tumor homing

In ovarian cancer, the omentum is the main site of metastasis. Nieman *et al.*<sup>455</sup> showed that omentum adipocytes favored tumor homing via secretion of cytokines. The most abundantly adipocyte-secreted cytokines included IL-6, IL-8, MCP-1, and tissue inhibitor of metalloproteinase-1 (TIMP-1). Antibody-mediated inhibition of these factors reduced ovarian cancer cell homing toward adipocytes *in vitro*. Neutralization of IL-6R and particularly IL-8R (CXCR1) reduced *in vivo* homing of ovarian cancer cells to the mouse omentum. Notably, the expression of IL-8R was strongly upregulated in ovarian cancer cells in coculture with adipocytes. In another study, Pramanik *et al.* showed that acute lymphoblastic leukemia (ALL) cells migrated into adipose tissues *in vivo* and exhibited chemotaxis towards adipocytes *in vitro*<sup>567</sup>. CXCL12/SDF-1 was identified as adipocyte-derived chemoattractant that was responsible for leukemia cell migration. Inhibition of SDF-1 receptor, CXCR4, in ALL cells abrogated their migration toward adipocytes. Together, these data illustrate that adipocytes can promote tumor metastasis and homing of cancer cells to adipose tissue.

#### 4.2.4. Adipocytes increase cancer resistance to therapies

Evading apoptosis is a main hallmark of cancer cells<sup>34</sup>. Despite the abundance and importance of adipocytes in the tumor microenvironment, little is known about their roles in survival and resistance of cancer cells to therapies. The first evidence comes from the observation of Iyengar *et al.*, which showed that 3T3-L1 adipocytes supported survival of transformed ductal epithelial cells *in vitro* under limiting serum conditions<sup>547</sup>. Indeed, adipocytes can promote anti-apoptosis and cancer resistance via different mechanisms: increasing cell-cell contact, cell-matrix adherence, and secretion of soluble factors.



#### 4.2.4.1. Adipocytes increase cell-cell contact and cell-matrix adherence

The cell-cell and matrix-cell contact provide survival signals whose withdrawal induces cell death<sup>568,569</sup>. As mentioned above, adipocytes are in close contact with cancer cells and secrete constituents for the tumor extracellular matrix. Collagen VI has been shown to confer resistance of ovarian cells to cisplatin *in vitro*<sup>570</sup>. Besides, preadipocytes can also participate into cancer resistance to cytotoxic agents via secretion of fibronectin<sup>571</sup> or via membrane exchange of multi-drug resistance proteins<sup>572</sup>.

#### 4.2.4.2. Adipocytes promote cancer resistance via soluble factors.

Different adipocyte-secreted soluble factors can mediate cancer resistance to chemotherapy. Behan *et al.* showed that *in vitro* 3T3-L1 adipocytes protected ALL cells from cytotoxic agents, including vincristine, dexamethasone, daunorubicin, and nilotinib<sup>573</sup>. This protection was independent of cell-cell contact and was associated with increased expression of pro-survival signals Bcl-2 and Pim-2<sup>573</sup>. Interestingly, the protective effect was also observed with the murine bone marrow-derived adipocyte OP-9 cells<sup>573</sup>, suggesting a role of the bone marrow adipose tissue in chemoresistance.

A recent study by Holland *et al.* showed that adiponectin inhibited apoptosis through stimulation of a ceramidase activity associated with its two receptors AdipoR1 and AdipoR2<sup>574</sup>. Ceramide induced cell death and its expression was increased in response to various chemotherapeutic agents<sup>575</sup>. Ceramide catabolism generates sphingosine-1-phosphate (S1P) which is an antiapoptotic metabolite. Thus, adipocytes may participate in chemoresistance via secretion of adiponectin. In addition, Chi *et al.* recently showed that adipocyte-secreted leptin contributed to resistance of melanoma cells to various therapeutic agents<sup>576</sup>. Bochet *et al.* also showed that cancer-associated adipocytes can promote breast tumor radioresistance, probably via the secretion of IL-6<sup>577</sup>.

On the other hand, bone marrow mesenchymal stem cells have been also shown to decrease the sensitivity of tumor cells to various cytotoxic agents, such as bortezomib, cisplatin, 5-FU and irinotecan<sup>578,579</sup>. This protective effect was mediated by secreted factors such as VEGF or fatty acids. Curiously, in a study by Roodhart *et al.*, the protective effect was observed with mouse BM-MSC and mouse embryonic fibroblasts (MEF) but not 3T3 fibroblasts, 3T3-L1 preadipocytes or 3T3-L1 differentiated adipocytes<sup>579</sup>. The authors argued that the multilineage differentiation potential of BM-MSC or MEF could determine their ability to secrete chemoprotective factors in response to platinum-based chemotherapy.

However, since the secretion profiles are different depending on the origin of the cells, further investigation on the protective effect of the same BM-MSC at different stages of differentiation is required to confirm this hypothesis.

Collectively, these data indicate that adipocytes and preadipocytes can promote cancer resistance to therapies. Moreover, as mentioned above, adipocytes and preadipocytes secrete factors that attract tumor homing to the adipose tissue, creating a protective tumor microenvironment for cancer cells. This underlines the importance of adipose tissue as a potential target in cancer treatment.

#### **4.2.5. Obesity and cancer**

Obesity is defined as an excess in body fat, with BMI values greater than 30 kg/m<sup>2</sup>. It is associated with an increase in adipocyte volume (hypertrophy) and to a lesser extent, an increase in adipocyte cell number (hyperplasia)<sup>196,241</sup>. Importantly, this expansion in obesity is associated with hypoxia, remodeling of ECM, dysregulation of adipocytokine secretion, adipocyte death, and chronic inflammation. Obesity is therefore considered as a pathological state which can further support tumorigenesis and cancer progression.

##### *4.2.5.1. Hypoxia, angiogenesis and remodeling of ECM*

Adipocyte hypertrophy was shown to be associated with local hypoxia in adipose tissue in obese mice and obese patients<sup>580,581</sup>. Hypoxia led to the expression of hypoxia induced factor 1  $\alpha$  (HIF-1 $\alpha$ ) and endoplasmic reticulum stress markers in adipocytes<sup>581,582</sup>. Besides, low oxygen levels increased glucose consumption and release of lactate from adipocytes<sup>583</sup>. Most importantly, hypoxia led to dysregulation of the secretion of adipocytokines. Hypoxia was shown to suppress adiponectin expression and secretion<sup>582,584,585</sup>, while it induced secretion of leptin, IL-6, VEGF and MMPs<sup>583,584</sup>. This was associated with enhanced angiogenesis<sup>586</sup> and remodeling of the adipose extracellular matrix<sup>587</sup>.

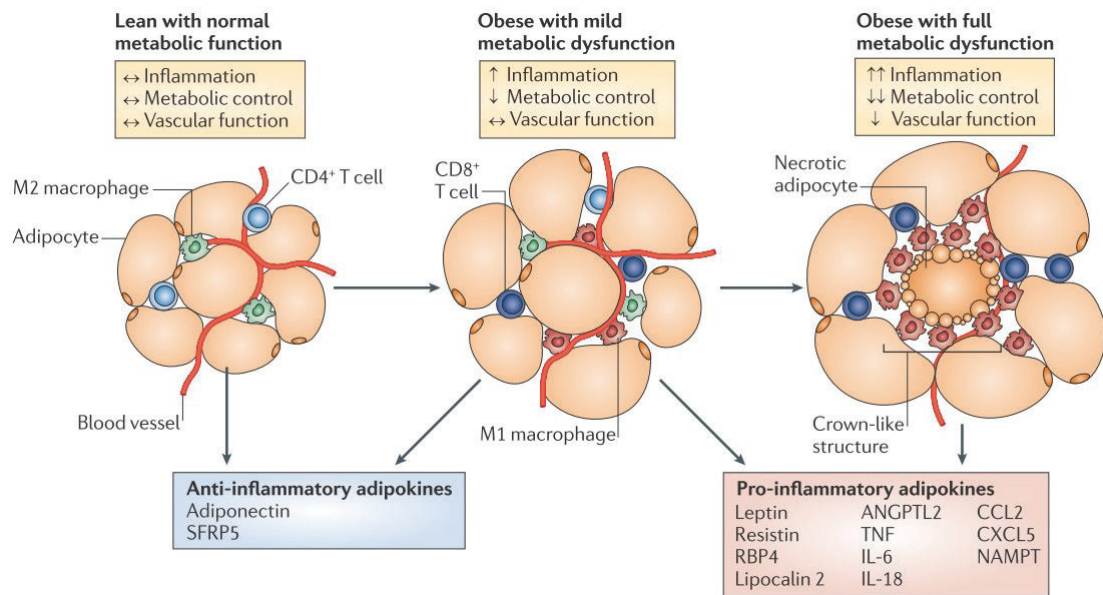
##### *4.2.5.2. Dysregulation of adipocytokines*

Obese adipose tissue displays a dysregulation of secreted adipocytokines<sup>242,588</sup>. Obesity was associated with increased adipose tissue expression and high plasma levels of several pro-inflammatory adipocytokines, including leptin<sup>589,590</sup>, resistin<sup>591</sup>, IL-6<sup>210,592,593</sup> and TNF- $\alpha$ <sup>594,595</sup>. By contrast, the plasma level of the anti-inflammatory adiponectin was decreased in obesity<sup>596</sup>. Interestingly, weight loss in obese individuals after a restricted calorie diet or exercises led to decreases of leptin<sup>589</sup>, IL-6<sup>597</sup> and TNF- $\alpha$ <sup>594</sup>, in parallel with increases of

adiponectin<sup>598</sup>. *In vitro* studies suggested IL-6 and TNF- $\alpha$  inhibited adiponectin<sup>598,599</sup>, while TNF- $\alpha$  increased expression of itself and of IL-6<sup>599</sup>. Moreover, IL-6 and TNF- $\alpha$  could promote the activity of aromatases, resulting in increased estrogen production<sup>600,601</sup>. Indeed, obesity was shown to be correlated with high levels of estrogen, notably in post-menopausal women<sup>602</sup>. Plasma levels of HGF, but not VEGF, were also shown to increase in obese individuals<sup>603</sup>. Together, these data indicate dysregulated secretion of adipocytokines and pro-inflammatory profile in obesity, which can promote metabolic disorders and tumorigenesis both locally and systemically.

#### 4.2.5.3. Adipocyte death, recruitment of macrophages and chronic inflammation

Obesity was shown to be unexpectedly associated with adipocyte death<sup>604-606</sup>. Using mice deficient for HLS, a major lipase in mature adipocytes, Cinti *et al.* demonstrated that adipocyte death was caused by cell hypertrophy and not by increased adipose mass<sup>604</sup>. The frequency of adipocyte cell death was positively correlated with adipocyte size. Interestingly, in this study, adipocyte death displayed ultrastructural features of necrosis, including rupture of plasma membrane and endoplasmic reticulum dilatation. In another study, Alkhoury *et al.* showed that adipocytes in obesity also displayed a pro-apoptotic phenotype, with activation of both extrinsic and intrinsic apoptotic pathways<sup>607</sup>. However, regardless of the mechanism, adipocyte death released lipid droplets into the medium<sup>241</sup>. It is thus reasonable to speculate that cancer cells can ingest these lipids and use them as source of energy for tumor growth. In addition, adipocyte death led to increased infiltration of macrophages which arranged around dead adipocytes and formed crown-like structures<sup>604,608</sup> (figure 18). These infiltrating macrophages abundantly secrete inflammatory cytokines such as IL-6 and TNF- $\alpha$ <sup>605,609</sup>, which further enhanced the local inflammation in obese adipose tissue.



**Figure 18. Phenotypic modulations of adipose tissue in obesity (Ouchi *et al.*, 2011)**

Lean adipose tissue possesses normal adipocytes with metabolic control and no inflammation. As the fat mass increases, adipocytes undergo hypertrophy but the tissue retains relatively sufficient vascular function, normal metabolic function and low levels of inflammation. Late obesity is associated with hypoxia, metabolic dysfunction, adipocyte death, recruitment of macrophages around dead adipocytes forming crown-like structures, and chronic inflammation with abundant secretion of pro-inflammatory factors, including leptin, resistin, retinol-binding protein 4 (RBP4), lipocalin 2, angiopoietin-like protein 2 (ANGPTL2), tumor necrosis factor (TNF), interleukin 6 (IL-6), IL-8, CC-chemokine ligand 2 (CCL2), CXC-chemokine ligand 5 (CXCL5) and nicotinamide phosphoribosyltransferase (NAMPT).

### **4.3. Adipocytes and NK cells: the fat and the killer**

Despite the fact that adipocytes are important components of the microenvironment and they actively secrete actors that act both locally and systemically, little is known about the impact of adipocytes on NK cells. However, some observations indicate the involvement of adipocytes in NK cell development, activation and cytotoxicity.

#### **4.3.1. NK cells are in close contact with adipocytes**

##### *4.3.1.1. Anatomical localization of NK cells related to adipose tissues*

NK cells derive from hematopoietic cells and their development mainly occurs in the bone marrow and lymph nodes<sup>610</sup>. It is well known that these organs are embedded in adipose tissue. In the bones of human adults, adipose tissue occupies over 50% volume of the marrow cavity. Adipocytes are the most abundant cell type in the bone marrow and they are in direct contact with hematopoietic cells<sup>172</sup>. By contrast, in the lymph nodes, the adipose tissue is separated from hematopoietic cells by a capsule which is composed of a thin, loose layer of collagenous material, and a layer of subcapsular sinus. Numerous fine lymph vessels pass through the adipose tissue before entering into the nodes. These vessels are permeable to large molecules and certain small cells<sup>611</sup>. Therefore substances secreted by the adipocytes can indirectly diffuse to hematopoietic cells via the afferent lymph vessels<sup>612</sup>.

Once mature, NK cells enter the blood circulation where is found a number of adipocytokines secreted by adipose tissue. Hence, NK cells in the peripheral blood are constantly in contact with adipocyte-secreted molecules. Moreover, in solid tumors, extravasating NK cells need to cross the tumor stroma before getting in contact with tumor cells. It was shown that infiltrating NK cells were often located in the surrounding tumor stroma and only few NK cells are in direct contact with tumor cells<sup>290</sup>. In the breast or the omentum where adipocytes constitute the major components of the tumor stroma, infiltrating NK cells are very likely in direct contact with adipocytes.

##### *4.3.1.2. Secretion of chemoattractants for NK cells*

Chemerin, a chemoattractant protein, was identified as an adipokine secreted by adipocytes both *in vitro* and *in vivo*<sup>613,614</sup>. It is synthesized under the inactive form prochemerin then becomes active after proteolytic cleavage<sup>615</sup>. Even if prochemerin expression is detectable in other tissues, adipose tissue is a main source of active chemerin, along with the liver<sup>613,616</sup>. Chemerin expression is induced during adipocyte differentiation

and acts in an autocrine/paracrine manner to regulate adipogenesis<sup>613,617,618</sup>. Additionally, chemerin synthesis and secretion is increased by TNF- $\alpha$ , IL-1 $\beta$  and free fatty acids<sup>619-621</sup>. In parallel, the chemerin receptor, ChemR23 (also known as CMKLR1 or CX<sub>3</sub>CR1) was shown to be present in human blood CD56<sup>low</sup>CD16<sup>+</sup>, but not CD56<sup>high</sup>CD16<sup>-</sup> NK cells, and notably in infiltrating NK cells<sup>622</sup>. Chemerin induced NK cell migration *ex vivo* in chemotaxis assays and in mice<sup>622</sup>. Recently, Pachynski *et al.* demonstrated that tumor-expressed chemerin inhibited *in vivo* tumor growth by recruiting NK cells to the tumor sites<sup>623</sup>. Knock-out of the host chemerin receptor CMKLR1 or depletion of NK cells abrogated the tumor inhibition, while intratumoral injection of chemerin inhibited tumor growth.

Other adipocyte-derived factors, such as IL-8 and lipids, can potentially promote NK cell homing to adipose tissue. Adipocytes are abundant sources of IL-8<sup>624</sup>. In parallel, human NK cells were shown to strongly express IL-8 receptors<sup>625,626</sup> and IL-8 could induce NK cell chemotaxis *in vitro*<sup>626,627</sup>. Besides, adipocytes secrete lysophospholipids, including lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P)<sup>628,629</sup>. The receptors for these molecules were found to be expressed on NK cells and both LPA and S1P induced human NK chemotaxis *in vitro*<sup>630,631</sup>. Moreover, S1P was shown to be required for *in vivo* NK cell mobilization to inflamed organs<sup>632</sup>. Interestingly, S1P generation can be upregulated by adiponectin via its receptors AdipoR1 and AdipoR2<sup>574</sup>.

Collectively, these data indicate the proximity between NK cells and adipocytes, which is further enhanced by adipocyte-secreted chemoattractants and infiltration of NK cells in case of tumor or inflammation. The close distance between these cells favors their crosstalk, either directly via cell-cell contact, or indirectly via secreted soluble factors.

#### **4.3.2. NK cells are regulated by adipocytokines**

NK cells express receptors for various adipocytokines. Multiple studies have demonstrated the importance of these adipocytokines in the development, activation and functions of NK cells.

##### *4.3.2.1. Leptin*

Human NK cell lines were shown to constitutively express leptin receptor (ObR), both the long form and the short form<sup>633</sup>. ObR is a single membrane-spanning protein which shares sequence homology and signal transduction similar to the IL-6 receptor<sup>634,635</sup>. Exposure of NK cell lines to recombinant human leptin led to phosphorylation of STAT3 and increased

transcription of IL-2 and perforin genes<sup>633</sup>. Several studies have demonstrated the importance of leptin in NK development and functions. In *db/db* mice lacking ObR and in *ob/ob* mice deficient for leptin, NK cells declined in number and displayed impaired cytotoxicity<sup>636,637</sup>. Indeed, Lo *et al.* suggested the role of leptin in enhanced survival of NK cells during development in mouse bone marrow<sup>638</sup>. Moreover, *in vitro* studies showed that leptin improved NK cell proliferation, activation and cytotoxicity<sup>633,636</sup>. Consistently with these results, *in vivo* administration of leptin reduced the growth of hepatocellular carcinoma in athymic or SCID mice, but not in NK-deficient SCID-beige mice<sup>639</sup>. However, in a recent article, Wrann *et al.* showed that only 5% of human NK cells from the peripheral blood of healthy individuals express ObR<sup>640</sup>. Additionally, these investigators showed different effects of leptin on NK cells depending on the length of leptin exposure<sup>640</sup>. In the case of short-term exposure, leptin induced NK cytotoxicity, while long-term exposure of leptin (more than 4 days) did not display any effect. Although in this study the authors did not check the stability of leptin in long-term exposure at 37°C, (leptin half life was found to be short in plasma<sup>641</sup>), this finding raised the impact of long-term leptin exposure in blood peripheral NK cells, notably in case of obesity where constantly elevated levels of leptin are found in the plasma<sup>589,590</sup>.

#### 4.3.2.2. Adiponectin

The expression of adiponectin receptors, AdipoR1 and AdipoR2, was reported on human NK cells from peripheral blood<sup>642,643</sup>. While Pang and Narendran reported that about 20% of CD56<sup>+</sup> NK cells express AdipoRs on their membrane<sup>642</sup>, Wilk *et al.* indicated AdipoR expression in 75% of CD56<sup>+</sup> NK population using the same primary antibody<sup>643</sup>. Interestingly, the membrane expression patterns seemed to depend on the CD56 status of NK cells: while most CD56<sup>dim</sup> expressed AdipoRs, only a minor portion of CD56<sup>bright</sup> cells were positive<sup>643</sup>. However, the CD56<sup>bright</sup> NK cells expressed both AdipoRs intracellularly like murine NK cells<sup>643</sup>. Since CD56<sup>bright</sup> NK cells are considered by some as precursors of the CD56<sup>dim</sup> subset<sup>644,645</sup>, Wilk *et al.* suggested the involvement of AdipoRs expression in NK cell maturation. Besides, Kim *et al.* demonstrated that *in vitro* exposition of murine NK cells to recombinant adiponectin suppressed IL-2-induced NK cell cytotoxicity by inhibiting the NF-κB signaling pathway and down-regulating IFN-γ, Fas ligand and TRAIL expression<sup>646</sup>. In line with this finding, recent *in vivo* studies by Han *et al.* showed that adiponecin-knockout mice presented high number and increased cytotoxicity of NK cells with and without IL-2 stimulation, which participated in the suppression of lymphoma growth<sup>647</sup>. However, the

inhibitory role of adiponectin in NK cytotoxicity was contradicted by Wilk *et al.*, whose studies indicated that adiponectin did not modify human NK cytotoxicity<sup>643</sup>. Moreover, they showed that adiponectin-knockout mice even displayed decreased NK-cell cytotoxicity with lower NK degranulation and NKG2D levels compared to wild-type mice<sup>643</sup>. Some elements may partly explain the differences between these results, such as the origins of the NK cells studied (human and murine), NK cell viability, and the concentrations of adiponectin used in *in vitro* studies (30 µg/mL vs 3 µg/mL in Kim's and Wilk's studies, respectively). However, further investigations are required to confirm the role of adiponectin in NK cell development and functions.

#### 4.3.2.3. IL-6

The receptor for IL-6 (IL-6R) is a heterodimer composed of one ligand-binding  $\alpha$  unit associated with a signal-transducing glycoprotein, gp130<sup>648-650</sup>. The expression of both IL-6R units was shown in murine NK cells<sup>651</sup>. However, whether IL-6R is expressed in human NK cells is not clear. Using the same technique of flow cytometry on cells preincubated with biotin-labeled IL-6, Smyth and Ortaldo detected FITC-avidin reactivity in CD3-CD56+ NK cells<sup>652</sup>, whereas Wognum *et al.* showed absence of IL-6 binding<sup>653</sup>. Likewise, the role of IL-6 in NK activity remains controversial. While some *in vitro* studies indicated IL-6 alone had no effect on human NK cytotoxicity<sup>654-657</sup>, others demonstrated enhancement of NK cytotoxicity by IL-6 alone<sup>652,658,659</sup> or in combination with IL-2 or IL-15<sup>652,654,657,659,660</sup>. Additionally, Rabinowich *et al.* also showed that IL-6 stimulated NK adhesion through upregulation of  $\beta$ -integrin<sup>661</sup>. In mice, expression of IL-6 by human tumor cells increased tumorigenesis, likely via NK dysfunction induced by high IL-6 levels at the tumor sites<sup>662</sup>. This result was further supported by the observation that in patients with advanced colon or pancreatic cancer, administration of IL-6 led to suppression of NK cytotoxicity<sup>663</sup>. Collectively, these findings suggest an enhancement of NK activity by IL-6 *in vitro* but the opposite effect *in vivo*.

#### 4.3.2.4. TGF- $\beta$

The receptor for TGF- $\beta$  is a membrane serine/threonine kinase associated with SMAD proteins. Binding of TGF- $\beta$  to its receptor leads to phosphorylation of SMAD which moves into the nucleus and activate target gene transcription<sup>664</sup>. Using radioactive TGF- $\beta$ , Rook *et al.* demonstrated the binding of TGF- $\beta$  to NK lymphocytes and suggested the presence of high-affinity TGF- $\beta$  receptor in these cells<sup>425</sup>. Recent study by Marcoe *et al.* showed that TGF- $\beta$  played important role in the maturation of NK cells *in vitro* in presence of bone



marrow stromal OP-9 cells<sup>665</sup>. Moreover, other studies have demonstrated the role of TGF- $\beta$  in the inhibition of NK cell functions. *In vitro* exposition of NK cells to TGF- $\beta$  inhibited IFN- $\gamma$  production, even in the presence of activating cytokines such as IFN- $\alpha$ , IL-2 or IL-12<sup>666-668</sup>. In addition, TGF- $\beta$  reduced NK cytotoxicity via down-regulation of NKp30 and NKG2D receptors<sup>422,424,425</sup>. TGF- $\beta$  was also shown to inhibit ADCC via SMAD3-mediated repression of granzyme A and B<sup>426</sup>. These findings were further confirmed *in vivo*. NK cells from *smad3* -/- mice produced more IFN- $\gamma$  in response to IL-12 and CD16 activation compared to wild-type mice<sup>426</sup>. Targeting TGF- $\beta$  by siRNA in human glioma abrogated tumorigenicity *in vivo* and enhanced NKG2D expression as well as NK cytotoxicity<sup>373</sup>. Particularly, Lee *et al.* observed an inverse correlation between plasma level of TGF- $\beta$  and NKG2D expression levels in patients with lung or colorectal cancer<sup>422</sup>.

#### 4.3.2.5. TNF- $\alpha$ , PGE2 and IL-10

Other adipocyte-secreted factors, such as TNF- $\alpha$  and PGE2, were shown to implicate in NK cell functions. Under certain circumstances, TNF- $\alpha$  can induce NK cell apoptosis<sup>669,670</sup>. PGE2 was shown to suppress cytokine production as well as NK cytotoxicity via binding to PGE2 receptors on NK cells<sup>671-673</sup>. By contrast, IL-10 induced stimulatory effect on NK cells, with enhancement of IFN- $\gamma$  production and NK cytotoxicity, notably when combined with IL-2 or IL-18<sup>674-677</sup>.

Overall, adipocytokines are largely implicated in NK cell development, activation and functions. Their effects can be mediated by both endocrine and paracrine manners. Thus, adipocytes and preadipocytes play important roles in the homeostasis and regulation of NK cells.

#### 4.3.3. NK cells are dysregulated in obesity

Obesity is associated with dysregulated secretion of adipocytokines. In murine and human obese subjects, adipose tissue expression and/or plasma levels of leptin<sup>678</sup>, IL-6<sup>592,593,595</sup>, TGF- $\beta$ <sup>679-682</sup>, TNF- $\alpha$ <sup>595</sup>, PGE2<sup>683,684</sup>, and IL-10<sup>685</sup> have been shown to be upregulated, while adiponectin is decreased<sup>598,596</sup>. This dysregulation may have impacts on NK cell development and functions.

The potential modifications of NK cells in obesity have been documented in several models. In genetically-disposed obese mice lacking either leptin (*ob/ob*) or leptin receptor (*db/db*), the NK cell number and cytotoxicity were both decreased<sup>636,637</sup>. However, no

modification of NK cell count as well as cytotoxicity was observed in diet-induced obese mice<sup>686</sup> or rats<sup>686</sup>. In humans, while Dovo and Nieman showed no difference between NK cell number and activity and in obese compared to lean subjects<sup>687,688</sup>, Lynch and Lautenbach demonstrated a decrease in both NK cell count and activity in obese individuals<sup>689-691</sup>. Curiously, Moriguchi *et al.* indicated higher NK activity and proliferation in obese subjects who were 39-60 years old compared to lean subjects, but an inverse variation in people who were 60-69 years old<sup>692</sup>. Despite the inconsistent data on number and activity of NK cells at basal levels in obese status, it is generally observed that NK cells from obese mice and individuals were less responsive to various challenges, including poly I:C<sup>636</sup>, cigarette smoke<sup>690</sup>, influenza infection<sup>693</sup>, and cancer<sup>637</sup>. Moreover, NK cells from diet-induced obese rats displayed leptin resistance: they did not respond to leptin-induced activation, contrary to NK cells from lean littermates<sup>686</sup>.

Interestingly, Lautenbach *et al.* suggested that altered phenotypes of NK cells from obese rats can be normalized by transfer into lean animals<sup>694</sup>, indicating the importance of the microenvironment in NK cell shaping. In humans, NK cell number and activity in obese individuals after weight loss by energy restricted diet were shown to be decreased in several studies<sup>695,696</sup>, while unchanged in others<sup>697</sup>. By contrast, acute nutritional deprivation by fasting enhanced NK cell activity in obese subjects<sup>698</sup>.

All together, these studies, in spite of some conflicting data, point out the susceptibility of NK cells to regulation by the adipose tissue, notably in case of obesity.

# AIMS OF THE STUDY

Adipocytes are the main actors of the breast tumor microenvironment. Due to their close localization to tumor cells and their abundant secretion of various factors, adipocytes could promote tumorigenesis. As mentioned above, some studies have suggested the involvement of adipocytes and preadipocytes in the resistance of cancer cells to chemotherapy and radiotherapy.

In this study, we investigated the implication of adipocytes in the resistance of breast cancer cells to targeted therapy with trastuzumab. We hypothesized that adipocytes, via their interactions with both cancer cells and NK cells, could alter the response of cancer cells to trastuzumab-mediated ADCC. This issue could be of major clinical importance, since it would clarify the mechanisms of resistance to trastuzumab. In addition, identification of the adipose tissue-derived factors that mediate the crosstalk between adipocytes and cancer cells or NK cells would open the fields to develop strategies to ameliorate the treatment of breast cancer patients. Furthermore, since obesity is an increasing healthcare problem and is linked with many types of cancers, our study could provide information on the biological mechanisms underlying the relationship between obesity and cancer.

# **RESULTS**

## **Part I**

# **Adipocytes inhibit trastuzumab-mediated ADCC via induction of GDF15**

(DUONG *et al.*, manuscript in preparation)

## **Adipocytes inhibit trastuzumab-mediated ADCC via induction of GDF15**

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### **Running title**

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### **Keywords**

Trastuzumab, adipocytes, breast cancer, resistance, GDF15

### **Conflict of interest**

The authors declare that no conflict of interest exists.

## **Abstract**

Trastuzumab has been used in the treatment of HER2-expressing breast cancer but its efficacy is limited. Little is known concerning the role of the tumor microenvironment in resistance to trastuzumab. Given the importance of antibody-dependent cellular cytotoxicity (ADCC) in the antitumor effect of trastuzumab and the abundance of adipose tissue in breast, we investigated the impact of adipocytes on ADCC. We found that adipocytes, as well as preadipocytes, inhibited trastuzumab-mediated ADCC in HER-2 expressing breast cancer cells. Co-culture studies showed that adipocytes secreted soluble factors that reduced the sensitivity of cancer cells to ADCC. Using a transcriptomic approach, we found GDF15 to be upregulated in cancer cells exposed to adipocyte-conditioned medium. Downregulation of GDF15 by siRNA reversed the adipocyte-induced inhibition of ADCC. Our findings underline the importance of adipose tissue in the resistance to trastuzumab, and suggest that development of approaches targeting GDF15 may sensitize cancer cells to trastuzumab-based therapy.

## **Significance**

Adipocytes mediate inhibition of trastuzumab-mediated tumor lysis by a direct protective effect on breast cancer cells. Targeting the crosstalk between adipocytes and cancer cells could represent a novel therapeutic approach to enhance the effect of trastuzumab-based therapy.

## Introduction

The human epidermal growth factor receptor 2 (HER2) is amplified in 15-20% of breast cancers and its over-expression is associated with adverse prognosis (1). Trastuzumab, a humanized monoclonal antibody directed against HER2, was approved in 1998 for the treatment of HER2-overexpressing breast cancer. Mechanisms of action of trastuzumab include inhibition of HER2 dimerization, direct induction of cell growth arrest and apoptotic cell death, inhibition of HER2 shedding, and recruitment of immune effector cells to mediate tumor lysis (2). This latter mechanism, designated as antibody-dependent cellular cytotoxicity (ADCC), has been shown to be dependent on innate immune cells expressing Fc receptors (3). Recent studies by Park *et al.* also demonstrated the involvement of adaptive immune cells in the action of anti-HER2/neu antibody (4). As a single agent or in combination with chemotherapy, trastuzumab has shown remarkable efficacy (5). However, not all patients respond to trastuzumab and several patients who initially respond to treatment will eventually progress, corresponding respectively to primary and acquired resistance to trastuzumab (5).

Different mechanisms of resistance to trastuzumab have been reported. Reduction of PTEN, a phosphatase whose activation contributes to trastuzumab activity, has been shown to confer trastuzumab resistance both *in vitro* and *in vivo* (6). Patients with PTEN-deficiency displayed poorer responses to trastuzumab-based therapy than those with active PTEN (6). Shedding of the extracellular domain of HER2 protein by proteolytic cleavage has been shown to neutralize the antitumor effects of trastuzumab (7). Elevated circulating levels of HER2 have also been correlated with disease progression in patients treated with trastuzumab-based therapy (8). Furthermore, masking of the HER2 antigens by the glycoprotein mucin-4 (MUC4) has been shown to reduce binding of anti-HER2 antibodies *in vitro* (9), while increased MUC4 levels have been observed *in vivo* in tumors that were resistant to anti-HER2 therapies (10). Additionally, activation of other signaling pathways, notably IGF-IR, has also been reported to inhibit trastuzumab-mediated growth inhibition in breast cancer cells (11).

The aforementioned mechanisms of resistance are related to alterations in the tumor cells themselves and do not take into account the impact of the tumor microenvironment. This latter is highly complex in terms of cellular composition with different cell types, including adipocytes, preadipocytes, endothelial cells, pericytes, and immune cells. Several studies have



shown that immune-suppressor cells, such as tumor-associated macrophages, myeloid-derived suppressor cells and regulatory T cells, are recruited to the tumor sites and promote immune evasion (12–14). However, the implication of resident cells, notably adipocytes, in tumor resistance to trastuzumab remains largely unknown.

Adipocytes are the most abundant cells in the breast adipose tissue. It has been shown that adipocytes are not simply energy storage depots, but also active sources of various paracrine and endocrine factors, termed adipocytokines, such as leptin, adiponectin, TNF-alpha and IL-6 (15). Given their abundance and their proximity with tumor cells, adipocytes have been shown to affect tumor behavior, including tumor growth and metastasis (16). Moreover, recent studies suggested the implication of adipocytes in resistance to radiotherapy (17) and chemotherapy (18). Furthermore, obesity, which is associated with increased fat mass and inflammatory adipose tissue phenotype, has been shown to be an important risk factor for breast cancer in post-menopausal women (19). Chemotherapy and endocrine therapy have also been observed to be less effective in breast cancer patients with obesity (20).

Adipocytes derive from precursors termed preadipocytes or mesenchymal stem cells through a tightly controlled differentiation process. These preadipocytes reside in the breast or may be recruited from distant adipose tissue such as bone marrow. Increased evidence has shown that preadipocytes contribute to tumor progression and resistance to chemotherapy (21).

In this study, we evaluated whether preadipocytes and adipocytes could contribute to resistance to trastuzumab. Using a co-culture system of human cell lines, we showed that preadipocytes and adipocytes inhibited trastuzumab-mediated ADCC in breast cancer cells via secretion of soluble factors, resulting in a direct protective effect on tumor cells.

## Results

### *hMADS and differentiated hMADS cells inhibit trastuzumab-mediated ADCC*

To investigate the role of preadipocytes and adipocytes, ADCC assays were performed on different human breast cancer cells in presence of undifferentiated hMADS or differentiated hMADS (#hMADS) (corresponding to models of preadipocytes and adipocytes, respectively) or their control media. As shown in Figure 1A, NK-92-CD16 cells strongly mediated lysis of HER2-overexpressing target cells in the presence of trastuzumab. Both hMADS and #hMADS cells reduced trastuzumab-mediated ADCC against HER2+/ER+ BT474 and HER2+/ER- MDA-MB-453 cells by approximately 30%. In contrast, no inhibition of ADCC was observed in the presence of immortalized human mammary epithelial HME1 cells. This result suggests that the inhibition of ADCC was not due to the physical presence of a feeder cell layer, but was specific to preadipocytes and adipocytes. The inhibition of ADCC by hMADS and #hMADS cells was also observed to a lesser extent with HER2+/ER- SKBR3 cells, but not with HER2+/ER+ MDA-MB-361 cells (Supplementary Fig. S1). This was not related to the difference in levels of HER2 expression between these cell lines (Supplementary Fig. S2) Therefore, we chose BT474 cells as target cells for later experiments. Interestingly, under hypoxic conditions with 1% O<sub>2</sub>, hMADS and #hMADS cells inhibited ADCC even more markedly (up to 60%) than under normoxic conditions (Fig. 1B). Collectively, these results suggest that preadipocytes and adipocytes protect tumor cells from antibody-mediated lysis of tumor cells by NK cells.

### *Inhibition of ADCC is mediated by soluble factors*

To investigate whether the inhibition of ADCC by hMADS and #hMADS cells was dependent on cell contact or mediated by soluble factors, we performed ADCC in the presence of the conditioned media from hMADS and #hMADS cells (hMADS-CM and #hMADS-CM, respectively). As shown in Figure 2A and 2B, inhibition of ADCC by conditioned media was similar to that observed in cell co-culture experiments. Moreover, similar results were obtained when using conditioned medium of primary human adipose-derived stem cells (ASCs), before and after *in vitro* differentiation (Fig. 2C). These data support the hypothesis that inhibition of ADCC by preadipocytes and adipocytes is mediated by soluble factors.

### *Inhibition of ADCC is not caused by antibody sequestration or degradation*

In order to determine whether preadipocytes and adipocytes inhibit ADCC by sequestering the antibody, we first determined the ability of trastuzumab to bind to these cells. As shown in Figure 3A, while trastuzumab bound efficiently to HER-2-expressing BT474 cells, no binding was observed with hMADS or #hMADS cells. In addition, hMADS and #hMADS cells showed no or very low expression of the Fc gamma receptors CD16, CD32 and CD64 (Supplementary Fig. S3). Furthermore, a ten-fold increase in the concentration of trastuzumab did not abolish the inhibition of ADCC by hMADS or #hMADS cells (Fig. 3B). Together, these results suggest that the inhibition of ADCC by hMADS and #hMADS cells was not due to a sequestration of the antibody.

Since adipose tissue has been shown to express and secrete metalloproteinases (22,23), enzymatic cleavage of trastuzumab is a possible mechanism of neutralization of the antitumor action of this antibody. For this reason, we investigated the possible alteration of trastuzumab by the conditioned media from hMADS and #hMADS cells. As shown in Figure 3C, FITC-conjugated trastuzumab that was previously pre-incubated with hMADS-CM or #hMADS-CM for 4 h bound to HER2-expressing BT474 cells and FcR-expressing NK-92-CD16 cells similarly to non-pre-incubated antibody. Moreover, trastuzumab pre-incubated overnight in either hMADS-CM or #hMADS-CM displayed ADCC activity as efficiently as unconditioned antibody (Fig. 3D). These results show that there is no degradation of trastuzumab, at least during the time of our studies.

### *NK cells are not altered by preadipocyte- or adipocyte-conditioned media*

To investigate whether hMADS- and #hMADS-secreted factors alter NK cells phenotype or functions, we analyzed NK cell markers at the end of the 4 h ADCC assay. As shown in Figure 4A, no modification of CD16 or CD107a expression levels was observed in the presence of hMADS-CM or #hMADS-CM. Additionally, the expression levels of the activation markers CD25 and CD69, and of the other NK receptors, NKG2D, NKp30, NKp44, and NKG2A, were unchanged (Fig. 4A). When NK-92-CD16 cells were pre-incubated overnight with hMADS-CM or #hMADS-CM, we did not observe any modification of NK cell viability nor of ability of NK cells to exert cytotoxicity in ADCC assays (Fig. 4B).

Furthermore, the conditioned media did not inhibit NK cytotoxicity towards K562 cells (Fig. 4C). Overall, these data suggest that NK-92-CD16 cells were not functionally altered by hMADS-CM or #hMADS-CM.

#### *Conditioned medium from adipocytes reduce sensitivity of tumor cells to ADCC*

We studied whether hMADS-CM and #hMADS-CM had a direct effect on tumor cells to reduce their sensitivity to ADCC. Since HER-2 is the target for trastuzumab, we evaluated HER-2 expression on BT474 cells exposed to conditioned media and observed no modification of HER-2 expression levels (Fig. 5A) nor change of HER-2 distribution on plasma membrane (Fig. 5B). However, BT474 cells pre-incubated overnight with #hMADS-CM were significantly less sensitive to lysis than cells pre-incubated with the control medium (Fig. 5C). By contrast, no change was observed with hMADS-CM-pre-incubated BT474 cells. Moreover, exposure of BT474 cells to #hMADS-CM rapidly induced the phosphorylation of Akt and MAPK (Fig 5D). The phosphorylation of Akt reached its maximal after 30 min and lasted for at least after exposure. These results show that conditioned-medium from #hMADS cells have a direct effect on tumor cells to reduce their sensitivity to ADCC.

#### *Adipocyte-conditioned medium modifies the gene expression profiles of breast cancer cells*

In order to gain insight into the changes induced in tumor cells by adipocyte-secreted factors, we used a transcriptomic approach on BT474 cells and SKBR3 exposed to #hMADS-CM for 2 h at 37 °C. As shown in Table I and Supplementary Table I, #hMADS-CM rapidly induced modification of the expression of several genes. *ADM*, *SERPINA3*, *NCOA7* were found to be up-regulated in both BT474 and SKBR3 cells, while *ID2*, *CYP1A1* and *ID1* were down-regulated in both cell lines. Interestingly, *GDF15*, *MYC*, *PMAIP1*, *EGR1* and *DKK1* were up-regulated only in BT474 cells but not in SKBR3 cells which were previously shown to be less sensitive to #hMADS-induced inhibition of ADCC. We further analyzed by RT-qPCR the kinetic induction by #hMADS-CM of the four most up-regulated genes (*ADM*, *GDF15*, *MYC* and *SERPINA3*), as well as *EGR1* in BT474 cells (Supplementary Fig. S4). While the expression levels of *ADM* and *EGR1* rapidly increased and reached their maxima at 1 h, *GDF15* and *MYC* were induced at maximal levels between 2 and 3 h after exposure. The

expression of *SERPINA3* increased more slowly but steadily, and reached 5-fold increase after 24 h of exposure (data not shown).

*Repression of GDF-15 reverses the inhibition of ADCC induced by #hMADS-CM*

Among the genes induced adipocyte-conditioned medium in only BT474 cells, we focused on two genes: *GDF15* and *SERPINA3*. GDF15 or growth differentiation factor 15, also known under the name of macrophage inhibitory cytokine 1 (MIC-1), belongs to the transforming growth factor beta (TGF- $\beta$ ) superfamily. GDF15 has been shown to play a role in anti-apoptosis (24). The other gene *SERPINA3*, also known as alpha 1-antichymotrypsin, encodes for a member of the serine protease inhibitor family that contains the granzyme B-inhibitor proteinase inhibitor-9 (PI-9)/SERPINB9 (25,26). It was previously shown that the murine *SERPINA3* homologue, *serpina3n*, can inhibit human granzyme B (27). Thus, upregulation of GDF15 or *SERPINA3* could confer resistance to tumor cells against lysis by NK cells. To confirm this hypothesis, we down-regulated the expression of GDF15 and *SERPINA3* in BT474 cells using siRNAs (Fig. 6A). Down-regulation of GDF15 but not *SERPINA3* reversed the #hMADS-CM-induced inhibition of ADCC (Fig. 6B), suggesting that GDF15 is involved in adipocyte-induced tumor resistance to NK lysis.

## Discussion

Adipocytes constitute the most abundant cell type in the adipose tissue proximal to breast cancer cells and actively participate in tumor progression through the paracrine secretion of various adipocytokines (28). Recent studies have suggested that adipocytes promote cancer resistance to chemotherapy and radiotherapy (17,18). However, the role of these cells in resistance to targeted therapy remains unknown. In this study, using an original co-culture system, we investigated the implication of adipocytes and preadipocytes in trastuzumab-mediated ADCC in HER2-overexpressing breast cancer cells.

We demonstrate for the first time that adipocytes and preadipocytes inhibit trastuzumab-mediated tumor lysis by NK cells. This result was further confirmed with primary adipose-derived stem cells (ASCs) and *in vitro* differentiated ASCs. Interestingly, the inhibition of ADCC by adipose cells was strongly enhanced under hypoxic conditions. Since hypoxia is one of the hallmarks of the tumor microenvironment (29), and has also been associated with obesity (30), this latter result underlines the potential clinical relevance of our findings on the impact of adipose tissue on breast cancer targeted therapy.

Inhibition of ADCC by adipose tissue was not due to an effect on the therapeutic antibody itself. We showed that adipocyte-mediated inhibition of ADCC was not due to a titration of the antibody nor to a cell-cell contact but was mediated via factors secreted into the conditioned media. Moreover, trastuzumab incubated in the conditioned media retained its capability to bind target cells and effector cells, as well as its ability to mediate ADCC.

Several studies have pointed out the alteration of NK phenotype or NK functions by the tumor microenvironment (31,32). Moreover, NK cells have been shown to express receptors for different adipokines and their cytotoxicity has been found to be modulated by factors such as leptin and adiponectin (33,34). Here, using the NK-92-CD16 cells, we did not observe any modification of NK cell markers as well as cytotoxicity when NK cells were incubated with adipocyte- or preadipocyte-conditioned media. These results are in contrast with the work by DelaRosa *et al.* who showed that human adipose-derived stem cells (ASCs) impaired NK cell cytotoxicity and NK cell markers, in particular decreased CD16 expression (35). However, in DelaRosa's studies, the NK cells were continuously co-incubated with ASCs for 72 h while in our studies, the incubation times were much shorter (4 h with the cells or overnight-18 h with the conditioned media). It is therefore unlikely that the short-term inhibition of ADCC by hMADS-CM and #hMADS-CM was mediated by an alteration of NK cytotoxicity.

Our results suggest that adipose-derived factors reduce the sensitivity of HER2+ breast tumor cells to trastuzumab-mediated ADCC. This is supported by the fact that the ability of adipose cells to inhibit ADCC was variable depending on the breast tumor cell lines studied, and that BT474 cells pre-incubated overnight in #hMADS-CM showed decreased sensitivity to ADCC. Curiously, pre-incubation of BT474 cells in #hMADS-CM but not hMADS-CM increased the resistance of cancer cells to trastuzumab-mediated ADCC. This result suggests that the protection by preadipocytes may be transient. It is also possible that adipocytes and preadipocytes mediate inhibition of ADCC via different mechanisms, since it is known that adipocytes and preadipocytes possess different secretion profiles.

We showed that exposure of BT474 cells to #hMADS-CM rapidly up-regulated the growth differentiation factor 15 (GDF15). This result is in keeping with the work by Kim *et al.*, in which they showed that the conditioned-medium from adipocytes stimulated the production of GDF15 in MDA-MB-231 breast cancer cells (36). The induction of GDF15 was likely mediated by EGR1 (37), of which the expression was shown in our studies to be induced before GDF15 in BT474 cells.

GDF15 has been shown to be over-expressed in various cancers, including breast, prostate and colorectal cancers (38). Although its role in tumorigenesis is still a matter of debate (39), our results suggest that down-regulation of GDF15 reverses the adipocyte-induced resistance of breast cancer cells to trastuzumab-mediated ADCC. These findings are in agreement with those of Roth *et al.*, in which depletion of GDF15 enhanced the susceptibility of mouse glioma cells to lysis by NK cells (40). Interestingly, GDF15 has also been shown to reduce trastuzumab-mediated growth inhibition in HER2-overexpressing breast cancer cells (41).

In an attempt to identify the adipocyte-secreted factor(s) involved in inhibition of ADCC, we tested the ability of known adipocytokines to inhibit ADCC under our experimental conditions. Neither leptin, adiponectin, IL-6, TNF- $\alpha$ , nor TGF- $\beta$  were found to reproduce the inhibition observed with the conditioned medium (data not shown). As the adipocyte-secretome is extremely diverse, a number of other adipocyte-derived factors could be tested. Previous studies by Kim *et al.* suggested that free fatty acids, notably palmitate, may be released by adipocytes and induce GDF15 expression in cancer cells (36). Alternatively, Ding *et al.* demonstrated that both adipocytes and preadipocytes also secrete GDF15 (42). Thus, adipocytes and preadipocytes could mediate the inhibition of ADCC via direct secretion of GDF15 or indirectly via the induction of GDF15 production by cancer cells. It is also noteworthy that in our short-time co-culture system, we did not observe any

delipidation of adipocytes by tumor cells, as described previously by Dirat *et al.* (43) (data not shown).

In conclusion, we have demonstrated the potential implication of adipocytes and preadipocytes in the resistance of breast cancer cells to trastuzumab-mediated ADCC. Although our results require further validations such as *in vivo* models using animals or human samples, they raise the possibility of a link between obesity and resistance to targeted therapy in breast cancer patients. Of interest, a recent study by Crozier *et al.* showed that increased body mass index (BMI) is associated with shorter disease-free survival in HER2-overexpressing breast cancer patients, although trastuzumab improved clinical outcome regardless of BMI (44). Further studies are required to elucidate the mechanisms of resistance mediated by GDF15 and to develop sensitizing strategies to circumvent adipose tissue mediated resistance to trastuzumab in breast cancer.

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## Materials and Methods

### *Cell culture*

The HER-2 overexpressing human breast cancer cell lines (BT474, SKBR3, MDA-MB-453, MDA-MB-361, all obtained from ATCC) were cultured in complete RPMI medium (RPMI supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin). The human multipotent adipose-derived stem cells (hMADS) were provided by Dr. Christian Dani, *UMR 6543 CNRS*, Nice, France. They were cultured in complete DMEM medium with 1 g/L glucose and supplemented with 2.5 ng/mL basic fibroblast growth factor (FGF2). In some experiments, the immortalized human mammary epithelial hTERT-HME1 cells were used as control. They were cultured in DMEM-F12 medium supplemented with 10% FCS, 2 mM L-glutamine, 1% nonessential amino acids, 50 µg/mL gentamycin, 3.5 µg/mL insulin, 100 ng/mL epidermal growth factor, 500 ng/mL hydrocortisone. All cells were maintained at 37°C in presence of 5% CO<sub>2</sub>, except in hypoxic experiments in which cells were maintained in 1% O<sub>2</sub>.

### *Retroviral transduction of NK-92 cells*

NK-92, the human NK cell line (45), generously provided by Conkwest (Del Mar, CA), was grown in complete RPMI culture medium. NK-92-CD16 cells were obtained by transduction of pMX/CD16 plasmid (46) using retroviral supernatant. Transient retroviral supernatants were produced by CaCl<sub>2</sub> precipitation (Invitrogen) with 15 µg pMX/CD16 plasmid using Phoenix-Ampho cells. The conditioned medium was collected 24-48 h post-transfection, filtered through 0.45-µm pore-size filters and kept at -80°C until use. The viral titer was determined by the transduction of Jurkat T cells (10<sup>6</sup> cells per well in 6-well plates) with serial dilutions. Retroviral supernatant titers were typically 1-5\*10<sup>5</sup> IU (Infectious Units)/ml. The NK-92 cell line was seeded at 10<sup>6</sup> cells into 6-well plates and exposed to 2\*2 ml of retroviral supernatant by spinoculation (2400 g, 1.5 h, 32°C) in the presence of 4 µg/ml polybrene (Sigma, St Quentin Fallavier, France) to generate the NK-92-CD16 cells. The culture medium was changed 24 h post-infection. Transduction efficiencies were assessed 5 days later by flow cytometry after staining with the CD16 PE-conjugated mouse anti-CD16 antibody (clone 3G8) (Beckman Coulter, Roissy, France). NK-92-CD16 cells were selected for higher CD16 expression by cell sorter flow cytometry using anti-CD16 antibody (Beckman Coulter, Roissy, France). Purity was >95%.

### *Induction of hMADS differentiation*

The differentiation process of hMADS cells was previously described (47,48). Briefly, hMADS cells were plated at high density (30,000 cells/cm<sup>2</sup>) in growth medium containing 2.5 ng/mL FGF2. Two days later, the medium was changed without FGF2 and cells were cultured to reach confluence for additional two days. Cells were induced for differentiation in DMEM/F12 medium supplemented with insulin (5 µg/mL), transferrin (10 µg/mL), T3 (0.2 nM), rosiglitazone (1 µM), 3-Isobutyl-1-methylxanthine (IBMX) (100 µM), and dexamethasone (1 µM) (day 0). At day 3, the medium was changed without IBMX nor dexamethasone. At day 12 of differentiation, the medium was replaced and supplemented with 10% FCS. Differentiated hMADS (#hMADS) cells were used at day 14 of differentiation. The differentiation yield was estimated to range between 70 and 80% and was verified by Oil Red O (Sigma) staining, as described previously (49). The conditioned media (CM) from hMADS and #hMADS cells were harvested after centrifugation at 300 g for 5 min and frozen at -20°C before use.

### *Isolation and differentiation of human adipose-derived stem cells (ASCs)*

Adipose tissues were provided by Dr Emmanuel Delay (Centre Léon Bérard, Lyon, France) and were obtained by liposuction from abdominal fat of patients undergoing plastic surgery. Written patient consent was obtained and this protocol was approved by the Lyon Ethics Committee. Adipose tissue samples were rapidly digested with 100 collagen digestion units (CDU)/mL collagenase (Sigma) at 37°C with agitation for 30 min. Digestion was stopped by addition of complete DMEM-F12 medium. After centrifugation at 300 g for 7 min, cells corresponding to the stromal vascular fraction (SVF) were seeded for adherence and amplification in complete DMEM/F12 medium supplemented with 10 ng/mL FGF2. Adipose-derived stem cells (ASCs) were used at passage 2 or 3, and verified by flow cytometry to be CD14-, CD45-, CD73+, CD90+, CD105+ and HLA-ABC+. Differentiation of ASCs into adipocytes was performed as described previously (50). Briefly, cells were seeded for adhesion at 40,000 cells/cm<sup>2</sup> in proliferation medium for 24 h. Cells were induced to differentiation by replacing the medium with complete DMEM/F12 medium supplemented with 2nM T3, 10 nM hydrocortisone, 0.5 µM dexamethasone, 0.5 mM IBMX, 1 µM rosiglitazone, and 5.7 µg/mL insulin. Differentiated ASCs (#ASCs) were used at day 14 of differentiation, with a differentiation yield more than 90% and verified by microscopy.

### *Antibody-dependent cellular cytotoxicity (ADCC) assay*

Hme-1 and hMADS cells were seeded at  $1.2 \times 10^5$  cells/1 mL/well in 12-well plates. The medium of 12-day #hMADS was changed and supplemented with 10% FCS. Two days later, target cells were labeled with 12.5  $\mu$ M calcein-AM (Sigma-Aldrich) for 30 min at 37°C and added at  $10^5$  cells/0.5 mL/well. NK-92-CD16 cells were added at  $5 \times 10^5$  cells/0.5 mL/well (effector : target (E:T) ratio = 5:1), with trastuzumab at 1  $\mu$ g/mL final. After 4 h of incubation at 37°C, the supernatants in each well were harvested and measured for the fluorescence signals at 485/535 nm. Triton X100 (Sigma-Aldrich) was added in control wells to estimate total cell lysis. Cytotoxicity was calculated using the formula: percent cytotoxicity = (experimental lysis – spontaneous lysis) / (maximal lysis – spontaneous lysis) \* 100.

### *Flow cytometry analyses*

Trypsinized cells or suspended cells were washed in PBS and incubated with corresponding antibodies for 15 min at room temperature. After wash, cells were analyzed with a BD LSRII cytometer using BD FACSDiva and Flowjo softwares. The antibodies used were purchased from BD Biosciences (anti-CD16 FITC clone 3G8, anti-CD25 FITC clone 2A3, anti-CD56 V450 clone B159, anti-CD69 PE clone FN50, anti-CD107a PE clone H4A3), Miltenyi (anti-CD159a (NKG2A) FITC clone REA110, anti-CD314 (NKG2D) APC clone BAT221, anti-CD336 (NKp44) APC clone 2.29, and anti-CD337 (NKp30) PE clone AF29-4D12). Anti-ErbB2 (HER2) affibody FITC was from Abcam.

### *Fluorescent microscopy*

BT474 cells were seeded overnight on Lab-Tek chambered coverglass. Conditioned media from #hMADS or hMADS cells were added and cells were incubated for additional 4 h at 37 °C before being labeled on ice with anti-ErbB2 affibody FITC (Abcam) for 15 min. After washing, cells were observed with a confocal Zeiss LSM 780 microscope.

### *Microarray analysis*

BT474 cells or SKBR3 cells were exposed to # hMAD-CM for 2 h at 37°C, 5% CO<sub>2</sub>. Cells were harvested and RNA was extracted using Qiamp RNeasy mini kit (Qiagen). After RNA amplification with totalprep RNA amplification kit (Ambion), cRNA was hybridized on humanHT-12 v4 beadchip (Illumina). Scan was performed with an Illumina iScan and data was analyzed with GeneSpring software.

### *Reverse transcription and quantitative PCR*

BT474 cells were exposed to # hMAD-CM or hMAD-CM for various times. Cells were harvested by trypsinization and RNA was extracted using RNeasy mini kit (Qiagen). Reverse transcription was performed using random primers (Invitrogen). Quantitative PCR was performed with primers (Quiagen) of indicated genes using a NANO light cycler (Roche).

### *siRNA transfection*

BT474 cells were seeded at  $2 \times 10^6$  cells/T25 flask. After 24 h, cells were transfected with 10 nM siRNA (Qiagen) against indicated genes using lipofectamin RNAiMAX (Invitrogen) according to the manufacturer's instructions. After 48 h post-transfection, cells were trypsinized and used for RT-qPCR to verify mRNA repression and for ADCC assays.

### *xCelligence assay*

The xCelligence system (ACEA Biosciences) provides a real time measure of the cell index, which takes into account cell number, cell morphology and cell adherence. The system measures electrical impedance across interdigitated microelectrodes integrated on the bottom of E-plates. Cells were seeded at  $10^4$  cells/100  $\mu$ L/well in E-plate 16 to adhere for 24 h. NK-92-CD16 cells were added in 25  $\mu$ L at E:T ratio = 2:1. The #hMADS-CM or hMADS-CM or their control media were added at 125  $\mu$ L/well, with trastuzumab final concentration of 1  $\mu$ g/mL. Cell index was monitored every 5 min and normalized (nCI) to the time point immediately preceding NK-92-CD16 and trastuzumab addition.

### *Statistical analysis*

Statistical significance was evaluated using paired Student's t test on the means of at least three independent experiments. P values below 0.05 (\*); 0.01 (\*\*) were deemed as significant while "ns" stands for not significant.

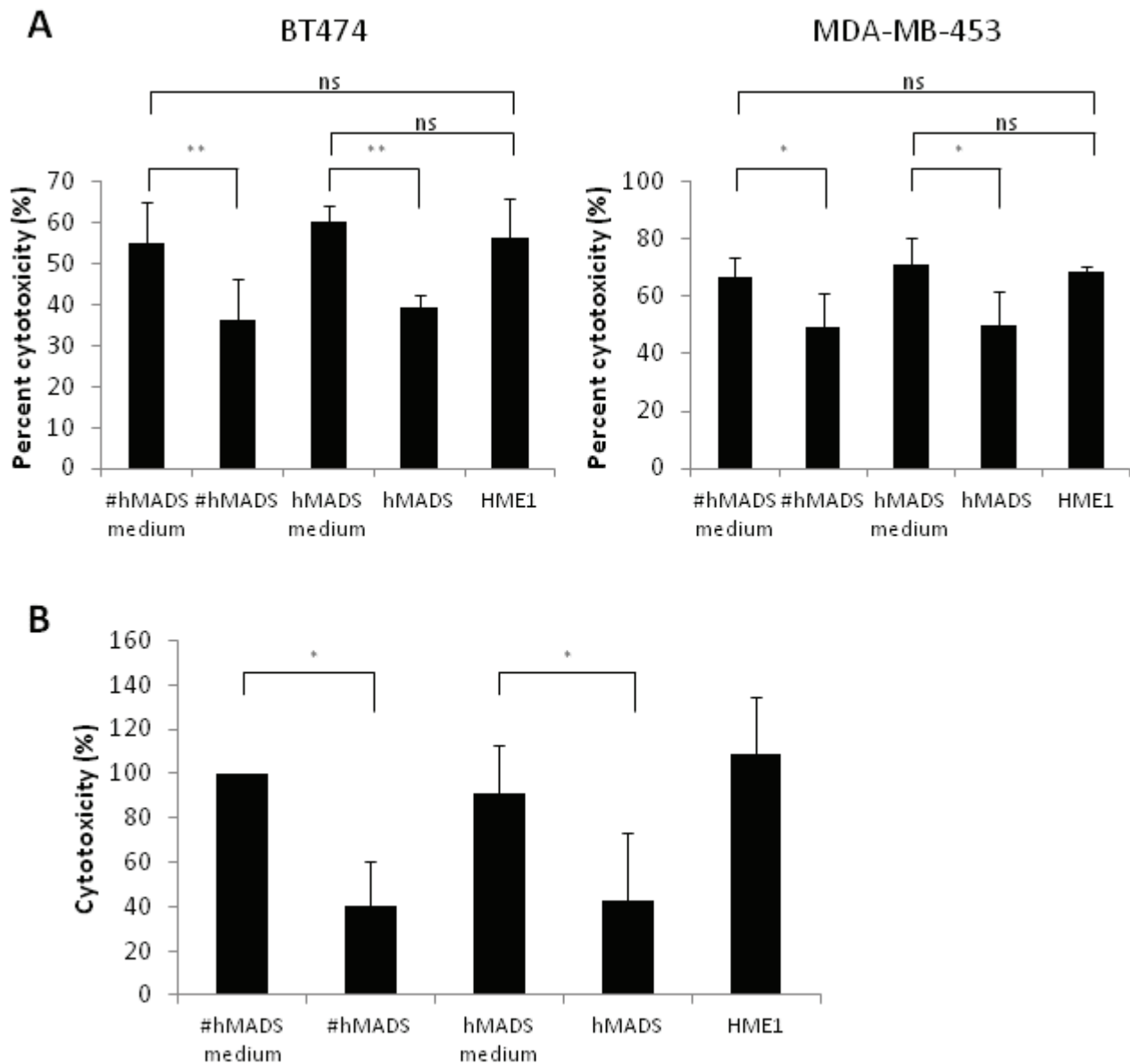
## References

1. Ross JS, Slodkowska EA, Symmans WF, Puzstai L, Ravdin PM, Hortobagyi GN. The HER-2 receptor and breast cancer: ten years of targeted anti-HER-2 therapy and personalized medicine. *The oncologist*. 2009 Apr;14(4):320–68.
2. Hudis CA. Trastuzumab--mechanism of action and use in clinical practice. *N Engl J Med*. 2007 Jul 5;357(1):39–51.
3. Musolino A, Naldi N, Bortesi B, Pezzuolo D, Capelletti M, Missale G, et al. Immunoglobulin G fragment C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2/neu-positive metastatic breast cancer. *J Clin Oncol*. 2008 Apr 10;26(11):1789–96.
4. Park S, Jiang Z, Mortenson ED, Deng L, Radkevich-Brown O, Yang X, et al. The therapeutic effect of anti-HER2/neu antibody depends on both innate and adaptive immunity. *Cancer Cell*. 2010 Aug 9;18(2):160–70.
5. Nahta R, Esteva FJ. HER2 therapy: Molecular mechanisms of trastuzumab resistance. *Breast Cancer Res*. 2006 Nov 6;8(6):215.
6. Nagata Y, Lan K-H, Zhou X, Tan M, Esteva FJ, Sahin AA, et al. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. *Cancer Cell*. 2004 Aug;6(2):117–27.
7. Brodowicz T, Wiltschke C, Budinsky AC, Krainer M, Steger GG, Zielinski CC. Soluble HER-2/neu neutralizes biologic effects of anti-HER-2/neu antibody on breast cancer cells in vitro. *Int J Cancer*. 1997 Dec 10;73(6):875–9.
8. Köstler WJ, Schwab B, Singer CF, Neumann R, Rücklinger E, Brodowicz T, et al. Monitoring of serum Her-2/neu predicts response and progression-free survival to trastuzumab-based treatment in patients with metastatic breast cancer. *Clin Cancer Res*. 2004 Mar 1;10(5):1618–24.
9. Price-Schiavi SA, Jepson S, Li P, Arango M, Rudland PS, Yee L, et al. Rat Muc4 (sialomucin complex) reduces binding of anti-ErbB2 antibodies to tumor cell surfaces, a potential mechanism for herceptin resistance. *Int J Cancer*. 2002 Jun 20;99(6):783–91.
10. Chen AC, Migliaccio I, Rimawi M, Lopez-Tarruella S, Creighton CJ, Massarweh S, et al. Upregulation of mucin4 in ER-positive/HER2-overexpressing breast cancer xenografts with acquired resistance to endocrine and HER2-targeted therapies. *Breast Cancer Res Treat*. 2012 Jul;134(2):583–93.
11. Lu Y, Zi X, Zhao Y, Mascarenhas D, Pollak M. Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin). *J Natl Cancer Inst*. 2001 Dec 19;93(24):1852–7.
12. Mantovani A, Schioppa T, Porta C, Allavena P, Sica A. Role of tumor-associated macrophages in tumor progression and invasion. *Cancer Metastasis Rev*. 2006 Sep 1;25(3):315–22.
13. Ostrand-Rosenberg S, Sinha P. Myeloid-Derived Suppressor Cells: Linking Inflammation and Cancer. *J Immunol*. 2009 Apr 15;182(8):4499–506.
14. Zou W. Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol*. 2006 Apr;6(4):295–307.
15. Waki H, Tontonoz P. Endocrine functions of adipose tissue. *Annu Rev Pathol*. 2007;2:31–56.
16. Hefetz-Sela S, Scherer PE. Adipocytes: Impact on tumor growth and potential sites for therapeutic intervention. *Pharmacol Ther*. 2013 Jan 24;
17. Bochet L, Meulle A, Imbert S, Salles B, Valet P, Muller C. Cancer-associated adipocytes promotes breast tumor radioresistance. *Biochem Biophys Res Commun*. 2011 Jul 22;411(1):102–6.
18. Behan JW, Yun JP, Proektor MP, Ehsanipour EA, Arutyunyan A, Moses AS, et al. Adipocytes impair leukemia treatment in mice. *Cancer Res*. 2009 Oct 1;69(19):7867–74.
19. Calle EE, Kaaks R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. *Nat Rev Cancer*. 2004 Aug;4(8):579–91.

20. Ewertz M, Jensen M-B, Gunnarsdóttir KÁ, Højris I, Jakobsen EH, Nielsen D, et al. Effect of obesity on prognosis after early-stage breast cancer. *J Clin Oncol*. 2011 Jan 1;29(1):25–31.
21. Houthuijzen JM, Daenen LGM, Roodhart JML, Voest EE. The role of mesenchymal stem cells in anti-cancer drug resistance and tumour progression. *Br J Cancer*. 2012 Jun 5;106(12):1901–6.
22. Maquoi E, Munaut C, Colige A, Collen D, Lijnen HR. Modulation of adipose tissue expression of murine matrix metalloproteinases and their tissue inhibitors with obesity. *Diabetes*. 2002 Apr;51(4):1093–101.
23. Andarawewa KL, Motrescu ER, Chenard M-P, Gansmuller A, Stoll I, Tomasetto C, et al. Stromelysin-3 is a potent negative regulator of adipogenesis participating to cancer cell-adipocyte interaction/crosstalk at the tumor invasive front. *Cancer Res*. 2005 Dec 1;65(23):10862–71.
24. Schiegnitz E, Kämmerer PW, Koch FP, Krüger M, Berres M, Al-Nawas B. GDF 15 as an anti-apoptotic, diagnostic and prognostic marker in oral squamous cell carcinoma. *Oral Oncol*. 2012 Jul;48(7):608–14.
25. Kaiserman D, Bird PI. Control of granzymes by serpins. *Cell Death Differ*. 2010 Apr;17(4):586–95.
26. Bird CH, Sutton VR, Sun J, Hirst CE, Novak A, Kumar S, et al. Selective regulation of apoptosis: the cytotoxic lymphocyte serpin proteinase inhibitor 9 protects against granzyme B-mediated apoptosis without perturbing the Fas cell death pathway. *Mol Cell Biol*. 1998 Nov;18(11):6387–98.
27. Sipione S, Simmen KC, Lord SJ, Motyka B, Ewen C, Shostak I, et al. Identification of a novel human granzyme B inhibitor secreted by cultured sertoli cells. *J Immunol Baltim Md 1950*. 2006 Oct 15;177(8):5051–8.
28. Tan J, Buache E, Chenard M-P, Dali-Youcef N, Rio M-C. Adipocyte is a non-trivial, dynamic partner of breast cancer cells. *Int J Dev Biol*. 2011;55(7-9):851–9.
29. Bertout JA, Patel SA, Simon MC. The impact of O<sub>2</sub> availability on human cancer. *Nat Rev Cancer*. 2008 Dec;8(12):967–75.
30. Kabon B, Nagele A, Reddy D, Eagon C, Fleshman JW, Sessler DI, et al. Obesity decreases perioperative tissue oxygenation. *Anesthesiology*. 2004 Feb;100(2):274–80.
31. Mamessier E, Sylvain A, Thibult M-L, Houvenaeghel G, Jacquemier J, Castellano R, et al. Human breast cancer cells enhance self tolerance by promoting evasion from NK cell antitumor immunity. *J Clin Invest*. 2011 Sep;121(9):3609–22.
32. Saito H, Osaki T, Ikeguchi M. Decreased NKG2D expression on NK cells correlates with impaired NK cell function in patients with gastric cancer. *Gastric Cancer*. 2012 Jan;15(1):27–33.
33. Zhao Y, Sun R, You L, Gao C, Tian Z. Expression of leptin receptors and response to leptin stimulation of human natural killer cell lines. *Biochem Biophys Res Commun*. 2003 Jan 10;300(2):247–52.
34. Kim K-Y, Kim JK, Han SH, Lim J-S, Kim KI, Cho DH, et al. Adiponectin is a negative regulator of NK cell cytotoxicity. *J Immunol Baltim Md 1950*. 2006 May 15;176(10):5958–64.
35. DelaRosa O, Sánchez-Correa B, Morgado S, Ramírez C, del Río B, Menta R, et al. Human adipose-derived stem cells impair natural killer cell function and exhibit low susceptibility to natural killer-mediated lysis. *Stem Cells Dev*. 2012 May 20;21(8):1333–43.
36. Kim JH, Kim K, Jeon JH, Lee SH, Hwang J-E, Lee JH, et al. Adipocyte culture medium stimulates production of macrophage inhibitory cytokine 1 in MDA-MB-231 cells. *Cancer Lett*. 2008 Mar 18;261(2):253–62.
37. Baek SJ, Kim J-S, Moore SM, Lee S-H, Martinez J, Eling TE. Cyclooxygenase inhibitors induce the expression of the tumor suppressor gene EGR-1, which results in the up-regulation of NAG-1, an antitumorigenic protein. *Mol Pharmacol*. 2005 Feb;67(2):356–64.
38. Welsh JB, Sapinoso LM, Kern SG, Brown DA, Liu T, Bauskin AR, et al. Large-scale delineation of secreted protein biomarkers overexpressed in cancer tissue and serum. *Proc Natl Acad Sci U S A*. 2003 Mar 18;100(6):3410–5.

39. Breit SN, Johnen H, Cook AD, Tsai VWW, Mohammad MG, Kuffner T, et al. The TGF- $\beta$  superfamily cytokine, MIC-1/GDF15: a pleiotropic cytokine with roles in inflammation, cancer and metabolism. *Growth Factors Chur Switz*. 2011 Oct;29(5):187–95.
40. Roth P, Junker M, Tritschler I, Mittelbronn M, Dombrowski Y, Breit SN, et al. GDF-15 contributes to proliferation and immune escape of malignant gliomas. *Clin Cancer Res*. 2010 Aug 1;16(15):3851–9.
41. Joshi JP, Brown NE, Griner SE, Nahta R. Growth differentiation factor 15 (GDF15)-mediated HER2 phosphorylation reduces trastuzumab sensitivity of HER2-overexpressing breast cancer cells. *Biochem Pharmacol*. 2011 Nov 1;82(9):1090–9.
42. Ding Q, Mracek T, Gonzalez-Muniesa P, Kos K, Wilding J, Trayhurn P, et al. Identification of macrophage inhibitory cytokine-1 in adipose tissue and its secretion as an adipokine by human adipocytes. *Endocrinology*. 2009 Apr;150(4):1688–96.
43. Dirat B, Bochet L, Dabek M, Daviaud D, Dauvillier S, Majed B, et al. Cancer-associated adipocytes exhibit an activated phenotype and contribute to breast cancer invasion. *Cancer Res*. 2011 Apr 1;71(7):2455–65.
44. Crozier JA, Moreno-Aspitia A, Ballman KV, Dueck AC, Pockaj BA, Perez EA. Effect of body mass index on tumor characteristics and disease-free survival in patients from the HER2-positive adjuvant trastuzumab trial N9831. *Cancer*. 2013 Jul 1;119(13):2447–54.
45. Gong JH, Maki G, Klingemann HG. Characterization of a human cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells. *Leukemia*. 1994 Apr;8(4):652–8.
46. Clémenceau B, Congy-Jolivet N, Gallot G, Vivien R, Gaschet J, Thibault G, et al. Antibody-dependent cellular cytotoxicity (ADCC) is mediated by genetically modified antigen-specific human T lymphocytes. *Blood*. 2006 Jun 15;107(12):4669–77.
47. Rodriguez A-M, Pisani D, Dechesne CA, Turc-Carel C, Kurzenne J-Y, Wdziekonski B, et al. Transplantation of a multipotent cell population from human adipose tissue induces dystrophin expression in the immunocompetent mdx mouse. *J Exp Med*. 2005 May 2;201(9):1397–405.
48. Zaragosi L-E, Ailhaud G, Dani C. Autocrine fibroblast growth factor 2 signaling is critical for self-renewal of human multipotent adipose-derived stem cells. *Stem Cells Dayt Ohio*. 2006 Nov;24(11):2412–9.
49. Abderrahim-Ferkoune A, Bezy O, Astri-Roques S, Elabd C, Ailhaud G, Amri E-Z. Transdifferentiation of preadipose cells into smooth muscle-like cells: role of aortic carboxypeptidase-like protein. *Exp Cell Res*. 2004 Feb 15;293(2):219–28.
50. Lequeux C, Auxenfans C, Mojallal A, Sergent M, Damour O. Optimization of a culture medium for the differentiation of preadipocytes into adipocytes in a monolayer. *Biomed Mater Eng*. 2009;19(4-5):283–91.

# Figure 1.

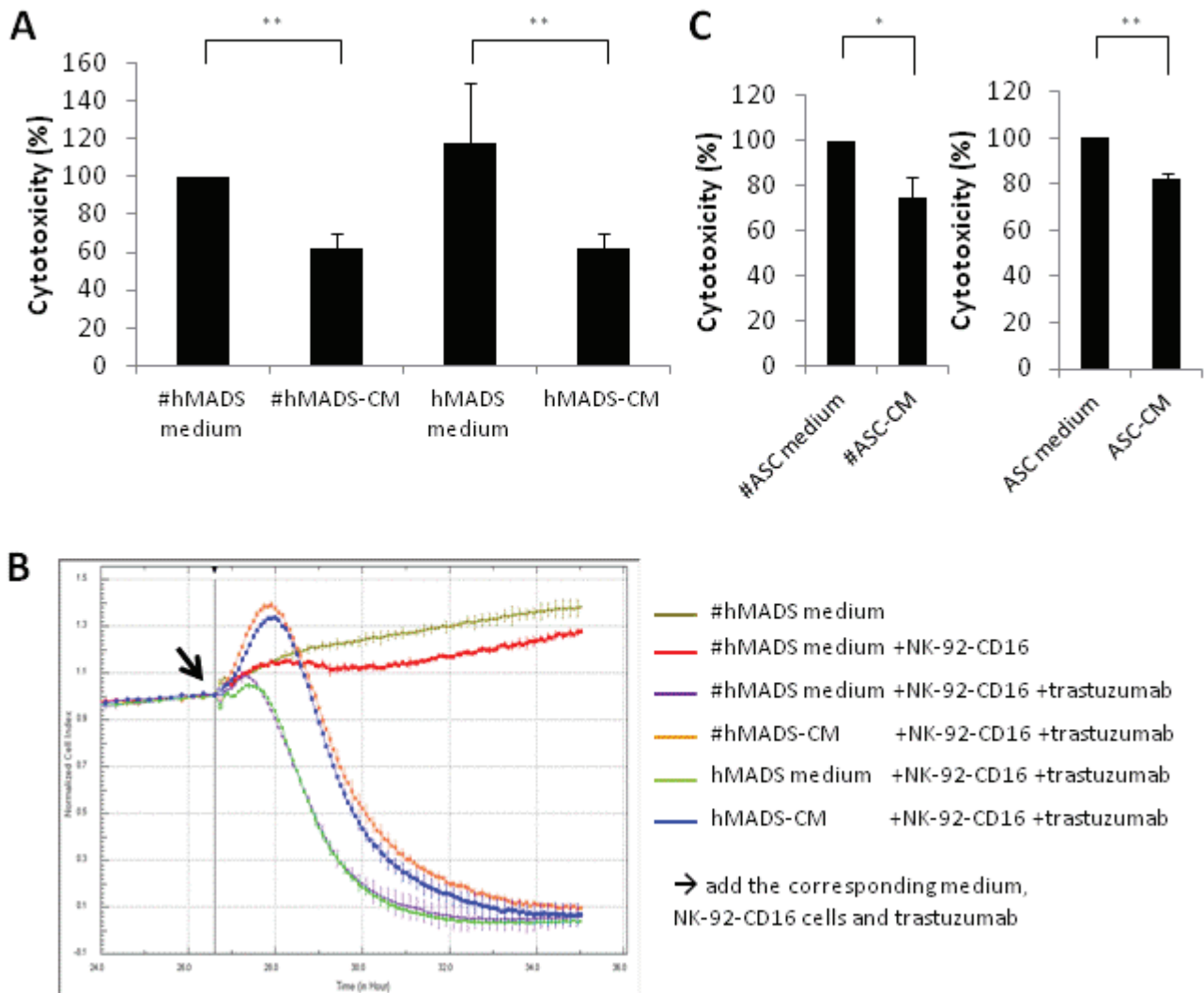


**Figure 1. hMADS and #hMADS cells inhibit ADCC.**

**A)** HER-2+/ER+ BT474 or HER-2+/ER- MDA-MB-453 human breast cancer cells were labeled with 12.5  $\mu$ M calcein and incubated with 1  $\mu$ g/mL trastuzumab and NK-92-CD16 cells at an effector to target (E:T) ratio = 5:1, in the presence of hMADS, #hMADS or HME1 cells. After 4 h of incubation, the percent cytotoxicity was calculated as indicated in the materials and methods. **B)** ADCC assay on BT474 cells was performed similarly as in A), but under hypoxic conditions with 1% O<sub>2</sub>. The cytotoxicity was normalized with the control #hMADS medium as 100%. Values shown are means  $\pm$  SD of at least 3 independent experiments.



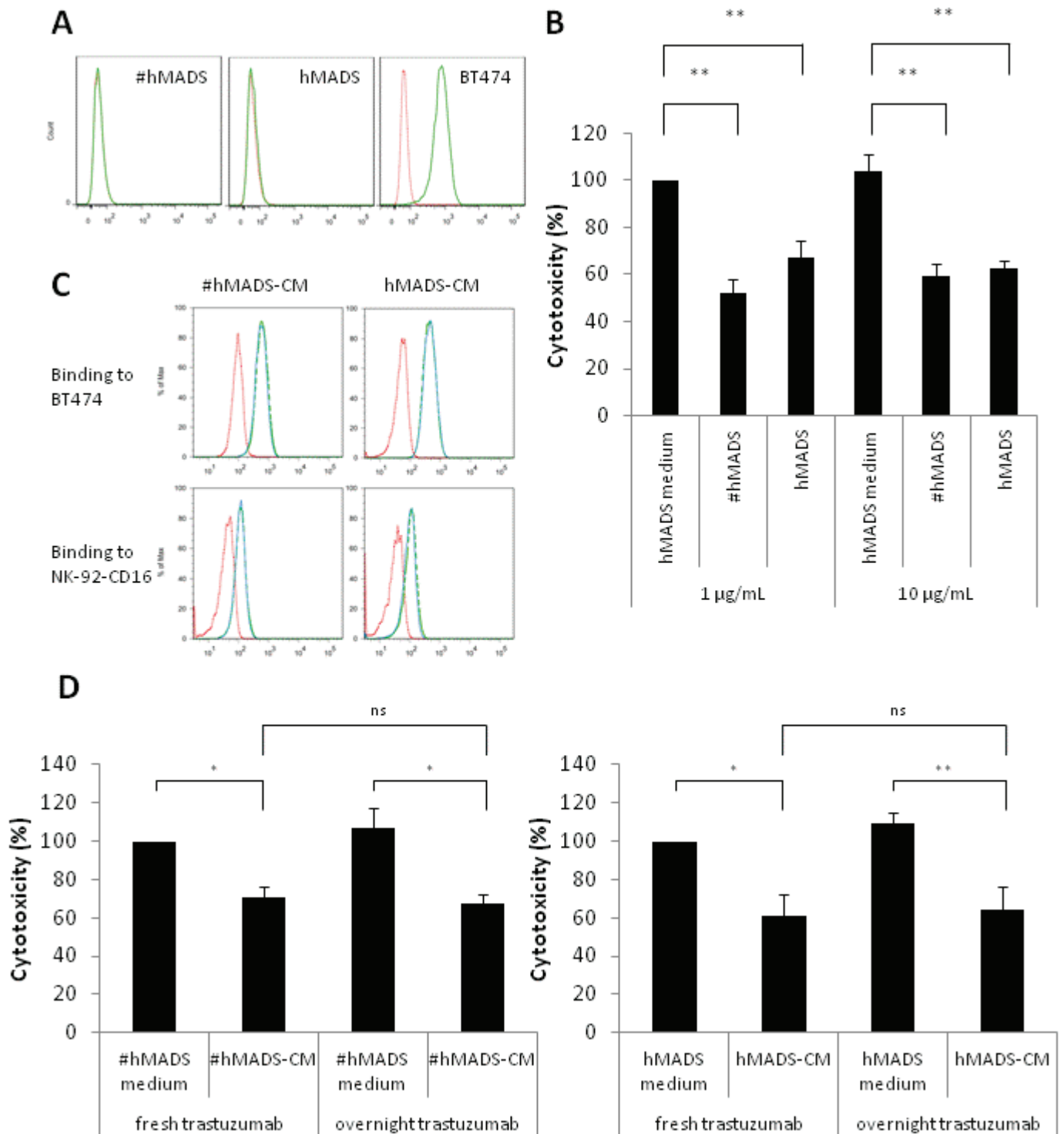
## Figure 2.



**Figure 2. Adipocyte- and preadipocyte-derived soluble factors inhibit ADCC.**

**A)** BT474 cells were labeled with 12.5  $\mu$ M calcein and co-incubated with NK-92-CD16 cells (E:T ratio = 5:1) and 1  $\mu$ g/mL trastuzumab, in presence of the conditioned media (CM) from hMADS or #hMADS cells (v/v=1/1). After 4 h, the cytotoxicity was calculated and normalized with the control medium as 100 %. **B)** BT474 cells were seeded for 24 h prior to adding NK-92-CD16 cells (E:T ratio = 2:1), trastuzumab (1  $\mu$ g/mL) and hMADS-CM or #hMADS-CM or their control media. Cell index was given by xCELLigence every 5 min and normalized with the cell index at the time of addition. **C)** Similar to A), but the conditioned media were from ASCs or #ASCs. Values shown in A and C are means  $\pm$  SD of at least 3 independent experiments, and a result representative of 3 independent experiments is shown in B.

## Figure 3.

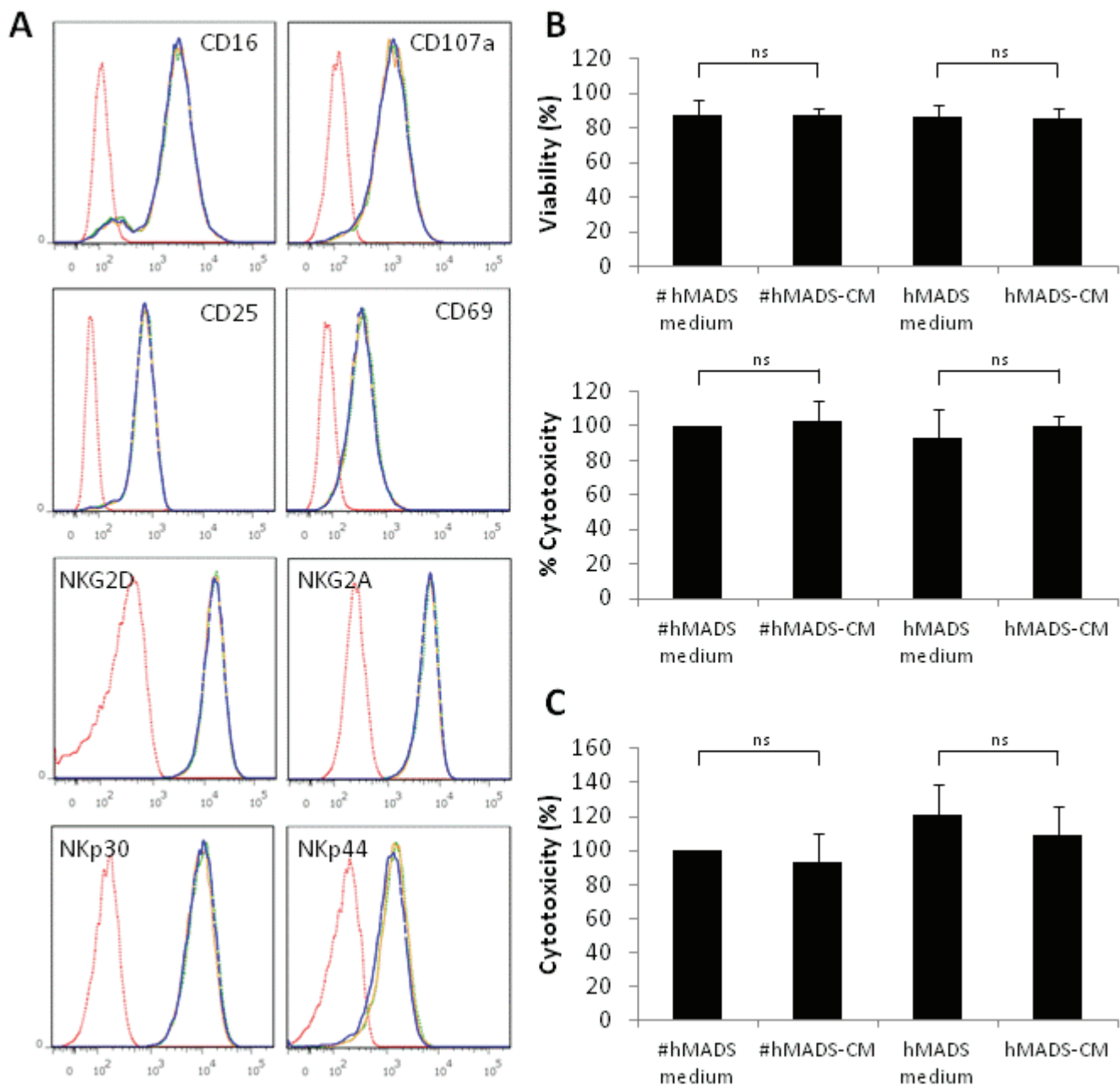


**Figure 3. hMADS and #hMADS cells do not alter trastuzumab.**

**A)** BT474, hMADS and #hMADS cells were incubated with 1  $\mu\text{g/mL}$  FITC-trastuzumab for 4 h at 37°C and analyzed by FACS. Dotted red lines indicate unstained cells and solid green lines indicate cells labeled with FITC-trastuzumab. **B)** BT474 cells were labeled with 12.5

$\mu$ M calcein and co-incubated with NK-92-CD16 cells (E:T ratio = 5:1) and 1  $\mu$ g/mL or 10  $\mu$ g/mL trastuzumab, in presence of hMADS or #hMADS cells. After 4 h, the cytotoxicity was calculated and normalized with the control medium as 100 %. **C)** FITC-trastuzumab was incubated in hMADS-CM or #hMADS-CM or the corresponding control media for 4 h at 37°C, then used to label BT474 cells at 1  $\mu$ g/mL (upper panels) or NK-92-CD16 cells at 10  $\mu$ g/mL (lower panels). Dotted red lines indicate unstained cells, dashed green lines indicate control media and solid blue lines indicate #hMADS-CM (left panels) or hMADS-CM (right panels). **D)** Trastuzumab was incubated in #hMADS-CM (left panel) or hMADS-CM (right panel) or their control media overnight. On the next day, calcein-labeled BT474 cells and NK-92-CD16 cells were added at E:T ratio = 5:1. The final concentration of trastuzumab in the wells was 1  $\mu$ g/mL. After 4 h, the percentages of cytotoxicity were calculated and normalized to the control medium. A result representative of 3 independent experiments is shown in A and C, and means  $\pm$  SD of at least 3 independent experiments are shown in B and D.

## Figure 4.

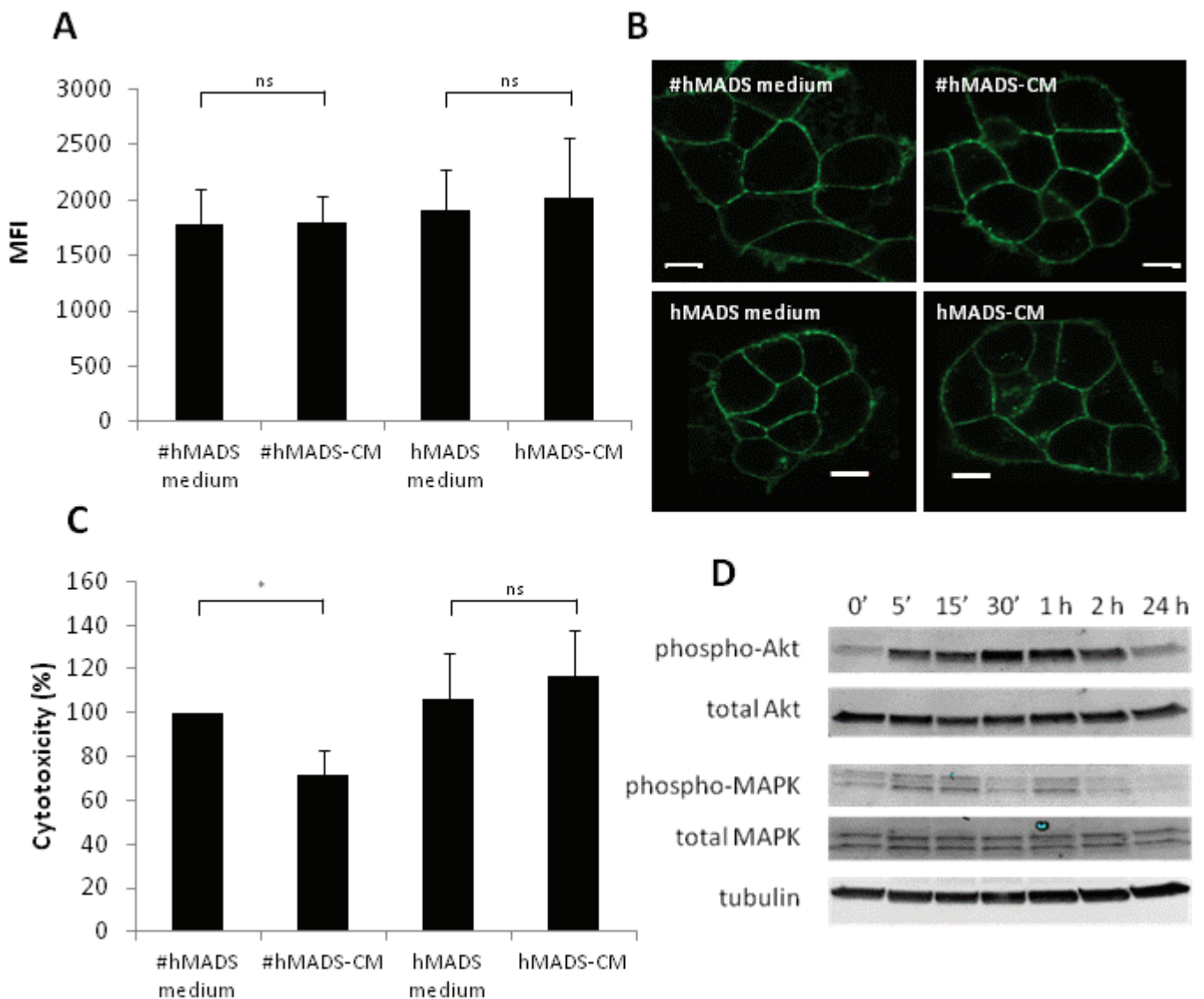


**Figure 4. hMADS-CM and #hMADS-CM do not alter NK cytotoxicity.**

**A)** NK-92-CD16 cells were co-incubated with BT474 cells (E:T ratio = 5:1) with 1  $\mu$ g/mL trastuzumab, in presence of hMADS-CM, #hMADS-CM or the control #hMADS medium. After 4 h, NK-92-CD16 cells were harvested, labeled with anti-CD56 and indicated fluorescent antibodies before analysis by flow cytometry. Dotted red lines, dotted green lines, solid coral lines and dashed blue line indicate unstained NK-92-CD16 cells, control #hMADS medium, #hMADS-CM and hMADS-CM conditions, respectively. **B)** NK-92-CD16 cells were pre-incubated overnight with #hMADS-CM or hMADS-CM or the control media,

washed, counted for viability and used in ADCC assay. C) K562 cells were labeled with 12.5  $\mu$ M calcein and incubated with NK-92-CD16 cells at E:T ratio = 5:1, in presence of hMADS-CM, #hMADS-CM or their control media. After 4 h, the percentages of cytotoxicity were calculated and normalized with #hMADS medium. A result representative of 3 independent experiments is shown in A), and means  $\pm$  SD of at least 3 independent experiments are shown in B) and C).

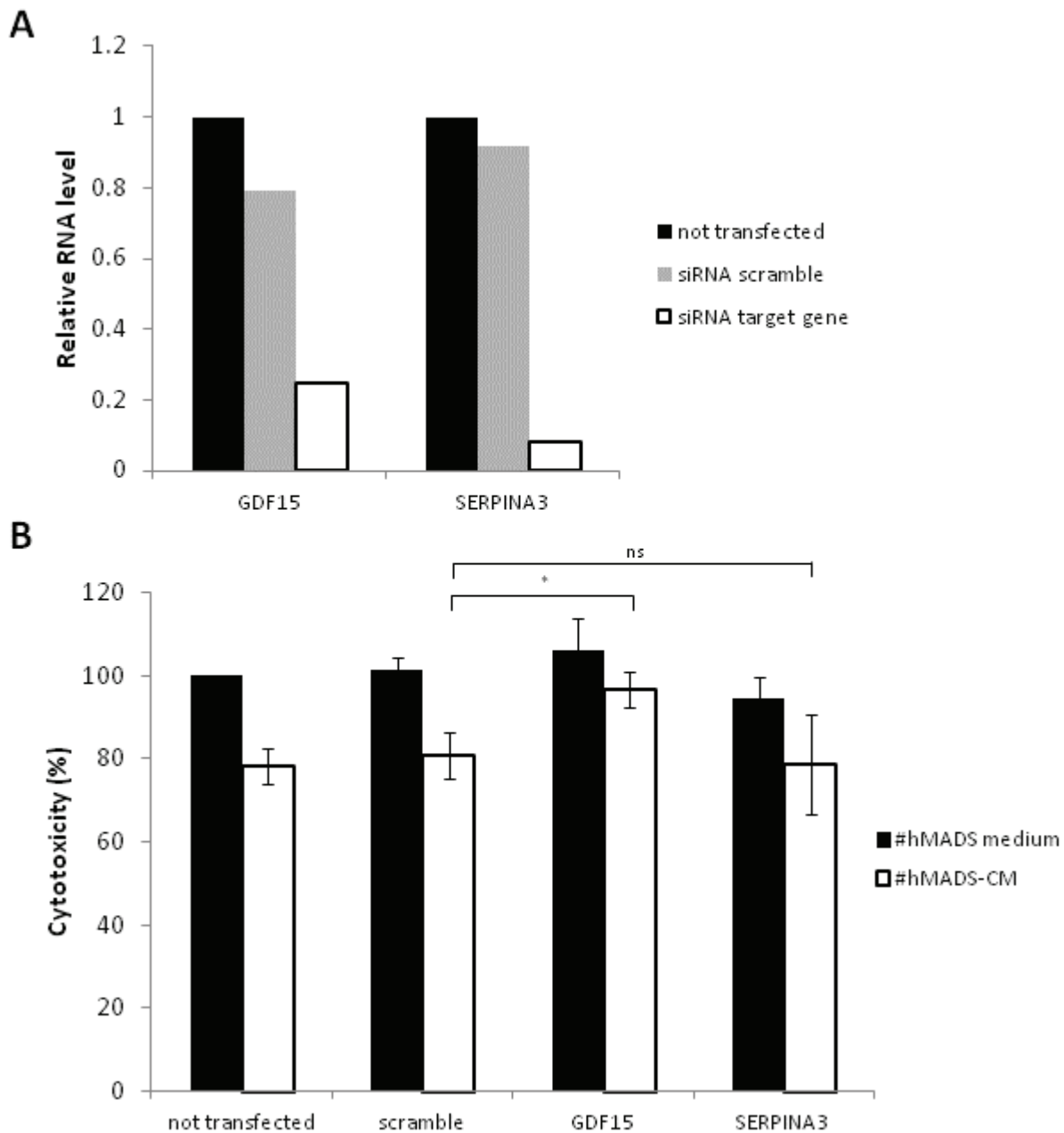
## Figure 5.



**Figure 5. #hMADS-CM reduces sensitivity of BT474 cells to ADCC.**

**A)** BT474 cells were incubated with hMADS-CM or #hMADS-CM or their control media. After 4 h, BT474 cells were harvested and labeled with HER2 antibody before analysis by flow cytometry. **B)** BT474 cells were incubated with hMADS-CM or #hMADS-CM or their control media. After 4 h, cells were labeled with HER2 antibody and visualized by confocal microscopy. Scale bars indicate 10 μm. **C)** BT474 cells were incubated overnight with hMADS-CM, #hMADS-CM or their control media, washed and used in ADCC assay. Percentages of cytotoxicity were normalized with #hMADS medium as 100 %. Means ± SD of at least 3 independent experiments are shown in A) and C). Results representative of 2 independent experiments are shown in B) and D).

## Figure 6.



**Figure 6. GDF15 mediated inhibition of ADCC.**

**A)** BT474 cells were transfected with 10 nM siRNA against indicated genes or control siRNA using lipofectamine RNAimax. After 48 h, BT474 cells were harvested, RNA was extracted and gene expression was accessed by RT-qPCR. A representative experiment of at least 3 independent experiments is shown. **B)** BT474 cells were transfected with 10 nM siRNA against *GDF15* or *SERPINA3* or control siRNA. After 48 h, BT474 cells were harvested and used in ADCC assays as described in figure 2C.

**Table I. List of genes up- or down-regulated by #hMADS-CM in BT474 cells**

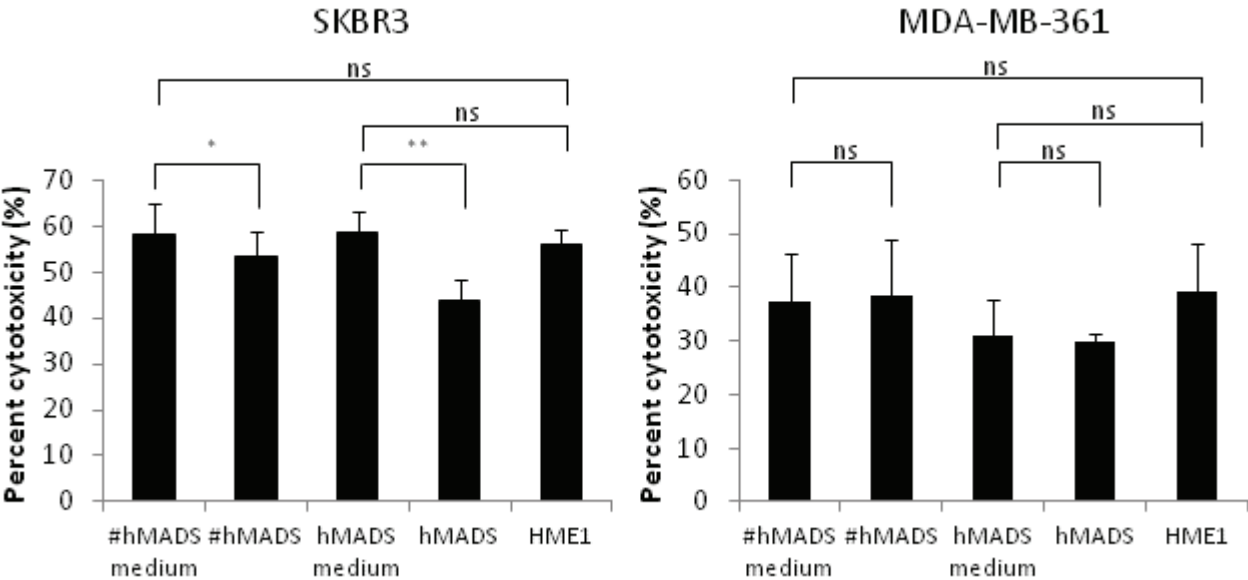
Gene name	Accession No	Fold change		Classification
		BT474	SKBR3	
ADM	NM_001124.1	2.54	2.28	Hormone activity
GDF15	NM_004864.1	2.52	1.09	Growth factor activity, cytokine activity
MYC	NM_002467.3	2.03	0.90	Transcription
SERPINA3	NM_001085.4	1.91	2.33	Serine protease inhibition
PMAIP1	NM_021127.1	1.78	0.92	Pro-apoptosis
NCOA7	NM_181782.2	1.76	3.62	Nuclear receptor coactivator activity
EGR1	NM_001964.2	1.74	1.17	Transcription
DKK1	NM_012242.2	1.73	0.88	Wnt pathway inhibition
CYP1B1	NM_000104.2	0.55	0.61	Metabolism
ID2	NM_002166.4	0.47	0.33	Transcription
CYP1A1	NM_000499.2	0.33	0.32	Metabolism
ID1	NM_181353.1	0.18	0.29	Transcription
ID3	NM_002167.2	0.16	0.79	Transcription

Only genes with more than 1.7-fold changes are shown



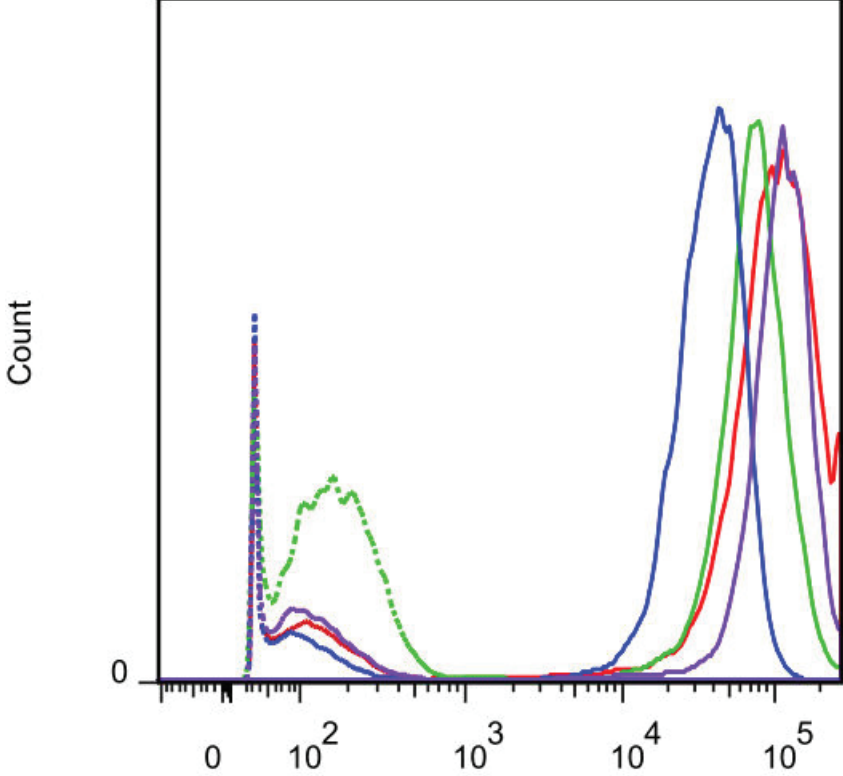
**Supplementary Figure S1. hMADS and #hMADS cells inhibit ADCC.**

HER-2+/ER- SKBR3 or HER-2+/ER+ MDA-MB-361 human breast cancer cells were labeled with 12.5 μM calcein and incubated with 1 μg/mL trastuzumab and NK-92-CD16 cells at effector to target (E:T) ratio = 5:1, in the presence of hMADS, #hMADS or hme-1 (control) cells. After 4 h of incubation, the percent cytotoxicity was calculated as indicated in the materials and methods. Mean ± SD of at least 3 independent experiments is shown.



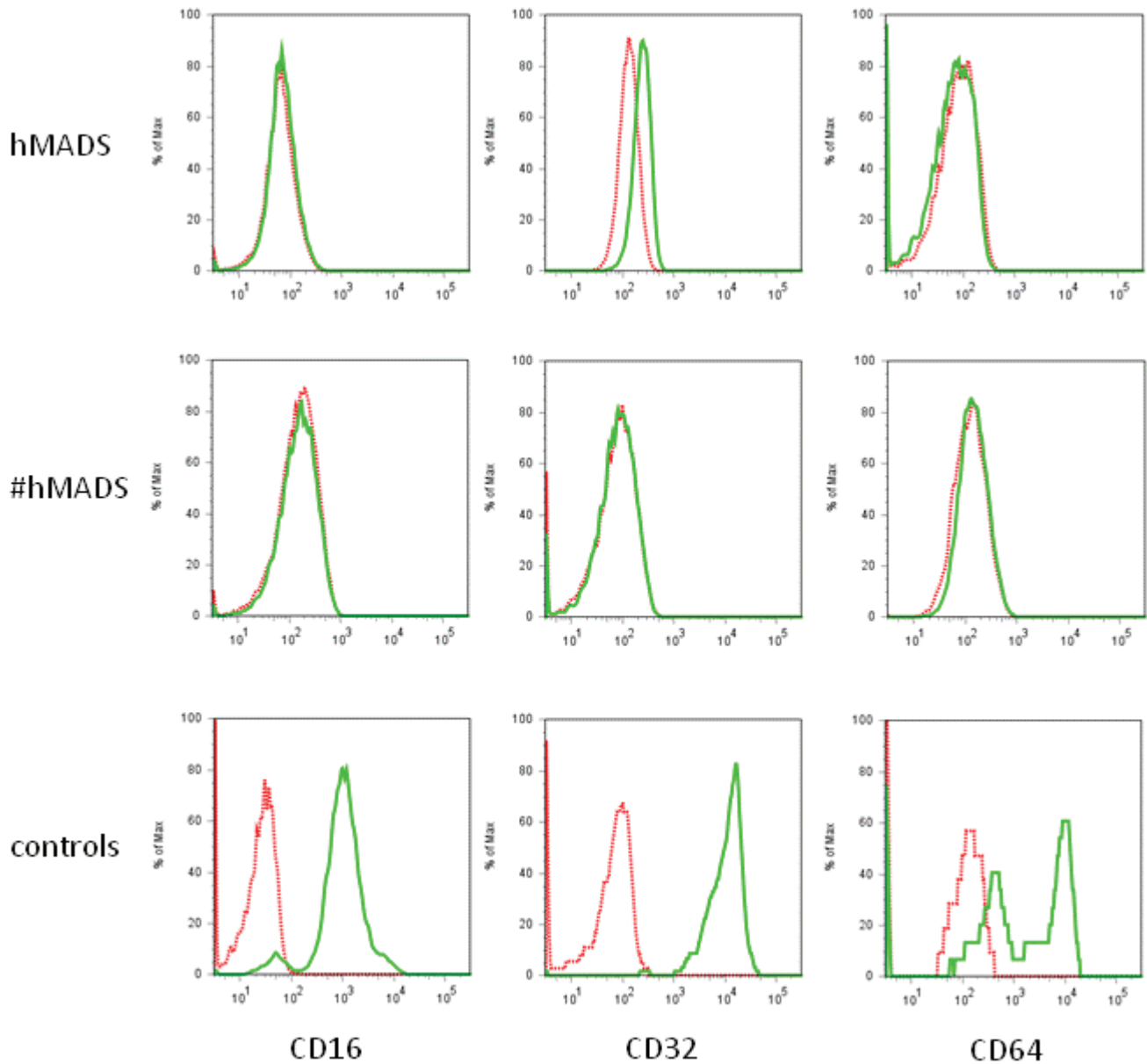
**Supplementary Figure S2. HER2 expression levels in the breast cancer cell lines studied.**

BT474 (red lines), MDA-MB-453 (blue lines), SKBR3 (violet lines) and MDA-MB-361 (green lines) cells were labeled with anti-HER2 affibody and analyzed by FACS. Dotted lines indicate unstained cells and solid lines indicate HER2-stained cells.



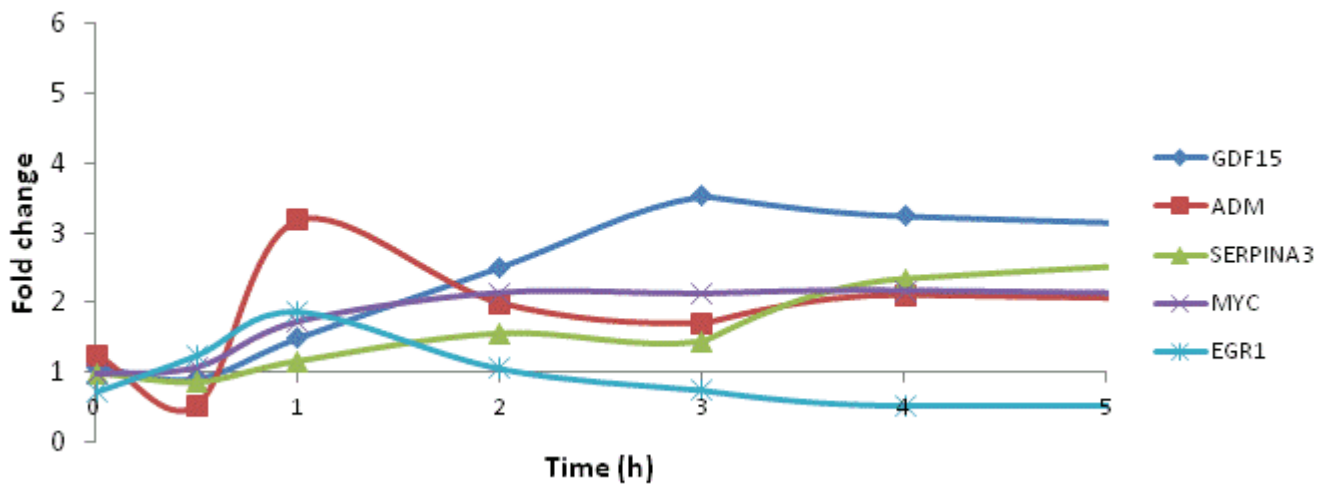
### Supplementary Figure S3. hMADS and #hMADS cells do not express FcR.

hMADS and #hMADS cells were labeled with anti-CD16 or anti-CD32 or anti-CD64 antibodies and analyzed by FACS. NK-92-CD16 cells were used as positive control of CD16 expression, and monocytes were used as positive control of CD32 and CD64 expression. Dotted red lines indicate unstained cells and solid green lines indicate corresponding antibodies. Result representative of 3 independent experiments is shown.



**Supplementary Figure S4. Kinetic expression of genes in BT474 cells induced by #hMADS-CM.**

BT474 cells were incubated with #hMADS-CM or the control medium for indicated times. Cells were harvested; RNA was extracted and used for RT-qPCR using primers for *GDF15*, *ADM*, *SERPINA3*, *MYC* and *EGR1*. A representative experiment of 3 independent experiments is shown.



**Supplementary Table I. List of genes regulated by #hMADS-CM in SKBR3 cells**

Gene name	Accession No	Fold change		Classification
		BT474	SKBR3	
FGG	NM_000509.4	1.07	7.33	Extracellular matrix composition
CEBPD	NM_005195.2	1.53	5.12	Transcription
CCL2	NM_002982.3	1.08	4.68	Cytokine activity
FGB	NM_005141.2	1.06	4.32	Extracellular matrix composition
NCOA7	NM_181782.2	1.76	3.62	Nuclear receptor coactivator activity
STEAP4	NM_024636.1	0.96	2.89	Oxidoreductase activity
TMEM2	NM_013390.1	1.11	2.76	Morphogenesis
IRF1	NM_002198.1	1.67	2.68	Transcription
PHLDA1	NM_007350.2	1.10	2.63	Anti-apoptosis
CDKN1A	NM_078467.1	1.20	2.59	Cell cycle regulation
NLF2	NM_001007595.1	1.69	2.52	Inflammation, adhesion
RGS16	NM_002928.2	1.52	2.52	Signal transduction
TFPI2	NM_006528.2	0.96	2.39	Serine protease inhibition
SOCS2	NM_003877.3	1.04	2.33	Signal transduction
SERPINA3	NM_001085.4	1.91	2.33	Serine protease inhibition
RASL11A	NM_206827.1	1.05	2.29	GTPase activity
ADM	NM_001124.1	2.54	2.28	Hormone activity
PPAP2B	NM_003713.3	1.04	2.27	Lipid phosphatase activity
LRG1	NM_052972.2	1.11	2.26	Signal transduction, adhesion
UGCG	NM_003358.1	1.20	2.24	Ceramide glucosyltransferase activity
FAM83A	NM_032899.4	0.95	2.23	Signal transduction
GPRC5A	NM_003979.3	1.39	2.22	Signal transduction
IER5	NM_016545.3	1.20	2.21	Transcription
F2RL1	NM_005242.3	1.02	2.21	Coagulation
ZFP36L2	NM_006887.3	1.39	2.18	Transcription
FHL2	NM_001450.3	1.44	2.15	Transcription
TM4SF1	NM_014220.2	1.04	2.11	Signal transduction
CEBPB	NM_005194.2	1.13	2.11	Transcription
RCAN1	NM_203418.1	1.10	2.08	Transcription
BCL3	NM_005178.2	1.28	2.07	Transcription
SOX9	NM_000346.2	1.25	2.07	Transcription
C17ORF96	NM_001130677.1	1.25	2.04	
CITED4	NM_133467.2	1.27	2.04	Transcription
GPR37	NM_005302.2	1.10	2.03	Signal transduction
HS.543887	Hs.543887	1.03	2.02	
ETS2	NM_005239.4	1.21	2.01	Transcription
EFNB2	NM_004093.2	1.22	2.00	Ephrin receptor
B4GALT5	NM_004776.2	1.13	1.99	Galactosyltransferase activity
ATF3	NM_001040619.1	1.04	1.99	Transcription
MAFF	NM_012323.2	1.33	1.97	Transcription
P2RY6	NM_176797.1	1.05	1.97	Pyrimidineric receptor
F2RL1	NM_005242.3	1.15	1.96	
SPRY4	NM_030964.2	1.40	1.94	Signal transduction
NAMPT	NM_182790.1	1.06	1.93	Metabolism
CDC42EP2	NM_006779.2	1.34	1.92	Rho GTPase activator activity
ZFP36	NM_003407.1	1.30	1.91	Transcription
TSC22D1	NM_006022.2	0.87	1.91	Transcription
LOC100129882	XM_001716882.1	0.71	1.90	
OBFC2A	NM_022837.1	1.15	1.89	RNA binding, single-stranded DNA binding
SLCO2A1	NM_005630.1	1.01	1.88	prostaglandin transporter

CRISPLD2	NM_031476.1	0.87	1.87	Heparin binding
IERSL	NM_203434.1	1.34	1.86	Transcription
PFKFB3	NM_004566.2	1.17	1.86	6-phosphofructo-2-kinase activity, ATP binding
FAM46C	NM_017709.2	1.07	1.83	
RND1	NM_014470.2	1.03	1.83	Rho GTPase activator activity
KIAA0247	NM_014734.2	1.12	1.82	Cell cycle regulation
LDLR	NM_000527.2	0.85	1.80	Low density lipoprotein receptor
MAT2A	NM_005911.4	0.97	1.79	Methionine adenosyltransferase activity
BHLHB2	NM_003670.1	1.49	1.78	Transcription
RCAN1	NM_203417.1	1.07	1.77	Transcription
YPEL2	NM_001005404.3	1.11	1.75	
NFIL3	NM_005384.2	1.15	1.74	Transcription
ARID5B	NM_032199.1	1.04	1.74	Transcription
IL20	NM_018724.3	0.99	1.73	
IL1RL1	NM_003856.2	0.90	1.71	Inflammation
CD14	NM_001040021.1	1.01	1.70	Inflammation
CCNG2	NM_004354.1	0.75	0.58	Cell cycle regulation
TXNIP	NM_006472.1	0.83	0.57	Transcription
EDN1	NM_001955.2	1.00	0.55	Vasoconstriction
HERPUD1	NM_001010990.1	0.84	0.53	Ubiquitination
CITED2	NM_006079.3	0.86	0.52	Transcription
FBXO32	NM_148177.1	0.80	0.49	Ubiquitination
FAM46B	NM_052943.2	0.82	0.42	
LOC643009	XM_932195.1	1.04	0.42	
ID2	NM_002166.4	0.47	0.33	Transcription
CYP1A1	NM_000499.2	0.33	0.32	Metabolism
ID1	NM_181353.1	0.18	0.29	Transcription

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Only genes with more than 1.7-fold changes are shown

## **Part II**

# **Identification of the adipocyte-derived inhibitory factors**

## **Objective of the study**

Adipocytes and preadipocytes are abundant in the breast and located in the proximity of tumor cells. They actively participate in the tumorigenesis via secretion of various substances, including fatty acids, growth factors, and adipokines and cytokines. In the previous part, we have shown that adipocytes and preadipocytes inhibited tumor cell killing by trastuzumab and NK cells, via soluble factors released into the conditioned media. This inhibition of ADCC may be mediated directly via inactivation of lytic agents released by NK cells, or indirectly via induction of tumor resistance. However, the inhibitory factors remain unknown. Their identification would be essential to target the crosstalk between adipocytes/preadipocytes and tumor cells to sensitize tumor cells to targeted therapy. Here, we tested several factors released by adipocytes for their capacity to inhibit ADCC.



## Materials and methods

### *Materials*

Human recombinant (rh) leptin, rh-adiponectin, rh-IL-6, ATP, ADP, AMP, and adenosine were purchased from Sigma-Aldrich. Rh-TNF- $\alpha$ , rh-IGF-I, pentostatin (deoxycoformycin) were obtained from Cetus corporation, Tercica, Inc. and Lederle respectively. TGF- $\beta$  was kindly given by Dr Julien Marie, CRCL, Lyon, France. Autotaxin was kindly given by Dr Olivier Peyruchaud, INSERM U1033, Lyon, France. LPA and S1P were purchased from Avanti Polar Lipids, Inc. Lactic acid solution was obtained from Santa Cruz. Anti-CD39 APC and anti-CD73 FITC antibodies were purchased from BD Biosciences.

### *Heating, ultracentrifugation and addition of albumin*

The conditioned medium from #hMADS adipocytes was harvested as described above. Proteins were denatured by heating at 95°C for 15 min. Exosomes were eliminated via ultracentrifugation at 100,000 g for 30 min, as previously described<sup>699</sup>. Free fatty acids were neutralized via preincubation of #hMADS-CM with 1% human fatty acid-free albumin (Sigma) for 1 h at 37°C.

### *ADCC assay*

BT474 cells were labeled with 12.5  $\mu$ M calcein-AM (Sigma-Aldrich) for 30 min at 37°C and added at  $10^5$  cells/well. NK-92-CD16 cells were added at  $5 \times 10^5$  cells/well (effector : target (E:T) ratio = 5:1), with trastuzumab at 1  $\mu$ g/mL final. Different molecules tested were added at indicated concentrations at the beginning of the assay. In the ADCC assay with APCP, the molecule was added in the #hMADS, hMADS or hme-1 cell culture everyday at 100  $\mu$ M during the two days before the assay, and also at the beginning of the assay. After 4 h of incubation at 37°C, triton X100 (Sigma-Aldrich) was added in control wells to have total cell lysis. The supernatants in each well were harvested and measured for the fluorescence signals at 485/535 nm. The cytotoxicity was calculated using the formula: percent cytotoxicity = (experimental lysis – spontaneous lysis) / (maximal lysis – spontaneous lysis) \* 100.

### *Measurement of CD73 activity by Malachite green phosphate assay*

Cells were seeded at  $2 \times 10^5$  cells/1 mL/well in 12-well plates and incubated at 37°C overnight. After two washes in CD73 buffer containing 2 mM MgCl<sub>2</sub>, 120 mM NaCl, 5 mM KCl, 10 mM dextrose, 20 mM HEPES pH7.4, cells were incubated in CD73 buffer for 30 min at 37°C

in presence or absence of 1 mM AMP, with or without 10  $\mu$ M APCP. The free phosphates in the supernatants were then measured by Malachite green phosphate assay kit (Bioassay Systems) according to the manufacturer's instructions.

#### *ATP assay*

The concentrations of ATP in the conditioned media from #hMADS adipocytes, hMADS preadipocytes and hme-1 cells were measured by the ATP determination kit (Molecular Probes) according to the manufacturer's instructions.

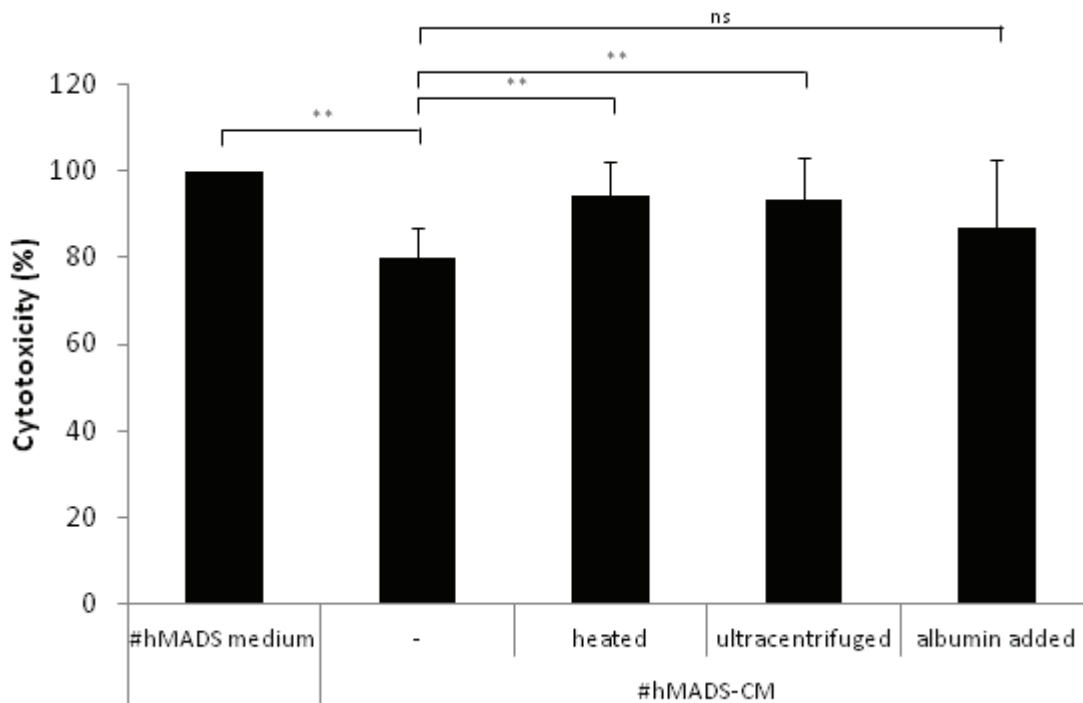
#### *Lactate assay*

The hydrazine-glycine buffer was prepared containing 37.5 g/L glycine, 1.26% hydrazine and adjusted to pH 9. The working enzyme solution was prepared containing 2 mM NAD<sup>+</sup> and lactic dehydrogenase. Glycine was purchased from Euromedex and other reagents were obtained from Sigma. 10  $\mu$ L of the conditioned media or lactate standards were added with 10  $\mu$ L or working enzyme solution into 100  $\mu$ L of hydrazine-glycine buffer. After 15 min of incubation at 37°C, the absorbance was read at 340 nm by Nanodrop device.

## Results

### *Identification of the biochemical nature of the inhibitors*

Adipocytes are known to secrete various factors, including adipocytokines<sup>212</sup>, fatty acids<sup>700</sup>, and exosomes<sup>701</sup>. In order to determine the biochemical nature of the adipocyte-secreted factors that mediated the inhibition of ADCC, we either denatured proteins by heating, or eliminated adiposomes by ultracentrifuging, or neutralized fatty acids by adding fatty acid-free albumin in the #hMADS-CM before using it in ADCC assay. As shown in Figure 8, heating and ultracentrifugation partially reversed the inhibition of ADCC by #hMADS-CM, suggesting that the inhibitors are proteins or exosomes.

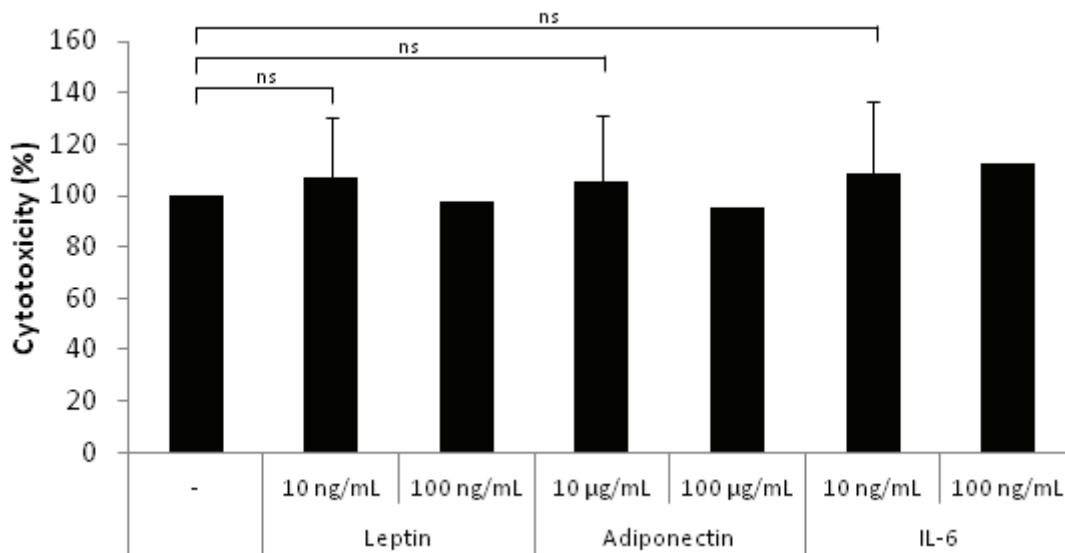


**Figure 8. Determination of the biochemical nature of the inhibitors**

#hMADS-CM was either heated at 95°C for 15 min, or ultracentrifuged at 100000 g for 30 min, or incubated in 1% of human fatty acid-free albumin for 1 h at 37°C, then used in ADCC assay in volume/volume (v/v) = 1/1. BT474 cells were labeled with 12.5 μM calcein and coincubated with NK-92-CD16 cells at E:T ratio = 5:1 and 1 μg/mL trastuzumab. After 4 h, the percentages of cytotoxicity were calculated and normalized with the cytotoxicity of #hMADS control medium as 100%. Means ± SD of at least three independent experiments are shown.

### *Leptin, adiponectin, and IL-6*

Leptin, adiponectin and IL-6 are the most documented adipocytokines secreted by adipocytes. In order to test their involvement in the adipocyte-induced resistance of cancer cells to ADCC, recombinant molecules have been directly added in ADCC assay. For each adipokine, two concentrations were used; the lower concentrations correspond to the ones found in the plasma of adults (for leptin or adiponectin<sup>702</sup>) or secreted by the adipocytes (for IL-6<sup>516</sup>). Surprisingly, none of these molecules at both concentrations tested modified the trastuzumab-mediated ADCC (Figure 9).

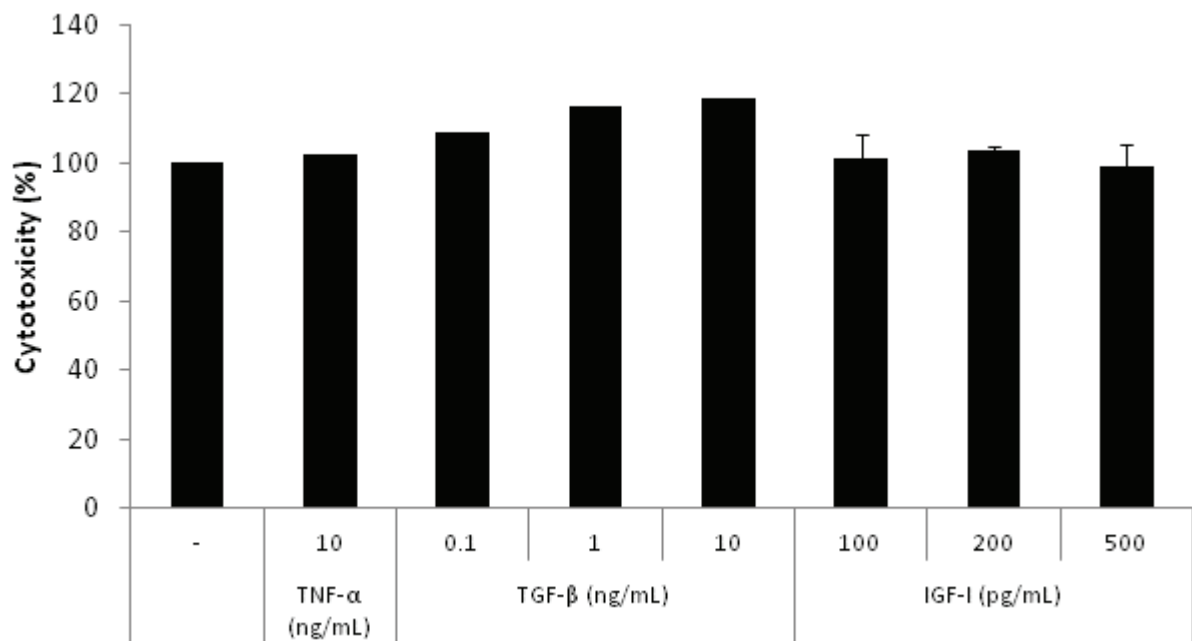


**Figure 9. No inhibition of ADCC by leptin, adiponectin or IL-6**

BT474 cells were labeled with 12.5 µM calcein and coincubated with NK-92-CD16 cells at E:T ratio = 5:1 and 1 µg/mL trastuzumab, in presence of indicated concentrations of leptin, adiponectin or IL-6. After 4 h, the percentages of cytotoxicity were calculated and normalized with the #hMADS control medium as 100%. Means ± SD of more than three independent experiments are shown for the low concentrations and just one experiment was performed at high concentrations.

### *TNF- $\alpha$ , TGF- $\beta$ , and IGF-I*

We next tested the involvement of other adipocytokines, TNF- $\alpha$ , TGF- $\beta$ , and IGF-I in the inhibition of ADCC. TNF- $\alpha$  is previously shown to induce NK cell death<sup>669,670</sup>, while TGF- $\beta$  is a well known immunosuppressor<sup>423</sup>. As for IGF-I, it is a survival factor in many cancers<sup>129</sup>. As shown in Figure 10, none of these molecules inhibited ADCC at all concentrations tested. It is noteworthy that the concentrations used are all similar or higher than the ones released by adipocytes as previously reported<sup>516</sup>.

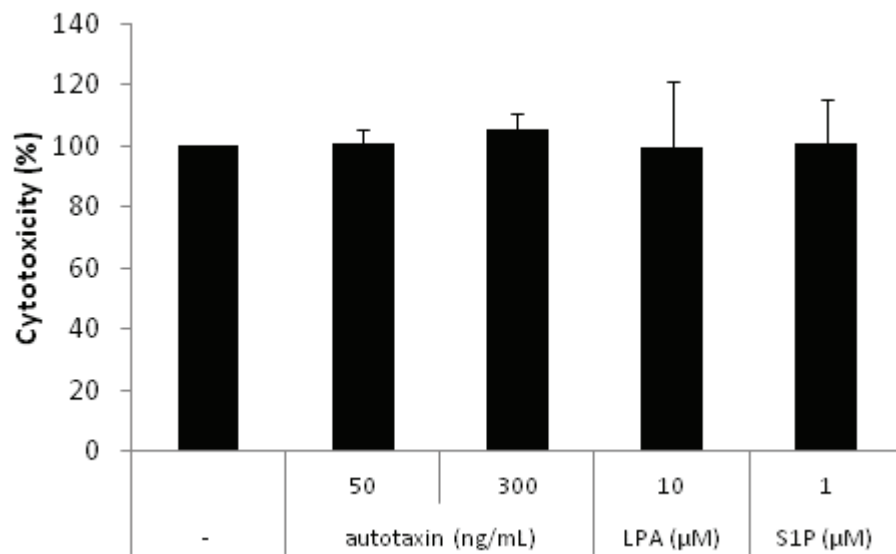


**Figure 10. No inhibition of ADCC by TNF- $\alpha$ , TGF- $\beta$  and IGF-I**

BT474 cells were labeled with 12.5  $\mu$ M calcein and coincubated with NK-92-CD16 cells at E:T ratio = 5:1 with 1  $\mu$ g/mL trastuzumab, in presence of indicated concentrations of TNF- $\alpha$ , TGF- $\beta$  and IGF-I. After 4 h, the percentages of cytotoxicity were calculated and normalized with the #hMADS control medium as 100%. Means  $\pm$  SD of 2 independent experiments is shown for IGF-I, and just one experiment was performed with TNF- $\alpha$  and TGF- $\beta$ .

*Autotaxin (ATX), lysophosphatidate (LPA) and sphingosine 1-phosphate (S1P)*

The lysophospholipids LPA and S1P have been shown to increase resistance of cancer cells to chemotherapy and radiotherapy<sup>703</sup>. Similarly, autotaxin or lysophospholipase D, the enzyme which generates LPA from lysophosphatidylcholine (LPC), has also been associated with tumor survival<sup>703,704</sup>. Since all these molecules are released by adipocytes<sup>628,629,705</sup>, we tested their implication in the inhibition of ADCC. As shown in Figure 11, none of these molecules inhibited ADCC. It is worth noting that the concentrations of LPA and S1P used are higher than the ones found in the human plasma<sup>706,707</sup>.

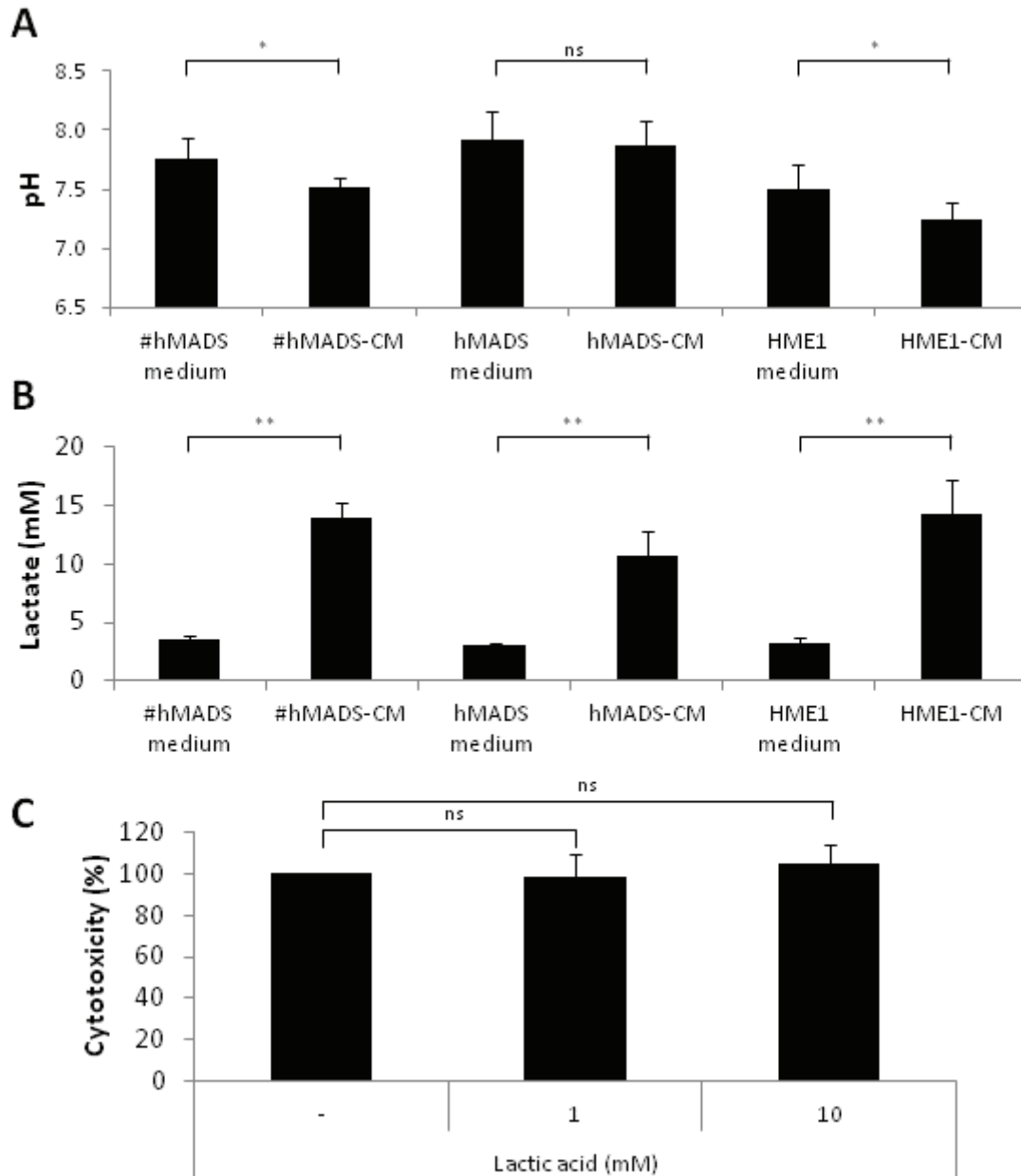


**Figure 11. No inhibition of ADCC by LPA or S1P**

BT474 cells were labeled with 12.5 µM calcein and coincubated with NK-92-CD16 cells at E:T ratio = 5:1 and 1 µg/mL trastuzumab, in presence of indicated concentrations of autotaxin, LPA or S1P. After 4 h, the percentages of cytotoxicity were calculated and normalized with the #hMADS control medium as 100%. Means ± SD of 2 independent experiments are shown.

### *Acidification of the conditioned media*

Hypoxia-induced extracellular acidification have been shown to promote aggressiveness and chemoresistance of cancer cells<sup>708</sup>. Although in our assays, the volume of the conditioned media was only half of the final volume, the acidification of #hMADS-CM or hMADS-CM may cause tumor cell resistance to ADCC. Therefore, we investigated the impact of the medium acidification on ADCC. As shown in Figure 12A, the conditioned media from #hMADS and HME1 cells were significantly more acid than the corresponding control media. By contrast, the pH of the hMADS-CM was only slightly lower than in the one of the control medium. Interestingly, HME1-CM was the most acid. Additionally, all three conditioned media were enriched in lactate compared to their control media (Figure 12B). Since we previously observed an inhibition of ADCC with #hMADS and hMADS cells, but not HME1 cells, it is unlikely that the pH or lactate interferes with ADCC. This was confirmed by the absence of inhibition of ADCC by lactic acid (Figure 12C). Altogether, these data suggest that the inhibition of ADCC by with #hMADS-CM and hMADS-CM was not due to an acidification of the conditioned media.



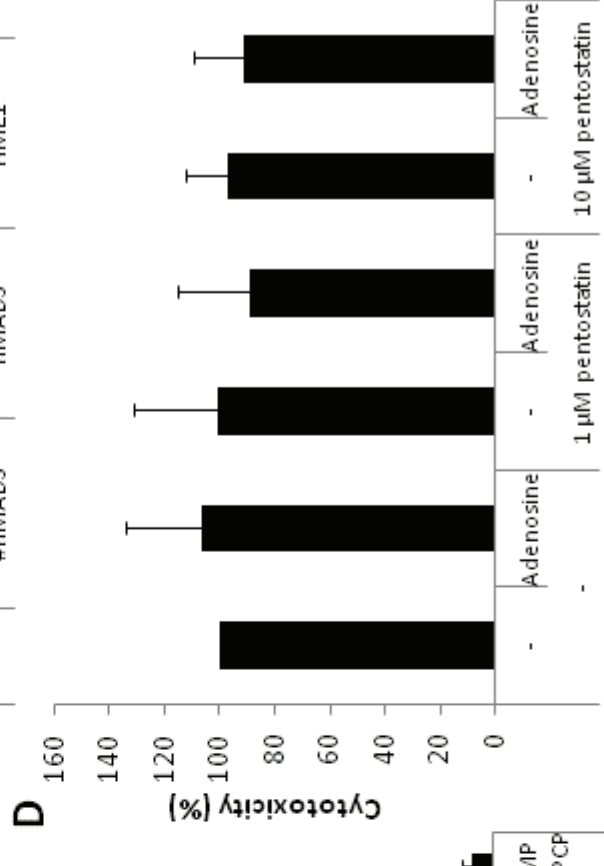
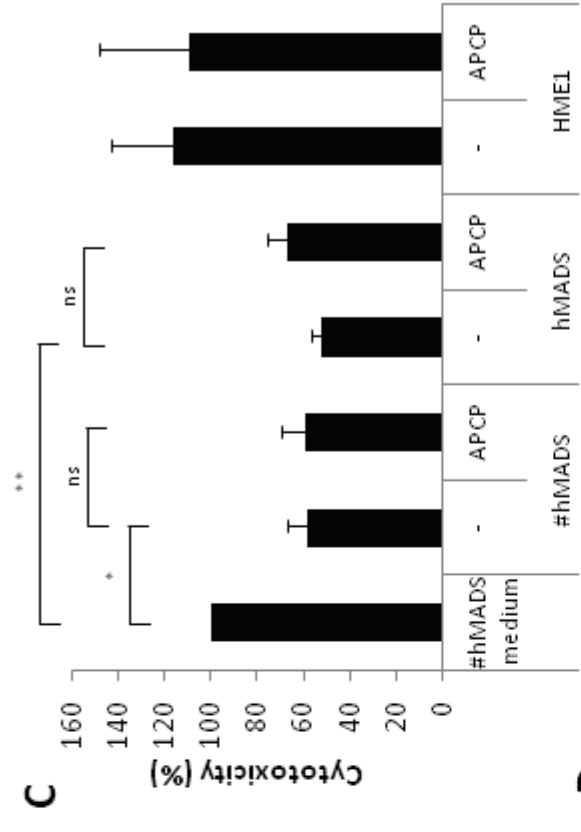
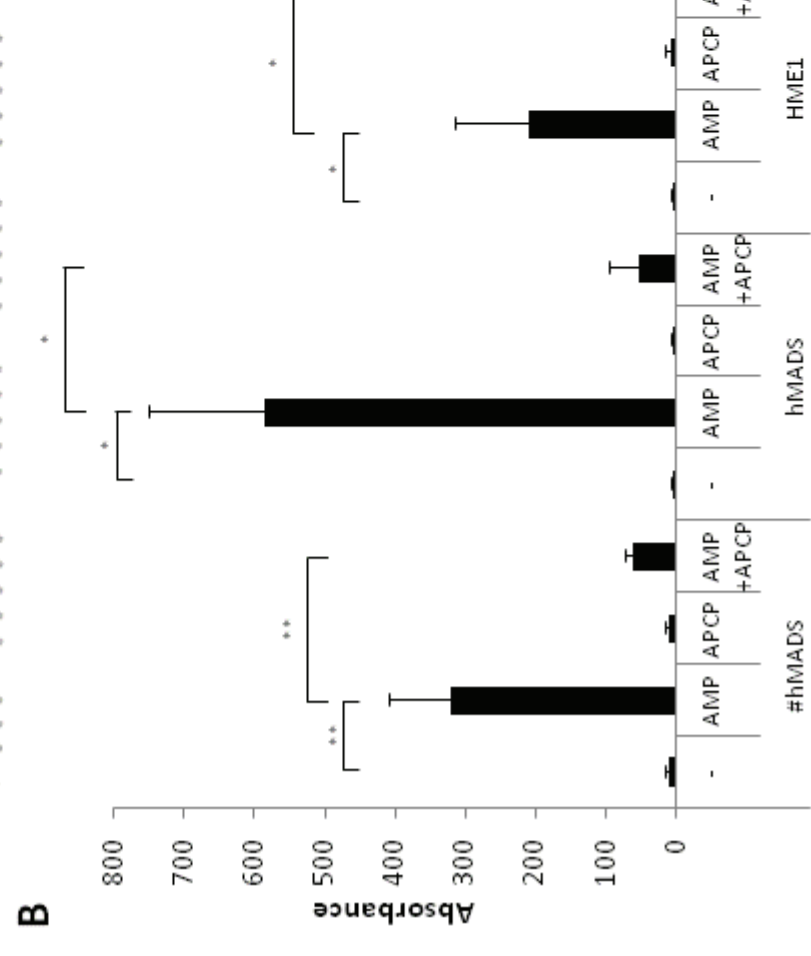
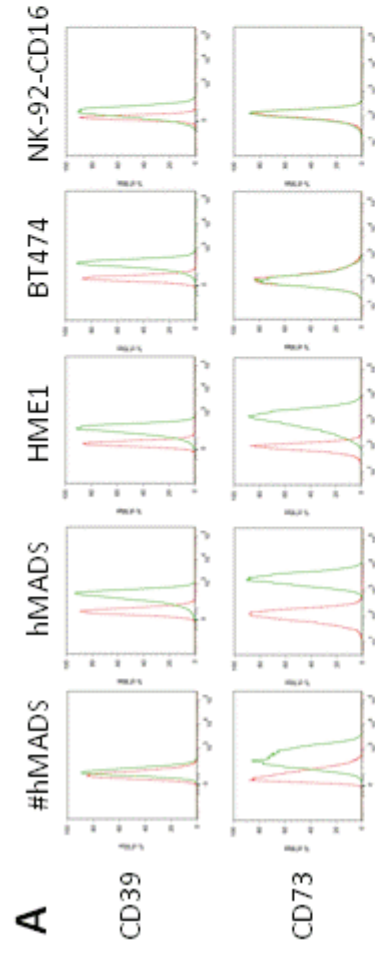
**Figure 12. No inhibition by acidification of extracellular medium**

**A)** pH of the conditioned media from #hMADS, hMADS and hme-1 cells as well as the control media were measured. **B)** The concentrations of lactate in #hMADS-CM, hMADS-CM or hme-1-CM were measured by lactate assay. **C)** BT474 cells were labeled with 12.5  $\mu$ M calcein and coincubated with NK-92-CD16 cells at E:T ratio = 5:1 and 1  $\mu$ g/mL trastuzumab, in presence of lactic acid at 1 mM or 10 mM. After 4 h, the percentages of cytotoxicity were calculated and normalized with the #hMADS control medium as 100%. Means  $\pm$  SD of at least 3 independent experiments is shown.



### *CD73 and adenosine*

Extracellular adenosine has been reported to be a suppressor of anti-tumor immune responses<sup>709</sup>. Adenosine is generated from the conversion of ATP into AMP via the ecto-enzyme CD39, and from AMP into adenosine via CD73. Therefore, we hypothesized that CD73 expressed by adipocytes and preadipocytes may play a role in the inhibition of ADCC. As shown in Figure 13A, #hMADS adipocytes, hMADS preadipocytes, as well as HME1 cells expressed high levels of CD73. hMADS and HME1 cells also expressed CD39, while the expression levels of CD39 was low in #hMADS cells. Both BT474 and NK-92-CD16 cells expressed CD39 but not CD73. The presence of CD73 correlated with the high nucleotidase activities on #hMADS, hMADS and HME1 cells, which were inhibited by adenosine 5'-( $\alpha,\beta$ -methylene) diphosphate (ADCP), an inhibitor of CD73 (Figure 13B). By contrast, BT474 and NK-92-CD16 presented very low nucleotidase activities (data not shown). Unexpectedly, ADCP did not reverse the inhibition of ADCC by #hMADS and hMADS cells (Figure 13C). Furthermore, addition of adenosine did not inhibit ADCC, even in the presence of pentostatin, an adenosine deaminase inhibitor (Figure 13D). Likewise, addition of AMP into #hMADS-CM or hMADS-CM did not enhance the inhibition of ADCC (data not shown). Collectively, these data suggest that adenosine is produced by adipocytes and preadipocytes are not involved in the inhibition of ADCC.

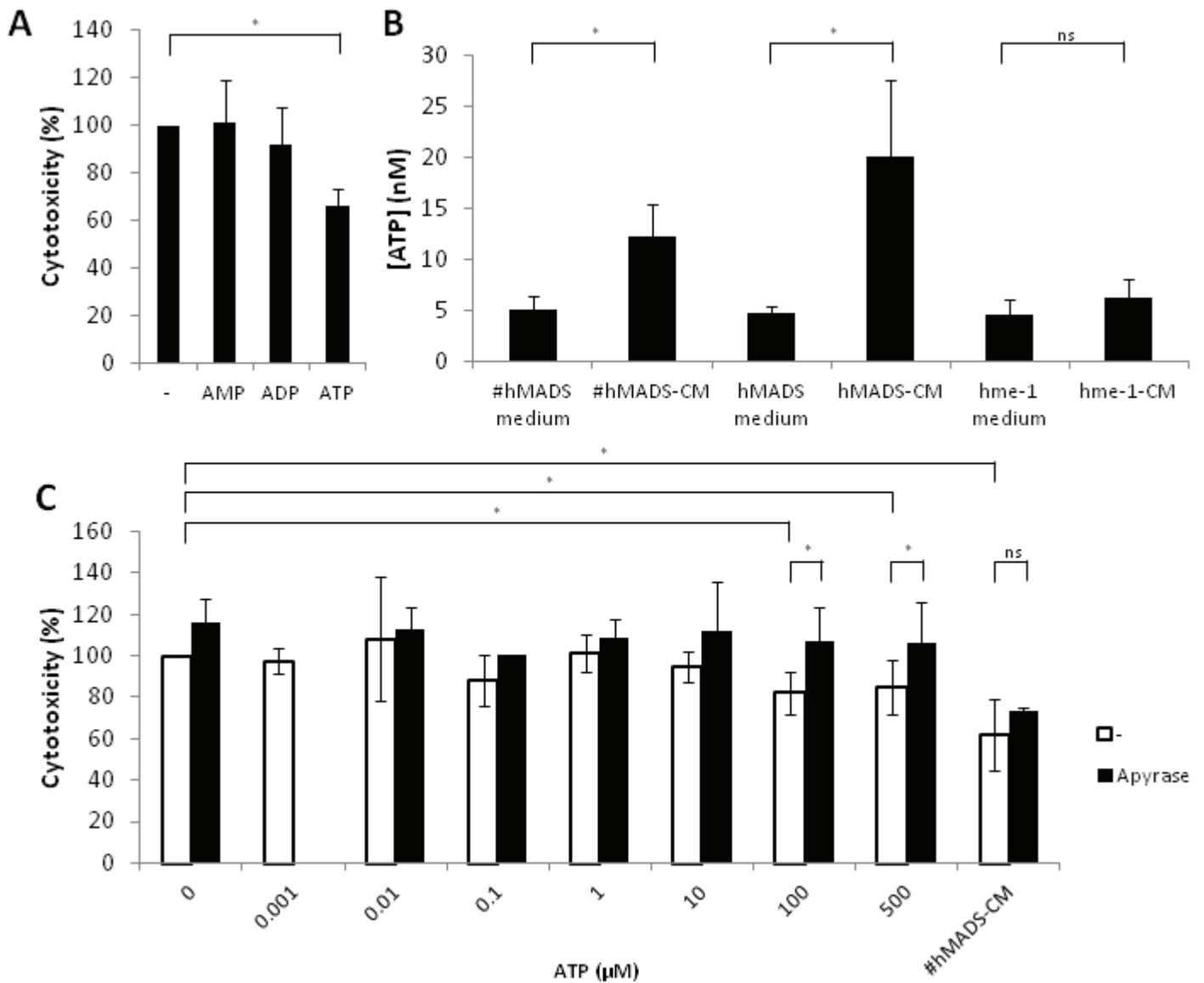


**Figure 13. No inhibition of ADCC by adenosine**

**A)** Cells were labeled with anti-CD39 or anti-CD73 fluorescent antibodies and analyzed by FACS. **B)** Cells were incubated with or without AMP and APCP, and the phosphates released in the supernatants were measured with the Malachite green assay. **C)** #hMADS, hMADS or hme-1 cells were pre-incubated with 100  $\mu$ M ADCP for 2 days and used in ADCC assay as described in Figure 1A. **D)** BT474 cells were labeled with 12.5  $\mu$ M calcein and coincubated with NK-92-CD16 cells at E:T ratio = 5:1 and 1  $\mu$ g/mL trastuzumab, in presence of 10  $\mu$ M adenosine with or without pentostatine at indicated concentrations. After 4 h, the percentages of cytotoxicity were calculated and normalized with the #hMADS control medium as 100%. Representative experiment of 3 independent experiments was shown in A), and means  $\pm$  SD of at least 3 independent experiments are shown in B), C) and D).

## *ATP*

We next tested the roles of adenosine precursors, AMP, ADP and ATP in the inhibition of ADCC. We showed that ATP at 1 mM inhibited ADCC (Figure 14A). Interestingly, this inhibitory effect was not observed with ADP or AMP, suggesting that the inhibition was not due to the conversion of ATP into adenosine. Besides, ATP was shown to be released by #hMADS adipocytes and hMADS preadipocytes into their conditioned media (Figure 14B), but at very low levels (nM range). At these low concentrations, ATP did not mediate inhibition of ADCC (Figure 14C). Furthermore, apyrase, an enzyme hydrolyzing ATP and ADP, reversed the ATP-mediated inhibition but not the #hMADS-CM-mediated inhibition of ADCC (Figure 14C). Overall, these data indicate that ATP is an inhibitor of ADCC but it is not the main inhibitory factor in #hMADS-CM.



**Figure 14. ATP is not the main inhibitory factor in #hMADS-CM**

A) BT474 cells were labeled with 12.5  $\mu\text{M}$  calcein and coincubated with NK-92-CD16 cells at E:T ratio = 5:1 and 1  $\mu\text{g}/\text{mL}$  trastuzumab, in presence of 1 mM of AMP or ADP or ATP. After 4 h, the percentages of cytotoxicity were normalized with the #hMADS control medium as 100%. B) Measure of ATP in the conditioned media from #hMADS, hMADS and hme-1 cells. C) Same in A, but at various concentrations of ATP in the presence or absence of apyrase. Means  $\pm$  SD of at least 3 independent experiments are shown.

## Discussion

So far more than fifty adipocytokines have been identified to be secreted by adipocytes. Several studies have shown the protective role of these factors on cancer cells against chemotherapy and radiotherapy<sup>573,577-579</sup>, but the mechanisms of resistance is not fully understood. We previously demonstrated that adipocytes inhibited tumor cell lysis by NK cells in presence of trastuzumab. This is mediated by adipocyte-derived factors that are secreted into the conditioned media. Here, we tried to identify these inhibitory factors.

By different approaches, we showed that heated-#hMADS-CM or ultracentrifugated #hMADS-CM displayed less inhibition of ADCC. This result suggests that the adipocyte-secreted inhibitors may be proteins or exosomes. This was not totally unexpected, since adipocytes are abundant sources of adipocytokines and exosomes<sup>212,701</sup>. Furthermore, the secretion of exosomes by adipocytes occurred at basal levels, but was strongly induced in response to physiological stimuli, such as palmitate, insulin, H<sub>2</sub>O<sub>2</sub><sup>701</sup>. Thus, we suggest that stimulated adipocytes would mediate stronger inhibition of ADCC. Further experiments would be required to confirm this hypothesis.

On the other hand, pre-incubated #hMADS-CM with fatty acid-free albumin did not reverse the inhibition of ADCC. It is known that cellular uptake of fatty acid requires the dissociation of the albumin-fatty acid complex, either passively or via albumin receptors located on the cell surface<sup>710</sup>. Addition of “uncomplexed” albumin was shown to inhibit fatty acid uptake by competing with the albumin-fatty acid complex for the albumin receptors<sup>711</sup>. However, fatty acids as well as triglycerides could be incorporated into adipocyte-derived exosomes<sup>701</sup>. In this case, albumin cannot prevent cellular uptake of fatty acids by cancer cells. Therefore, it is possible that the inhibition of ADCC was mediated via the delivery of fatty acids to cancer cells via adipocyte-derived exosomes.

We investigated several adipocytokines for their implication in the inhibition of ADCC. Despite a broad number of relevant adipocytokines tested, including leptin, adiponectin, IL-6, TNF- $\alpha$ , TGF- $\beta$ , IGF-I, ATX, LPA, and S1P, none of them displayed inhibitory effects on ADCC. The concentrations used for each molecule in our studies were equal or higher than the ones found in human plasma or secreted by adipocytes. Thus, the absence of effects was unlikely due to an underdose. It is also important to note that many adipocytokines, such as leptin, adiponectin, IL-6, TGF- $\beta$  were previously shown to alter NK cytotoxicity<sup>640,646,652,667,712</sup>. However, in most of these studies, the effects were observed when

NK cells were pre-incubated with indicated molecules for 24-48 h, and no effects were obtained when the molecules were only added during the 4 h of cytotoxicity assays.

Interestingly, we showed that #hMADS adipocytes and hMADS preadipocytes highly expressed CD73, the membrane 5'-ectonucleotidase responsible for the generation of extracellular adenosine. CD73 has been observed in several types of cancer, and is correlated with poor prognosis in breast cancer<sup>709,713</sup>. CD73 has been implicated in both immune evasion as well as tumor invasion<sup>714,715</sup>. Recent study by Loi *et al.* showed that CD73 expression in triple breast tumor cells promoted anthracycline resistance by suppressing adaptive antitumor immune response<sup>716</sup>. The expression of CD73 by adipocytes and preadipocytes strongly suggest the implication of adipose tissue in the promotion of tumorigenesis. This is in line with several studies showing that CD73-deficient mice were resistant to chemically induced carcinogenesis<sup>717</sup> or tumor transplantation<sup>718-720</sup>. Particularly, the resistance was largely attributed to the non-hematopoietic host compartment<sup>719,720</sup>.

Extracellular adenosine generated from the activity of CD73 has been shown to mediate immunosuppressive function. Several studies have shown that adenosine or its analogues inhibit NK cytotoxicity *in vitro*<sup>721,428-430</sup>, even when adenosine was added at the beginning of the 4 h-cytotoxicity assay. This effect is likely mediated via the adenosine receptor AdoRA2 expressed on NK cells, which led to an increase of intracellular cAMP and an inhibition of both granzyme- and FasL-mediated cytotoxicity<sup>430,721</sup>. However, in our studies, we did not observe any inhibition of ADCC by adenosine at similar concentrations used in the literature. It is unlikely that adenosine was rapidly metabolized by adenosine deaminase, since pentostatin was also added at the same or higher concentrations than the ones previously reported<sup>428</sup>. Moreover, APCP, the inhibitor of CD73, did not reverse the inhibition of ADCC by #hMADS-CM. Taken together, these data indicate that CD73-generated adenosine is not the main inhibitor in the #hMADS-CM in ADCC.

Interestingly, we observed that ATP inhibited ADCC. This result was in line with Miller *et al.* who showed that ATP, but not adenosine, strongly inhibited NK cytotoxicity towards K562 cells<sup>722</sup>. ATP acts directly via binding to the ATP receptors P2XR and P2YR expressed on the plasma membrane of NK cells. Interestingly, high concentrations of extracellular ATP have been observed in the tumor microenvironment<sup>723</sup>. This extracellular ATP could be released from cancer cell death or from cells in the tumor stroma. We showed that ATP was enriched in the conditioned media from #hMADS and hMADS cells. This is supported by the study by Kim *et al.*, who showed that adipocytes released extracellular ATP via ATP synthase

located on the cell surface<sup>724</sup>. However, when ATP was added directly in the ADCC assay, no inhibition of ADCC was observed at the concentrations measured in #hMADS- and hMADS-CM. Moreover, apyrase did not reverse the #hMADS-CM-mediated inhibition of ADCC. Altogether, these results suggest the inhibition of ADCC by #hMADS-CM is not principally due to the release of extracellular ATP.

In summary, our study demonstrated that the inhibition of ADCC by adipocytes was mediated via proteins or exosomes secreted into the adipocyte-conditioned medium. Although we tested different relevant adipocytokines, none of them was responsible of the inhibition alone. This data suggests that the inhibition of ADCC by #hMADS and/or hMADS cells was mediated via a combination of several factors. Further investigations combining these factors would be necessary to understand the mechanisms of inhibition.



# **DISCUSSION**

## Implication of adipocytes in resistance to therapies

In 1989, Stephen Paget proposed the “seed and soil” theory, in which cancer cells tend to metastasize in a location with similar characteristics as their region of origin<sup>725</sup>. This theory underlines the crucial roles of the tumor microenvironment during tumorigenesis at not only metastatic sites but also primary sites. In breast cancer, adipocytes constitute the major components of the breast stroma and are localized at the proximity of tumor cells. Raising data have pointed out the roles of adipocytes in promoting tumor growth and invasion<sup>726,727</sup>. Moreover, several studies have indicated that adipocytes are also implicated in the resistance of cancer cells to chemotherapy and radiotherapy<sup>573,577</sup>.

In the scope of this study, we demonstrated that adipocytes inhibit trastuzumab-mediated ADCC in HER2+ breast cancer cells. Since ADCC is one major mechanism of action of trastuzumab<sup>79,85</sup>, our data suggests that adipocytes could induce the resistance of cancer cells to trastuzumab-based therapy. This is in keeping with the results by Nahta’s group who showed that adipocytokines mediated resistance of HER2+ breast cancer cell lines to trastuzumab-induced apoptosis<sup>728</sup>. However, further *in vivo* experiments are required to validate the involvement of adipocytes in cancer resistance to trastuzumab. We are currently testing this hypothesis using subcutaneous injections of human adipose tissue which forms lipomas in SCID mice.

Recently, Dirat *et al.* described that adipocytes cultivated with tumor cells displayed altered phenotypes and these cancer-associated adipocytes (CAA), but not “normal” adipocytes, promoted the invasion of breast cancer cells<sup>451</sup>. Although in our studies, we did not observe any delipidation of adipocytes during the short coculture time, it would be very interesting to know whether the protection of cancer cells against ADCC could be enhanced by CAA.

Besides breast cancer, in many other cancers such as ovarian cancer, melanoma or myeloma, tumor cells are located at the proximity of omental or subcutaneous or bone marrow adipocytes. Recent studies have demonstrated that adipocytes contribute to resistance of leukemia to L-asparaginase<sup>729</sup> and to resistance of melanoma to cisplatin, docetaxel and histone deacetylase inhibitor SAHA<sup>576</sup>. Therefore, it is tempting to speculate that adipocytes induce cancer resistance to various types of therapy. Further investigations on which therapeutic agents (conventional cytotoxic agents, hormone therapy or targeted therapy) are sensitive to the effect of adipocytes would be necessary to better understand the roles of the tumor microenvironment in induction of cancer resistance.

## Obesity and breast cancer resistance to therapies

Obesity has been clearly associated with poor prognosis in breast cancer. Obese breast cancer patients present more aggressive tumors, increased risk of recurrence and higher mortality<sup>730,731</sup>. Obesity is associated with a chronic inflammatory microenvironment and an increase in estrogen plasma levels<sup>732</sup>. Therefore, several data suggested a link between obesity and cancer resistance to therapies.

Recent study by Bochet *et al.* suggested that cancer-associated adipocytes could promote breast tumor radioresistance<sup>733</sup>. However, no correlation between BMI and radioresistance has been reported in any cancer.

A study by Behan *et al.* showed that adipocytes increased chemoresistance of leukemia in mice. However, like radiotherapy, no correlation between BMI and chemoresistance has been reported in cancer. This is partly due to the underdosing of obese patients by clinicians because of fear of toxicity might contribute to the decrease in treatment efficacy.

In hormone therapy, the link between BMI and the efficacy of the treatment seems to be not clear. While several studies indicated that aromatase inhibitors had lower efficacy in obese patients<sup>734–736</sup>, others showed no difference of efficacy between obese and nonobese women treated with tamoxifen or aromatase inhibitors<sup>737,738</sup>.

Our study suggested that obesity may have a negative impact on the efficacy of trastuzumab treatment. However, a recent study by Crozier *et al.* in a cohort with more than 3500 patients indicated that adjuvant trastuzumab improved the clinical outcome in all patients regardless of their BMI<sup>739</sup>.

Overall, the impact of BMI on cancer resistance to different therapies requires further investigations. Of interest, several studies suggested that weight gain after diagnosis appeared to be associated with greater disease mortality<sup>730</sup>. However, whether weight loss after diagnosis may improve prognosis remains unclear<sup>730</sup>.

## GDF15 and cancer resistance

In this study, we found that GDF15 was rapidly induced in cancer cells exposed to the conditioned medium from adipocytes. This result is in keeping with the work by Kim *et al.* who showed that the conditioned-medium from mouse bone marrow adipocytes stimulated the production of GDF15 in MDA-MB-231 breast cancer cells<sup>740</sup>. It is worth noting that under physiological conditions, GDF15 is expressed in low amounts in normal tissues except from the placenta. However, in pathological conditions such as inflammation and cancer, GDF15 expression is dramatically increased<sup>741</sup>. Although the over-expression of GDF15 has been reported in many cancers, including breast, prostate, and colon cancers<sup>742</sup>, its histological localization in tumor sites in relation to the adipose tissue remains unclear. Therefore, it would be interesting to investigate the evolution of GDF15 expression as the tumor invades the adjacent stroma to better understand the role of adipocytes in the induction of GDF15 expression in cancer.

Previous studies have shown that the expression of GDF15 in cancer cells could be up-regulated in response to various factors, including iron depletion<sup>743</sup>, hypoxia<sup>743</sup>, nonsteroidal anti-inflammatory drugs as well as dietary compounds<sup>744</sup>. Interestingly, it has been shown that GDF15 could be induced by palmitate<sup>740</sup>, IL-1 $\beta$ , TNF- $\alpha$  and IL-6<sup>745</sup>, which were all reported to be secreted by adipocytes<sup>213,620,740</sup>. The GDF15 promoter presents binding sites for the transcriptional factors SP1, p53 and EGR-1<sup>744</sup>. Coherently, we observed at mRNA levels a rapid up-regulation of EGR-1 that preceded the up-regulation of GDF15 in cancer cells after exposure to the adipocyte-conditioned medium. Current experiments are going to validate the GDF15 expression at protein levels.

GDF15 is a divergent member of the TGF- $\beta$  superfamily. It is synthesized and secreted under either the mature form after a cleavage of the dimeric propeptide or the unprocessed precursor<sup>746</sup>. Upon secretion, the mature GDF15 is diffused to the circulation while the propeptide binds to the extracellular matrix<sup>747</sup>. High levels of GDF15 serum levels have been reported in obese women<sup>748</sup>. Although the receptor for GDF15 remains unknown, there are some studies suggesting that GDF15 mediates its effect via the TGF- $\beta$  RII<sup>749,750</sup>. GDF15 was shown to activate different intracellular signaling pathways including SMAD, EGFR, AKT and ERK1/2<sup>750-752</sup>. In our studies, we observed a rapid phosphorylation of AKT and ERK1/2 (5-15 mins) before the induction of GDF15 in cancer cells. Therefore, adipocytokines other than cancer-secreted GDF15 would be responsible for the early activation of the survival AKT and MAPK pathways. Interestingly, Ding *et al.* has demonstrated that GDF15 could also be secreted by adipocytes as well as preadipocytes<sup>753</sup>, which suggests a direct effect by

adipocyte- or preadipocyte-secreted GDF15. Further experiments consisting of dosing GDF15 in the conditioned media of adipocytes and of cancer cells by Elisa would clarify the origin of GDF15.

The roles of GDF15 in cancer development and progression are complex<sup>741,746</sup>. While some *in vitro* and *in vivo* experimental evidence suggest anti-tumorigenic effects of GDF15, other data indicate that it has oncogenic activity. *In vitro*, over-expression of GDF15 in cancer cell lines resulted in growth arrest and increase in apoptosis<sup>754,755</sup>. However, Lee *et al.* reported that the forced expression of GDF15 in gastric cancer cell lines did not alter cell proliferation but increased its invasiveness<sup>756</sup>. *In vivo*, GDF15 over-expression in HCT116<sup>754</sup>, MCF-7<sup>757</sup>, PC-3<sup>758</sup> and glioblastoma cell lines<sup>759</sup> inhibited the growth of tumors in nude mice and xenograft models. By contrast, Boyle *et al.* showed that knock-down of GDF15 by shRNA in melanoma cells significantly decreased tumorigenicity in mice<sup>760</sup>. Additionally, orthotopically implemented PC-3 cells over-expressing GDF15 enhanced metastasis in mice compared to the PC-3 vector cells<sup>761</sup>. GDF15 was also shown to mediate anorexia/cachexia in mice with xenografted prostate tumors, which could be reversed by administration of anti-GDF15 antibody<sup>750</sup>. In patients, high levels of GDF15 are in general associated with tumor progression in many cancers, including melanoma, colon, pancreatic, thyroid and prostate cancer<sup>741</sup>. In breast cancer, no correlation between GDF15 expression and tumor progression has been so far reported. Overall, it is possible that the anti-tumorigenic or pro-tumorigenic effects of GDF15 are dependent on the types of cancer. Moreover, like TGF- $\beta$ , GDF15 may inhibit tumor growth in early stages of cancer, whereas in advanced cancer, it favors tumor progression<sup>762</sup>.

In our study, down-regulation of GDF15 by siRNA in cancer cells reversed the adipocyte-induced inhibition of ADCC. These results suggest that GDF15 is involved in adipocyte-promoting resistance of breast cancer cells to lysis by NK cells. This is supported by the work of Roth *et al.* who showed that depletion of GDF15 enhanced the susceptibility of mouse glioma cells to the lysis by NK cells<sup>763</sup>. Studies by Joshi *et al* also showed that GDF15 induced resistance to trastuzumab-mediated growth inhibition in breast cancer cells<sup>764</sup>. Besides, both over-expression of GDF15 or exogenous GDF15 conferred resistance to various chemotherapeutic agents in prostate cancer cells<sup>765</sup> and myeloma cells<sup>766</sup>. Altogether, these data suggest that GDF15 plays an important role in cancer resistance. Better understanding of the mechanisms of actions of GDF15 as well as the contribution by tumor cells or the tumor stroma would be necessary to develop strategies targeting GDF15 in future therapies.

## MYC, ADM and EGR1 as potential inducers of resistance

Our studies have identified several genes other than *Serpina3* and *Gdf15* that were rapidly up-regulated in cancer cells upon exposure to the conditioned medium from adipocytes. These genes include *Myc*, *Adm*, and *Egr1*.

*c-Myc* is a well known oncogene and a transcriptional factor that is deregulated in many cancers<sup>767</sup>. In breast cancer, MYC target genes are involved in cell growth, transformation, angiogenesis and cell-cycle control<sup>768</sup>. *c-Myc* amplification was shown to strongly correlated with *Her2* amplification in breast cancer<sup>769</sup>. However, since MYC has been shown to induce apoptosis<sup>770</sup>, it was unexpected to observe the up-regulation of *c-myc* in cancer cells that were resistant to ADCC. Further experiments are necessary to understand this apparent paradox.

*Adm* encodes for adrenomedullin (ADM), a pluripotent hormone expressed in many cancers, including breast, ovarian cancers and leukemia<sup>771</sup>. Interestingly, ADM has been shown to protect cancer cells against apoptosis induced by hypoxia, serum deprivation or chemotherapeutic agent<sup>772-774</sup>. However, the role of ADM in the resistance of tumor cells to ADCC remains unknown. Therefore, it would be interesting to examine whether adipocyte-induced ADM allows protection of tumor cells against lysis by NK cells.

*Egr1* encodes for a transcriptional factor which is rapidly induced by different stimuli, including growth factors, hypoxia or cytokines<sup>775</sup>. While *Egr1* has been shown to be a tumor suppressor gene in breast cancer cells<sup>776,777</sup>, it was implicated in resistance to apoptosis via inhibition of CD95 (Fas) expression in prostate cancer cells<sup>778</sup>. Therefore, whether adipocyte-induced EGR1 is implicated in the inhibition of ADCC requires further investigations.

## Alterations of NK cells by adipocytes

It was previously reported that NK cells were altered in the breast tumor microenvironment compared to NK cells in the blood<sup>277</sup>. Mamessier *et al.* showed that breast cancer cells altered NK cell phenotypes and functions via secretion of soluble factors<sup>289</sup>. However, the alteration of NK cells by the tumor stroma remains unclear. Given that infiltrating NK cells are often found within the stroma surrounding the tumors<sup>290</sup>, and that NK cells expressed receptors for many adipocytokines, it would be reasonable to speculate about an alteration of NK cells by adipocytes and/or preadipocytes.

Previous studies have demonstrated the phenotypic alteration of NK cells when NK cells were co-cultured with either peripheral ASCs or bone marrow-derived mesenchymal stem cells (BM-MSCs) for 3 to 6 days. Co-cultured NK cells displayed decreased expression of NKG2D, NKp30 and NKp44<sup>779-781</sup>. However, the modification of CD16 expression was inconsistent between studies. While CD16 expression was reported to be decreased in DelaRosa's study<sup>779</sup>, it was shown to be unchanged in Sortiropoulou's<sup>780</sup> and Spaggiari's<sup>781</sup> studies. Nevertheless, all these studies indicated that NK cytotoxicity was decreased when NK cells were co-cultured with MSCs. This is supported by a recent study by Ljujic *et al.* who showed that intravenous injection of human MSCs derived from the peripheral blood into mice led to decrease in cytotoxicity of NK cells in tumor-bearing mice<sup>782</sup>. Together, these data underline the importance of adipocyte-derived progenitors in the modulation of NK cell functions.

As for mature adipocytes, their roles in NK cell phenotype and functions remain largely unknown. In our study, we did not detect any change in NK cell surface receptors as well as NK cytotoxicity by adipocytes but also preadipocytes during the ADCC assay. This can be explained by the short time of co-incubation of adipocytes/preadipocytes and NK cells in the coculture system. Moreover, using the human NK-92 cell line over-expressing CD16 has limited pertinence. Further experiments using purified human NK cells are required. Given the abundance of adipocytes in the tumor microenvironment, as well as their relevance not only in targeted therapy but also in the immune-surveillance role of NK cells, further investigations would be necessary to understand the mechanism of alteration of NK cells by adipocytes.

## Identification of the adipocyte-secreted inhibitory factors

We demonstrated that the inhibition of ADCC by adipocytes and preadipocytes was mediated by factors secreted into the conditioned medium. We showed that these factors were likely proteins or exosomes in nature. Although they remain to be clearly determined, we speculate that the inhibition of ADCC observed was a result of a combination of effects of different factors secreted by adipocytes.

Adipocyte-derived factors can inhibit ADCC by direct inhibition of lytic granules secreted by NK cells. It was previously reported that both adipocytes and preadipocytes secrete a number of serine proteinase inhibitors (serpins), such as SERPINA3 ( $\alpha$ 1-antitrypsin inhibitor) and SERPINA12 (vaspin)<sup>783</sup>. While the targets and mechanisms of action of vaspin remain unknown<sup>784</sup>, Sipione *et al.* demonstrated that the murine homologue of SERPINA3, Serpina3n, could bind and inhibit granzyme B activity<sup>391</sup>. However, the addition of SERPINA3 purified from human serum did not inhibit ADCC activity (data not shown). Therefore, further investigations are required to understand the implication of adipocyte-secreted serpins in the inhibition of ADCC.

We tested several relevant adipocytokines for their implication in the inhibition of ADCC, including leptin, adiponectin, IL-6 and TGF- $\beta$ . Unexpectedly, none of these molecules displayed inhibitory effect at any concentration tested. It is possible that the adipocyte-mediated inhibition of ADCC is a combined effect of various adipocytokines. Therefore, experiments using neutralizing antibodies may provide better understanding on the contribution of each adipocytokine in the crosstalk between adipocytes and cancer cells.



## CD73 as target in anticancer treatment

CD73 has been largely recognized as a potent immune suppressor<sup>709</sup>. Its expression CD73 has been shown to be increased in many cancers, including breast cancer<sup>709</sup>. Previous studies suggested that CD73 expression was negatively associated with ER<sup>713,785</sup>. However, we did not detect any expression of CD73 in our four HER2-positive breast cancer cell lines regardless of their ER statuses: positive (BT474 and MDA-MB-361 cells) or negative (SKBR3 and MDA-MB-453 cells). These results are in agreement with the ones of Spychala *et al.* for BT474 and SKBR3 cells, while MDA-MB-361 and MDA-MB-453 cells were not tested in their work<sup>785</sup>.

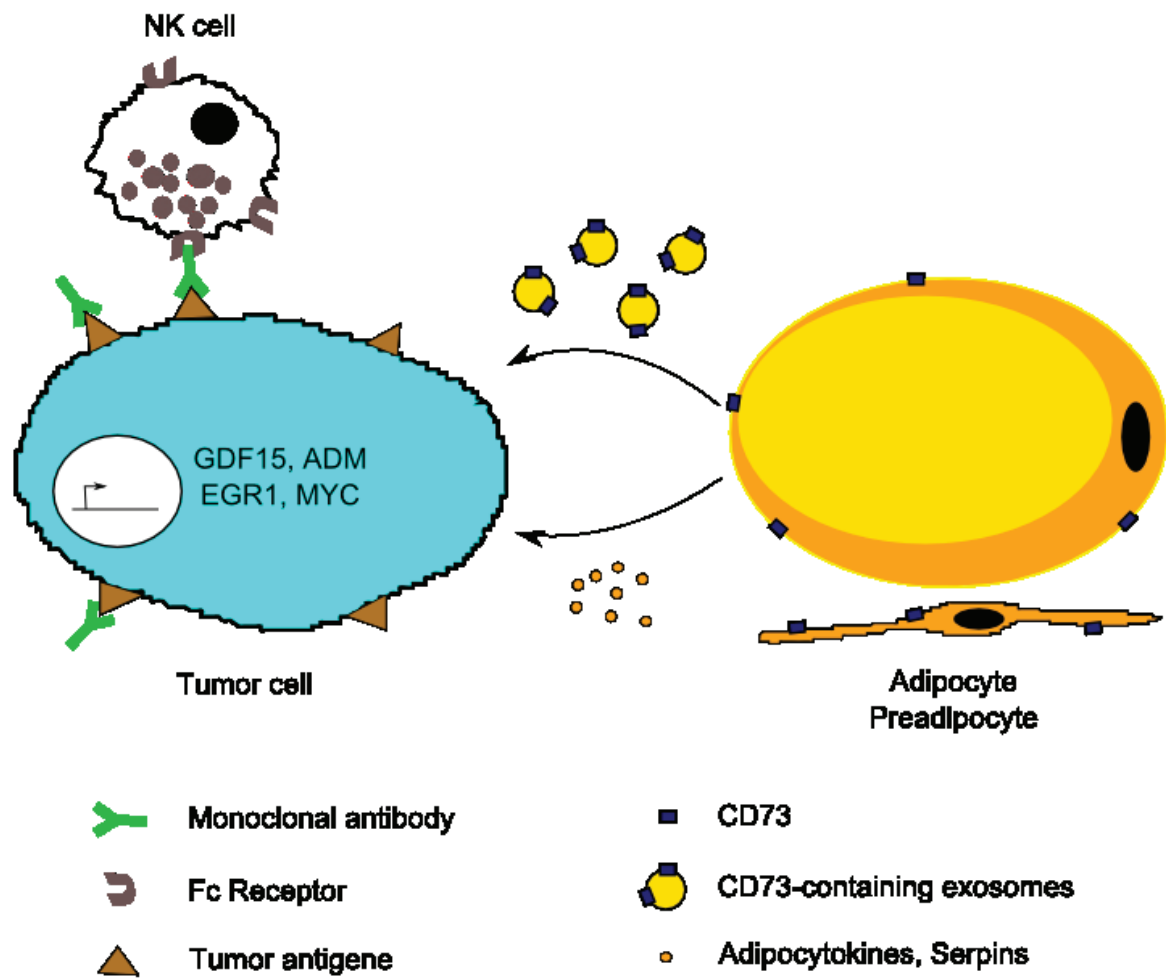
Although the expression of CD73 has been mainly attributed to lymphocytes, endothelial and epithelial cells<sup>709</sup>, it has been known that mesenchymal stem cells also express CD73<sup>257</sup>. In this study, we confirmed the expression as well as the enzymatic activity of CD73 on both preadipocytes and adipocytes. Interestingly, Krüger *et al.* have shown that in breast cancer carcinoma, CD73 was strongly expressed in the stromal compartment, particularly in the fibroblast-like population, but not in the tumor cells<sup>786</sup>. These fibroblasts are partly derived from the dedifferentiation of adipocytes in contact with tumor cells<sup>787</sup>. Furthermore, previous studies have demonstrated that non-hematopoietic host compartment promoted tumorigenesis<sup>719,720</sup>. Together, these data suggest the implication of adipose CD73 in the immunosuppressive function of the tumor microenvironment.

Besides the expression on the cell surface, CD73 has been shown to be incorporated into adipocyte-derived exosomes<sup>701</sup>. Curiously, this phenomenon was also similarly observed in cancer cells, with the release of CD73-expressing cancer exosomes that mediated immune-suppression<sup>788</sup>. Since cancer cells are permanently in interactions with adipocytes, it would be interesting to know whether the secretion of CD73-expressing exosomes could be induced or enhanced in both cell types in coculture. Moreover, Müller *et al.* demonstrated that CD73 could be transferred via exosomes from adipocytes to other adipocytes or stromal vascular cells<sup>789</sup>. Additionally, CD73-containing exosomes stimulated lipid synthesis in receptor cells<sup>789</sup>. Since cancer cells have been shown to accumulate lipids when co-cultivated with adipocytes<sup>455</sup>, we hypothesize that adipocytes could transfer CD73 to cancer cells, which may enhance the immune evasion of cancer cells. However, our preliminary experiments measuring the presence of CD73 on cancer cells from a 3-day coculture with adipocytes did not support this hypothesis (data not shown).

Although the immune suppression of CD73 was thought to be mediated via the generation of adenosine<sup>790</sup>, we did not observe the inhibition of ADCC by adenosine or

AMP. Therefore, we speculate that besides the generation of adenosine, the immune suppression of CD73 may be partly independent of its catalytic activity. This is supported by a very recent finding by Terp *et al.*, in which the authors showed that their anti-CD73 AD2 antibody inhibited metastasis of CD73-expressing breast cancer without altering CD73 catalytic activity<sup>791</sup>. These authors suggested that CD73 could also act as an adhesion and/or signaling molecule.

Collectively, these data suggest that adipocytes could participate into the immune evasion of cancer cells via the expression of CD73, or the secretion of CD73-expressing exosomes. Although the role of CD73 in the inhibition of ADCC has not been able to be validated in our experiments, further investigations would be required to understand the contribution of adipocyte-derived adenosine and the potential signaling pathway of CD73 in cancer resistance to other therapies.



**Figure 19. Adipocyte/preadipocyte-mediated increase in resistance of cancer cells to ADCC**

Adipocytes and preadipocytes express high levels of CD73. They also secrete various soluble factors, including adipocytokines, serpins and CD73-containing exosomes. Serpins may directly inhibit cytotoxic granzymes released by NK cells. Adipocytokines up-regulate the expression of genes that may be implicated in tumor survival. CD73 which is expressed on adipocytes or on preadipocytes, or on adipocyte-derived exosomes, or is transferred to tumor cells, can mediate immune evasion via the generation of adenosine. CD73 could also act as an adhesion or signaling molecule.

# CONCLUSION AND PERSPECTIVES

In conclusion, our study has demonstrated the involvement of adipocytes and preadipocytes in the resistance of breast cancer cells to trastuzumab-mediated ADCC. This illustrates the importance of the tumor microenvironment that needs to be taken into account during antibody-based therapy. Further investigations would be necessary to better understand the roles of adipose tissue in cancer resistance to other therapies and in other types of cancer. Interestingly, we have identified the adipocyte-induced genes in cancer cells and suggested that GDF15 may play an important role in the cancer resistance. In the next steps, we will focus on determining the signalization pathway of GDF15 and on identifying the adipocytokines that induce the expression of GDF15. A critical limitation of our study is the requirement of validation of our results *in vivo*, such as in mouse models or in tumor samples, which is ongoing. Our work on the crosstalk between adipocytes and cancer cells suggests a link between obesity and cancer resistance to therapies. This is of particular interest since obesity has been identified as an important risk and associated with poor prognosis in many cancers. Identification of the mediators between adipocytes and cancer cells will allow the development of sensitizing strategies to circumvent adipose tissue-mediated resistance of cancer to therapies.

# REFERENCES

1. Hill, T. D., Khamis, H. J., Tyczynski, J. E. & Berkel, H. J. Comparison of male and female breast cancer incidence trends, tumor characteristics, and survival. *Ann. Epidemiol.* **15**, 773–780 (2005).
2. Ferlay, J. *et al.* Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int. J. Cancer J. Int. Cancer* **127**, 2893–2917 (2010).
3. Elston, C. W. & Ellis, I. O. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* **41**, 154–161 (2002).
4. Rakha, E. A. *et al.* Prognostic significance of Nottingham histologic grade in invasive breast carcinoma. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **26**, 3153–3158 (2008).
5. Galea, M. H., Blamey, R. W., Elston, C. E. & Ellis, I. O. The Nottingham Prognostic Index in primary breast cancer. *Breast Cancer Res. Treat.* **22**, 207–219 (1992).
6. D'Eredita', G., Giardina, C., Martellotta, M., Natale, T. & Ferrarese, F. Prognostic factors in breast cancer: the predictive value of the Nottingham Prognostic Index in patients with a long-term follow-up that were treated in a single institution. *Eur. J. Cancer Oxf. Engl.* **1990** **37**, 591–596 (2001).
7. Okugawa, H. *et al.* Prognostic factors in breast cancer: the value of the Nottingham Prognostic Index for patients treated in a single institution. *Surg. Today* **35**, 907–911 (2005).
8. *World Health Organization Classification of Tumors. Pathology and Genetics of Tumours of the Breast and Female Genital Organs*, (IARC Press, 2003).
9. Pereira, H. *et al.* Pathological prognostic factors in breast cancer. IV: Should you be a typer or a grader? A comparative study of two histological prognostic features in operable breast carcinoma. *Histopathology* **27**, 219–226 (1995).
10. Patani, N., Martin, L.-A. & Dowsett, M. Biomarkers for the clinical management of breast cancer: international perspective. *Int. J. Cancer J. Int. Cancer* **133**, 1–13 (2013).
11. Heldring, N. *et al.* Estrogen receptors: how do they signal and what are their targets. *Physiol. Rev.* **87**, 905–931 (2007).
12. Nelson, L. R. & Bulun, S. E. Estrogen production and action. *J. Am. Acad. Dermatol.* **45**, S116–124 (2001).
13. Liang, J. & Shang, Y. Estrogen and cancer. *Annu. Rev. Physiol.* **75**, 225–240 (2013).
14. Dunnwald, L. K., Rossing, M. A. & Li, C. I. Hormone receptor status, tumor characteristics, and prognosis: a prospective cohort of breast cancer patients. *Breast Cancer Res. BCR* **9**, R6 (2007).
15. Horwitz, K. B., Koseki, Y. & McGuire, W. L. Estrogen control of progesterone receptor in human breast cancer: role of estradiol and antiestrogen. *Endocrinology* **103**, 1742–1751 (1978).
16. Li, X. & O'Malley, B. W. Unfolding the action of progesterone receptors. *J. Biol. Chem.* **278**, 39261–39264 (2003).
17. Rakha, E. A. *et al.* Biologic and clinical characteristics of breast cancer with single hormone receptor positive phenotype. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **25**, 4772–4778 (2007).
18. Viale, G. *et al.* Prognostic and predictive value of centrally reviewed expression of estrogen and progesterone receptors in a randomized trial comparing letrozole and tamoxifen adjuvant therapy for postmenopausal early breast cancer: BIG 1-98. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **25**, 3846–3852 (2007).
19. Dowsett, M. *et al.* Relationship between quantitative estrogen and progesterone receptor expression and human epidermal growth factor receptor 2 (HER-2) status with recurrence in the Arimidex, Tamoxifen, Alone or in Combination trial. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **26**, 1059–1065 (2008).
20. Arpino, G. *et al.* Estrogen receptor-positive, progesterone receptor-negative breast cancer: association with growth factor receptor expression and tamoxifen resistance. *J. Natl. Cancer Inst.* **97**, 1254–1261 (2005).

21. Bardou, V.-J., Arpino, G., Elledge, R. M., Osborne, C. K. & Clark, G. M. Progesterone receptor status significantly improves outcome prediction over estrogen receptor status alone for adjuvant endocrine therapy in two large breast cancer databases. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **21**, 1973–1979 (2003).
22. Early Breast Cancer Trialists' Collaborative Group (EBCTCG) *et al.* Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. *Lancet* **378**, 771–784 (2011).
23. Dowsett, M. *et al.* Benefit from adjuvant tamoxifen therapy in primary breast cancer patients according oestrogen receptor, progesterone receptor, EGF receptor and HER2 status. *Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. ESMO* **17**, 818–826 (2006).
24. Olivotto, I. A. *et al.* Time to stop progesterone receptor testing in breast cancer management. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **22**, 1769–1770 (2004).
25. Colomer, R. *et al.* It is not time to stop progesterone receptor testing in breast cancer. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **23**, 3868–3869; author reply 3869–3870 (2005).
26. Fuqua, S. A. W., Cui, Y., Lee, A. V., Osborne, C. K. & Horwitz, K. B. Insights into the role of progesterone receptors in breast cancer. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **23**, 931–932; author reply 932–933 (2005).
27. Colozza, M., Larsimont, D. & Piccart, M. J. Progesterone receptor testing: not the right time to be buried. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **23**, 3867–3868; author reply 3869–3870 (2005).
28. Moasser, M. M. The oncogene HER2: its signaling and transforming functions and its role in human cancer pathogenesis. *Oncogene* **26**, 6469–6487 (2007).
29. Slamon, D. J. *et al.* Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **235**, 177–182 (1987).
30. Dandachi, N., Dietze, O. & Hauser-Kronberger, C. Chromogenic in situ hybridization: a novel approach to a practical and sensitive method for the detection of HER2 oncogene in archival human breast carcinoma. *Lab. Investig. J. Tech. Methods Pathol.* **82**, 1007–1014 (2002).
31. Chia, S. *et al.* Human epidermal growth factor receptor 2 overexpression as a prognostic factor in a large tissue microarray series of node-negative breast cancers. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **26**, 5697–5704 (2008).
32. Ross, J. S. *et al.* The Her-2/neu gene and protein in breast cancer 2003: biomarker and target of therapy. *The oncologist* **8**, 307–325 (2003).
33. Gerdes, J., Schwab, U., Lemke, H. & Stein, H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *Int. J. Cancer J. Int. Cancer* **31**, 13–20 (1983).
34. Hanahan, D. & Weinberg, R. A. The hallmarks of cancer. *Cell* **100**, 57–70 (2000).
35. Chang, J. *et al.* Prediction of clinical outcome from primary tamoxifen by expression of biologic markers in breast cancer patients. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **6**, 616–621 (2000).
36. Veronese, S. M. *et al.* Proliferation index as a prognostic marker in breast cancer. *Cancer* **71**, 3926–3931 (1993).
37. Urruticoechea, A., Smith, I. E. & Dowsett, M. Proliferation marker Ki-67 in early breast cancer. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **23**, 7212–7220 (2005).
38. Dowsett, M. *et al.* Assessment of Ki67 in breast cancer: recommendations from the International Ki67 in Breast Cancer working group. *J. Natl. Cancer Inst.* **103**, 1656–1664 (2011).
39. Gnant, M., Harbeck, N. & Thomssen, C. St. Gallen 2011: Summary of the Consensus Discussion. *Breast Care Basel Switz.* **6**, 136–141 (2011).
40. Perou, C. M. *et al.* Molecular portraits of human breast tumours. *Nature* **406**, 747–752 (2000).
41. Sørli, T. *et al.* Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 10869–10874 (2001).
42. Sorlie, T. *et al.* Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 8418–8423 (2003).

43. Sotiriou, C. *et al.* Breast cancer classification and prognosis based on gene expression profiles from a population-based study. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 10393–10398 (2003).
44. Herschkowitz, J. I. *et al.* Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. *Genome Biol.* **8**, R76 (2007).
45. Prat, A. *et al.* Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. *Breast Cancer Res. BCR* **12**, R68 (2010).
46. Callagy, G. *et al.* Molecular classification of breast carcinomas using tissue microarrays. *Diagn. Mol. Pathol. Am. J. Surg. Pathol. Part B* **12**, 27–34 (2003).
47. Jacquemier, J. *et al.* Protein expression profiling identifies subclasses of breast cancer and predicts prognosis. *Cancer Res.* **65**, 767–779 (2005).
48. Abd El-Rehim, D. M. *et al.* High-throughput protein expression analysis using tissue microarray technology of a large well-characterised series identifies biologically distinct classes of breast cancer confirming recent cDNA expression analyses. *Int. J. Cancer J. Int. Cancer* **116**, 340–350 (2005).
49. Blows, F. M. *et al.* Subtyping of Breast Cancer by Immunohistochemistry to Investigate a Relationship between Subtype and Short and Long Term Survival: A Collaborative Analysis of Data for 10,159 Cases from 12 Studies. *PLoS Med* **7**, e1000279 (2010).
50. Hugh, J. *et al.* Breast cancer subtypes and response to docetaxel in node-positive breast cancer: use of an immunohistochemical definition in the BCIRG 001 trial. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **27**, 1168–1176 (2009).
51. Cheang, M. C. U. *et al.* Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. *J. Natl. Cancer Inst.* **101**, 736–750 (2009).
52. Ryan, Q. *et al.* FDA drug approval summary: lapatinib in combination with capecitabine for previously treated metastatic breast cancer that overexpresses HER-2. *The oncologist* **13**, 1114–1119 (2008).
53. Nelson, M. H. & Dolder, C. R. Lapatinib: a novel dual tyrosine kinase inhibitor with activity in solid tumors. *Ann. Pharmacother.* **40**, 261–269 (2006).
54. Bolduan, C. & Ehrlich, P. *Collected studies on immunity.* (J. Wiley & sons; [etc., etc.], 1906).
55. Köhler, G. & Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**, 495–497 (1975).
56. Brekke, O. H. & Sandlie, I. Therapeutic antibodies for human diseases at the dawn of the twenty-first century. *Nat. Rev. Drug Discov.* **2**, 52–62 (2003).
57. Weiner, L. M., Surana, R. & Wang, S. Monoclonal antibodies: versatile platforms for cancer immunotherapy. *Nat. Rev. Immunol.* **10**, 317–327 (2010).
58. Presta, L. G. Engineering of therapeutic antibodies to minimize immunogenicity and optimize function. *Adv. Drug Deliv. Rev.* **58**, 640–656 (2006).
59. Reichert, J. M., Rosensweig, C. J., Faden, L. B. & Dewitz, M. C. Monoclonal antibody successes in the clinic. *Nat. Biotechnol.* **23**, 1073–1078 (2005).
60. Spano, J. P. *et al.* Epidermal growth factor receptor signaling in colorectal cancer: preclinical data and therapeutic perspectives. *Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. ESMO* **16**, 189–194 (2005).
61. Hicklin, D. J. & Ellis, L. M. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **23**, 1011–1027 (2005).
62. Knuefermann, C. *et al.* HER2/PI-3K/Akt activation leads to a multidrug resistance in human breast adenocarcinoma cells. *Oncogene* **22**, 3205–3212 (2003).
63. Baselga, J. & Mendelsohn, J. Receptor blockade with monoclonal antibodies as anti-cancer therapy. *Pharmacol. Ther.* **64**, 127–154 (1994).
64. Ludwig, D. L., Pereira, D. S., Zhu, Z., Hicklin, D. J. & Bohlen, P. Monoclonal antibody therapeutics and apoptosis. *Oncogene* **22**, 9097–9106 (2003).
65. Trauth, B. C. *et al.* Monoclonal antibody-mediated tumor regression by induction of apoptosis. *Science* **245**, 301–305 (1989).

66. Yonehara, S., Ishii, A. & Yonehara, M. A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. *J. Exp. Med.* **169**, 1747–1756 (1989).
67. Itoh, N. *et al.* The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* **66**, 233–243 (1991).
68. Heinemann, V., Stintzing, S., Kirchner, T., Boeck, S. & Jung, A. Clinical relevance of EGFR- and KRAS-status in colorectal cancer patients treated with monoclonal antibodies directed against the EGFR. *Cancer Treat. Rev.* **35**, 262–271 (2009).
69. Normanno, N. *et al.* Implications for KRAS status and EGFR-targeted therapies in metastatic CRC. *Nat. Rev. Clin. Oncol.* **6**, 519–527 (2009).
70. Qiu, L.-X. *et al.* Predictive and prognostic value of KRAS mutations in metastatic colorectal cancer patients treated with cetuximab: a meta-analysis of 22 studies. *Eur. J. Cancer Oxf. Engl.* **1990** **46**, 2781–2787 (2010).
71. Gelderman, K. A., Tomlinson, S., Ross, G. D. & Gorter, A. Complement function in mAb-mediated cancer immunotherapy. *Trends Immunol.* **25**, 158–164 (2004).
72. Reff, M. E. *et al.* Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. *Blood* **83**, 435–445 (1994).
73. Di Gaetano, N. *et al.* Complement activation determines the therapeutic activity of rituximab in vivo. *J. Immunol. Baltim. Md* **1950** **171**, 1581–1587 (2003).
74. Graziano, R. F. & Guyre, P. M. in *eLS* (John Wiley & Sons, Ltd, 2001). at <<http://onlinelibrary.wiley.com/doi/10.1038/npg.els.0000498/abstract>>
75. Iannello, A. & Ahmad, A. Role of antibody-dependent cell-mediated cytotoxicity in the efficacy of therapeutic anti-cancer monoclonal antibodies. *Cancer Metastasis Rev.* **24**, 487–499 (2005).
76. Daëron, M. Fc receptor biology. *Annu. Rev. Immunol.* **15**, 203–234 (1997).
77. Strome, S. E., Sausville, E. A. & Mann, D. A mechanistic perspective of monoclonal antibodies in cancer therapy beyond target-related effects. *The oncologist* **12**, 1084–1095 (2007).
78. Nimmerjahn, F. & Ravetch, J. V. Fcγ receptors as regulators of immune responses. *Nat. Rev. Immunol.* **8**, 34–47 (2008).
79. Clynes, R. A., Towers, T. L., Presta, L. G. & Ravetch, J. V. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nat. Med.* **6**, 443–446 (2000).
80. Takai, T. Roles of Fc receptors in autoimmunity. *Nat. Rev. Immunol.* **2**, 580–592 (2002).
81. Hernandez-Ilizaliturri, F. J. *et al.* Neutrophils contribute to the biological antitumor activity of rituximab in a non-Hodgkin's lymphoma severe combined immunodeficiency mouse model. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **9**, 5866–5873 (2003).
82. Metes, D. *et al.* Expression and function of Fc γ RII on human natural killer cells. *Nat. Immun.* **13**, 289–300 (1994).
83. Cartron, G. *et al.* Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor Fcγ RIIIa gene. *Blood* **99**, 754–758 (2002).
84. Weng, W.-K. & Levy, R. Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **21**, 3940–3947 (2003).
85. Musolino, A. *et al.* Immunoglobulin G fragment C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2/neu-positive metastatic breast cancer. *J. Clin. Oncol.* **26**, 1789–1796 (2008).
86. Manches, O. *et al.* In vitro mechanisms of action of rituximab on primary non-Hodgkin lymphomas. *Blood* **101**, 949–954 (2003).
87. Akewanlop, C. *et al.* Phagocytosis of breast cancer cells mediated by anti-MUC-1 monoclonal antibody, DF3, and its bispecific antibody. *Cancer Res.* **61**, 4061–4065 (2001).
88. Uchida, J. *et al.* The innate mononuclear phagocyte network depletes B lymphocytes through Fc receptor-dependent mechanisms during anti-CD20 antibody immunotherapy. *J. Exp. Med.* **199**, 1659–1669 (2004).



89. Tamura, K. *et al.* FcγR2A and 3A polymorphisms predict clinical outcome of trastuzumab in both neoadjuvant and metastatic settings in patients with HER2-positive breast cancer. *Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. ESMO* **22**, 1302–1307 (2011).
90. Zhang, W. *et al.* FCGR2A and FCGR3A polymorphisms associated with clinical outcome of epidermal growth factor receptor expressing metastatic colorectal cancer patients treated with single-agent cetuximab. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **25**, 3712–3718 (2007).
91. Carter, P. Improving the efficacy of antibody-based cancer therapies. *Nat. Rev. Cancer* **1**, 118–129 (2001).
92. King, C. R., Kraus, M. H. & Aaronson, S. A. Amplification of a novel v-erbB-related gene in a human mammary carcinoma. *Science* **229**, 974–976 (1985).
93. Valabrega, G., Montemurro, F. & Aglietta, M. Trastuzumab: mechanism of action, resistance and future perspectives in HER2-overexpressing breast cancer. *Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. ESMO* **18**, 977–984 (2007).
94. Colomer, R., Lupu, R., Bacus, S. S. & Gelmann, E. P. erbB-2 antisense oligonucleotides inhibit the proliferation of breast carcinoma cells with erbB-2 oncogene amplification. *Br. J. Cancer* **70**, 819–825 (1994).
95. Roh, H., Pippin, J. & Drebin, J. A. Down-regulation of HER2/neu expression induces apoptosis in human cancer cells that overexpress HER2/neu. *Cancer Res.* **60**, 560–565 (2000).
96. Choudhury, A. *et al.* Small interfering RNA (siRNA) inhibits the expression of the Her2/neu gene, upregulates HLA class I and induces apoptosis of Her2/neu positive tumor cell lines. *Int. J. Cancer J. Int. Cancer* **108**, 71–77 (2004).
97. Faltus, T. *et al.* Silencing of the HER2/neu gene by siRNA inhibits proliferation and induces apoptosis in HER2/neu-overexpressing breast cancer cells. *Neoplasia N. Y. N* **6**, 786–795 (2004).
98. Juhl, H., Downing, S. G., Wellstein, A. & Czubayko, F. HER-2/neu is rate-limiting for ovarian cancer growth. Conditional depletion of HER-2/neu by ribozyme targeting. *J. Biol. Chem.* **272**, 29482–29486 (1997).
99. Messerle, K., Schlegel, J., Hynes, N. E. & Groner, B. NIH/3T3 cells transformed with the activated erbB-2 oncogene can be phenotypically reverted by a kinase deficient, dominant negative erbB-2 variant. *Mol. Cell. Endocrinol.* **105**, 1–10 (1994).
100. Beerli, R. R., Wels, W. & Hynes, N. E. Intracellular expression of single chain antibodies reverts ErbB-2 transformation. *J. Biol. Chem.* **269**, 23931–23936 (1994).
101. Deshane, J. *et al.* Targeted tumor killing via an intracellular antibody against erbB-2. *J. Clin. Invest.* **96**, 2980–2989 (1995).
102. Baasner, S., von Melchner, H., Klenner, T., Hilgard, P. & Beckers, T. Reversible tumorigenesis in mice by conditional expression of the HER2/c-erbB2 receptor tyrosine kinase. *Oncogene* **13**, 901–911 (1996).
103. Schiffer, I. B. *et al.* Switching off HER-2/neu in a tetracycline-controlled mouse tumor model leads to apoptosis and tumor-size-dependent remission. *Cancer Res.* **63**, 7221–7231 (2003).
104. Xie, W., Chow, L. T., Paterson, A. J., Chin, E. & Kudlow, J. E. Conditional expression of the ErbB2 oncogene elicits reversible hyperplasia in stratified epithelia and up-regulation of TGFα expression in transgenic mice. *Oncogene* **18**, 3593–3607 (1999).
105. Moody, S. E. *et al.* Conditional activation of Neu in the mammary epithelium of transgenic mice results in reversible pulmonary metastasis. *Cancer Cell* **2**, 451–461 (2002).
106. Montemurro, F. & Aglietta, M. Incorporating trastuzumab into the neoadjuvant treatment of HER2-overexpressing breast cancer. *Clin. Breast Cancer* **6**, 77–80 (2005).
107. Cobleigh, M. A. *et al.* Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J. Clin. Oncol.* **17**, 2639–2648 (1999).
108. Baselga, J. Clinical trials of single-agent trastuzumab (Herceptin). *Semin. Oncol.* **27**, 20–26 (2000).
109. Vogel, C. L. *et al.* Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **20**, 719–726 (2002).

110. Sawaki, M. *et al.* Efficacy and safety of trastuzumab as a single agent in heavily pretreated patients with HER-2/neu-overexpressing metastatic breast cancer. *Tumori* **90**, 40–43 (2004).
111. Montemurro, F., Valabrega, G. & Aglietta, M. Trastuzumab-based combination therapy for breast cancer. *Expert Opin. Pharmacother.* **5**, 81–96 (2004).
112. Hudis, C. A. Trastuzumab--mechanism of action and use in clinical practice. *N. Engl. J. Med.* **357**, 39–51 (2007).
113. Nielsen, D. L., Andersson, M. & Kamby, C. HER2-targeted therapy in breast cancer. Monoclonal antibodies and tyrosine kinase inhibitors. *Cancer Treat. Rev.* **35**, 121–136 (2009).
114. Slamon, D. J. *et al.* Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N. Engl. J. Med.* **344**, 783–792 (2001).
115. Marty, M. *et al.* Randomized phase II trial of the efficacy and safety of trastuzumab combined with docetaxel in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer administered as first-line treatment: the M77001 study group. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **23**, 4265–4274 (2005).
116. Gasparini, G. *et al.* Randomized Phase II Trial of weekly paclitaxel alone versus trastuzumab plus weekly paclitaxel as first-line therapy of patients with Her-2 positive advanced breast cancer. *Breast Cancer Res. Treat.* **101**, 355–365 (2007).
117. Delord, J. P. *et al.* Selective inhibition of HER2 inhibits AKT signal transduction and prolongs disease-free survival in a micrometastasis model of ovarian carcinoma. *Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. ESMO* **16**, 1889–1897 (2005).
118. Nagata, Y. *et al.* PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. *Cancer Cell* **6**, 117–127 (2004).
119. Cooley, S., Burns, L. J., Repka, T. & Miller, J. S. Natural killer cell cytotoxicity of breast cancer targets is enhanced by two distinct mechanisms of antibody-dependent cellular cytotoxicity against LFA-3 and HER2/neu. *Exp. Hematol.* **27**, 1533–1541 (1999).
120. Park, S. *et al.* The therapeutic effect of anti-HER2/neu antibody depends on both innate and adaptive immunity. *Cancer Cell* **18**, 160–170 (2010).
121. Christianson, T. A. *et al.* NH2-terminally truncated HER-2/neu protein: relationship with shedding of the extracellular domain and with prognostic factors in breast cancer. *Cancer Res.* **58**, 5123–5129 (1998).
122. Molina, M. A. *et al.* NH(2)-terminal truncated HER-2 protein but not full-length receptor is associated with nodal metastasis in human breast cancer. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **8**, 347–353 (2002).
123. Molina, M. A. *et al.* Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells. *Cancer Res.* **61**, 4744–4749 (2001).
124. Köstler, W. J. *et al.* Monitoring of serum Her-2/neu predicts response and progression-free survival to trastuzumab-based treatment in patients with metastatic breast cancer. *Clin. Cancer Res.* **10**, 1618–1624 (2004).
125. Fornier, M. N. *et al.* Serum HER2 extracellular domain in metastatic breast cancer patients treated with weekly trastuzumab and paclitaxel: association with HER2 status by immunohistochemistry and fluorescence in situ hybridization and with response rate. *Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. ESMO* **16**, 234–239 (2005).
126. Yen, L. *et al.* Heregulin selectively upregulates vascular endothelial growth factor secretion in cancer cells and stimulates angiogenesis. *Oncogene* **19**, 3460–3469 (2000).
127. Izumi, Y., Xu, L., di Tomaso, E., Fukumura, D. & Jain, R. K. Tumour biology: herceptin acts as an anti-angiogenic cocktail. *Nature* **416**, 279–280 (2002).
128. Klos, K. S. *et al.* Combined trastuzumab and paclitaxel treatment better inhibits ErbB-2-mediated angiogenesis in breast carcinoma through a more effective inhibition of Akt than either treatment alone. *Cancer* **98**, 1377–1385 (2003).
129. Kurmasheva, R. T. & Houghton, P. J. IGF-I mediated survival pathways in normal and malignant cells. *Biochim. Biophys. Acta* **1766**, 1–22 (2006).

130. Khandwala, H. M., McCutcheon, I. E., Flyvbjerg, A. & Friend, K. E. The effects of insulin-like growth factors on tumorigenesis and neoplastic growth. *Endocr. Rev.* **21**, 215–244 (2000).
131. Hankinson, S. E. *et al.* Circulating concentrations of insulin-like growth factor-I and risk of breast cancer. *Lancet* **351**, 1393–1396 (1998).
132. Pollak, M. Insulin and insulin-like growth factor signalling in neoplasia. *Nat. Rev. Cancer* **8**, 915–928 (2008).
133. Yee, D. *et al.* Analysis of insulin-like growth factor I gene expression in malignancy: evidence for a paracrine role in human breast cancer. *Mol. Endocrinol. Baltim. Md* **3**, 509–517 (1989).
134. Paik, S. Expression of IGF-I and IGF-II mRNA in breast tissue. *Breast Cancer Res. Treat.* **22**, 31–38 (1992).
135. Gebauer, G., Jäger, W. & Lang, N. mRNA expression of components of the insulin-like growth factor system in breast cancer cell lines, tissues, and metastatic breast cancer cells. *Anticancer Res.* **18**, 1191–1195 (1998).
136. Lu, Y., Zi, X., Zhao, Y., Mascarenhas, D. & Pollak, M. Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin). *J. Natl. Cancer Inst.* **93**, 1852–1857 (2001).
137. Nahta, R., Yuan, L. X. H., Zhang, B., Kobayashi, R. & Esteva, F. J. Insulin-like growth factor-I receptor/human epidermal growth factor receptor 2 heterodimerization contributes to trastuzumab resistance of breast cancer cells. *Cancer Res.* **65**, 11118–11128 (2005).
138. Valabrega, G. *et al.* TGF $\alpha$  expression impairs Trastuzumab-induced HER2 downregulation. *Oncogene* **24**, 3002–3010 (2005).
139. Austin, C. D. *et al.* Endocytosis and sorting of ErbB2 and the site of action of cancer therapeutics trastuzumab and geldanamycin. *Mol. Biol. Cell* **15**, 5268–5282 (2004).
140. Murphy, C. G. & Morris, P. G. Recent advances in novel targeted therapies for HER2-positive breast cancer. *Anticancer. Drugs* **23**, 765–776 (2012).
141. Valabrega, G. *et al.* HER2-positive breast cancer cells resistant to trastuzumab and lapatinib lose reliance upon HER2 and are sensitive to the multitargeted kinase inhibitor sorafenib. *Breast Cancer Res. Treat.* **130**, 29–40 (2011).
142. Brodowicz, T. *et al.* Soluble HER-2/neu neutralizes biologic effects of anti-HER-2/neu antibody on breast cancer cells in vitro. *Int. J. Cancer* **73**, 875–879 (1997).
143. Carney, W. P. *et al.* Monitoring the circulating levels of the HER2/neu oncoprotein in breast cancer. *Clin. Breast Cancer* **5**, 105–116 (2004).
144. Price-Schiavi, S. A. *et al.* Rat Muc4 (sialomucin complex) reduces binding of anti-ErbB2 antibodies to tumor cell surfaces, a potential mechanism for herceptin resistance. *Int. J. Cancer* **99**, 783–791 (2002).
145. Nagy, P. *et al.* Decreased accessibility and lack of activation of ErbB2 in JIMT-1, a herceptin-resistant, MUC4-expressing breast cancer cell line. *Cancer Res.* **65**, 473–482 (2005).
146. Chen, A. C. *et al.* Upregulation of mucin4 in ER-positive/HER2-overexpressing breast cancer xenografts with acquired resistance to endocrine and HER2-targeted therapies. *Breast Cancer Res. Treat.* **134**, 583–593 (2012).
147. Wajchenberg, B. L. Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. *Endocr. Rev.* **21**, 697–738 (2000).
148. Exercise, A. C. on. *ACE Personal Trainer Manual: The Ultimate Resource for Fitness Professionals.* (American Council on Exercise, 2003).
149. Shen, W. *et al.* Adipose Tissue Quantification by Imaging Methods: A Proposed Classification. *Obes. Res.* **11**, 5 (2003).
150. Cinti, S. Between brown and white: novel aspects of adipocyte differentiation. *Ann. Med.* **43**, 104–115 (2011).
151. Ricquier, D. & Bouillaud, F. The uncoupling protein homologues: UCP1, UCP2, UCP3, StUCP and AtUCP. *Biochem. J.* **345**, 161 (2000).
152. Gimble, J. M., Katz, A. J. & Bunnell, B. A. Adipose-derived stem cells for regenerative medicine. *Circ. Res.* **100**, 1249–1260 (2007).
153. Cypess, A. M. *et al.* Identification and importance of brown adipose tissue in adult humans. *N. Engl. J. Med.* **360**, 1509–1517 (2009).

154. Van Marken Lichtenbelt, W. D. *et al.* Cold-activated brown adipose tissue in healthy men. *N. Engl. J. Med.* **360**, 1500–1508 (2009).
155. Virtanen, K. A. *et al.* Functional brown adipose tissue in healthy adults. *N. Engl. J. Med.* **360**, 1518–1525 (2009).
156. Cinti, S. Adipocyte differentiation and transdifferentiation: plasticity of the adipose organ. *J. Endocrinol. Invest.* **25**, 823–835 (2002).
157. Cinti, S. Transdifferentiation properties of adipocytes in the adipose organ. *Am. J. Physiol. Endocrinol. Metab.* **297**, E977–986 (2009).
158. Loncar, D. Convertible adipose tissue in mice. *Cell Tissue Res.* **266**, 149–161 (1991).
159. English, J. T., Patel, S. K. & Flanagan, M. J. Association of pheochromocytomas with brown fat tumors. *Radiology* **107**, 279–281 (1973).
160. Huttunen, P., Hirvonen, J. & Kinnula, V. The occurrence of brown adipose tissue in outdoor workers. *Eur. J. Appl. Physiol.* **46**, 339–345 (1981).
161. Seale, P. *et al.* Prdm16 determines the thermogenic program of subcutaneous white adipose tissue in mice. *J. Clin. Invest.* **121**, 96–105 (2011).
162. Vegiopoulos, A. *et al.* Cyclooxygenase-2 controls energy homeostasis in mice by de novo recruitment of brown adipocytes. *Science* **328**, 1158–1161 (2010).
163. Madsen, L. *et al.* UCP1 induction during recruitment of brown adipocytes in white adipose tissue is dependent on cyclooxygenase activity. *PLoS One* **5**, e11391 (2010).
164. Hansen, J. B. *et al.* Retinoblastoma protein functions as a molecular switch determining white versus brown adipocyte differentiation. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 4112–4117 (2004).
165. Calo, E. *et al.* Rb regulates fate choice and lineage commitment in vivo. *Nature* **466**, 1110–1114 (2010).
166. Wu, J. *et al.* Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell* **150**, 366–376 (2012).
167. Seale, P. *et al.* PRDM16 controls a brown fat/skeletal muscle switch. *Nature* **454**, 961–967 (2008).
168. Petrovic, N. *et al.* Chronic peroxisome proliferator-activated receptor gamma (PPARgamma) activation of epididymally derived white adipocyte cultures reveals a population of thermogenically competent, UCP1-containing adipocytes molecularly distinct from classic brown adipocytes. *J. Biol. Chem.* **285**, 7153–7164 (2010).
169. Moore, S. G. & Dawson, K. L. Red and yellow marrow in the femur: age-related changes in appearance at MR imaging. *Radiology* **175**, 219–223 (1990).
170. Sadie-Van Gijzen, H., Hough, F. S. & Ferris, W. F. Determinants of bone marrow adiposity: The modulation of peroxisome proliferator-activated receptor- $\gamma$ 2 activity as a central mechanism. *Bone* **56**, 255–265 (2013).
171. Naveiras, O. *et al.* Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment. *Nature* **460**, 259–263 (2009).
172. Gimble, J. M., Robinson, C. E., Wu, X. & Kelly, K. A. The function of adipocytes in the bone marrow stroma: an update. *Bone* **19**, 421–428 (1996).
173. Gesta, S., Tseng, Y.-H. & Kahn, C. R. Developmental origin of fat: tracking obesity to its source. *Cell* **131**, 242–256 (2007).
174. Liu, L.-F., Shen, W.-J., Ueno, M., Patel, S. & Kraemer, F. B. Characterization of age-related gene expression profiling in bone marrow and epididymal adipocytes. *BMC Genomics* **12**, 212 (2011).
175. Krings, A. *et al.* Bone marrow fat has brown adipose tissue characteristics, which are attenuated with aging and diabetes. *Bone* **50**, 546–552 (2012).
176. Crossno, J. T., Jr, Majka, S. M., Grazia, T., Gill, R. G. & Klemm, D. J. Rosiglitazone promotes development of a novel adipocyte population from bone marrow-derived circulating progenitor cells. *J. Clin. Invest.* **116**, 3220–3228 (2006).
177. Majka, S. M. *et al.* De novo generation of white adipocytes from the myeloid lineage via mesenchymal intermediates is age, adipose depot, and gender specific. *Proc. Natl. Acad. Sci.* **107**, 14781–14786 (2010).

178. Greenberg, A. S. *et al.* Perilipin, a major hormonally regulated adipocyte-specific phosphoprotein associated with the periphery of lipid storage droplets. *J. Biol. Chem.* **266**, 11341–11346 (1991).
179. Smas, C. M. & Sul, H. S. Pref-1, a protein containing EGF-like repeats, inhibits adipocyte differentiation. *Cell* **73**, 725–734 (1993).
180. Smas, C. M., Chen, L. & Sul, H. S. Cleavage of membrane-associated pref-1 generates a soluble inhibitor of adipocyte differentiation. *Mol. Cell. Biol.* **17**, 977–988 (1997).
181. Strutz, F. *et al.* Identification and characterization of a fibroblast marker: FSP1. *J. Cell Biol.* **130**, 393–405 (1995).
182. Franke, W. W., Schmid, E., Osborn, M. & Weber, K. Different intermediate-sized filaments distinguished by immunofluorescence microscopy. *Proc. Natl. Acad. Sci. U. S. A.* **75**, 5034–5038 (1978).
183. Tomasek, J. J., Gabbiani, G., Hinz, B., Chaponnier, C. & Brown, R. A. Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat. Rev. Mol. Cell Biol.* **3**, 349–363 (2002).
184. Schmid, E. *et al.* Distribution of vimentin and desmin filaments in smooth muscle tissue of mammalian and avian aorta. *Exp. Cell Res.* **137**, 329–340 (1982).
185. Rettig, W. J. *et al.* Regulation and heteromeric structure of the fibroblast activation protein in normal and transformed cells of mesenchymal and neuroectodermal origin. *Cancer Res.* **53**, 3327–3335 (1993).
186. Mosher, D. F. Physiology of Fibronectin. *Annu. Rev. Med.* **35**, 561–575 (1984).
187. Goldberg, B. & Green, H. AN ANALYSIS OF COLLAGEN SECRETION BY ESTABLISHED MOUSE FIBROBLAST LINES. *J. Cell Biol.* **22**, 227 (1964).
188. Fredriksson, K. *et al.* Red blood cells increase secretion of matrix metalloproteinases from human lung fibroblasts in vitro. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **290**, L326–333 (2006).
189. Mias, C. *et al.* Mesenchymal stem cells promote matrix metalloproteinase secretion by cardiac fibroblasts and reduce cardiac ventricular fibrosis after myocardial infarction. *Stem Cells Dayt. Ohio* **27**, 2734–2743 (2009).
190. Nishida, N., Yano, H., Nishida, T., Kamura, T. & Kojiro, M. Angiogenesis in Cancer. *Vasc. Health Risk Manag.* **2**, 213 (2006).
191. Caspar-Bauguil S, Cousin B, Bour S, Casteilla L, Penicaud L, Carpené C. Adipose tissue lymphocytes: types and roles. *J Physiol Biochem* **65**, 423–36. (2009).
192. Coussens, L. M. & Werb, Z. Inflammation and cancer. *Nature* **420**, 860–867 (2002).
193. De Visser, K. E., Eichten, A. & Coussens, L. M. Paradoxical roles of the immune system during cancer development. *Nat. Rev. Cancer* **6**, 24–37 (2006).
194. Cristancho, A. G. & Lazar, M. A. Forming functional fat: a growing understanding of adipocyte differentiation. *Nat. Rev. Mol. Cell Biol.* **12**, 722–734 (2011).
195. Gregoire, F. M., Smas, C. M. & Sul, H. S. Understanding adipocyte differentiation. *Physiol. Rev.* **78**, 783–809 (1998).
196. Rosen, E. D. & MacDougald, O. A. Adipocyte differentiation from the inside out. *Nat. Rev. Mol. Cell Biol.* **7**, 885–896 (2006).
197. Tseng, Y.-H. *et al.* New role of bone morphogenetic protein 7 in brown adipogenesis and energy expenditure. *Nature* **454**, 1000–1004 (2008).
198. Matsumoto, T. *et al.* Mature adipocyte-derived dedifferentiated fat cells exhibit multilineage potential. *J. Cell. Physiol.* **215**, 210–222 (2008).
199. Poloni, A. *et al.* Human Dedifferentiated Adipocytes Show Similar Properties to Bone Marrow-Derived Mesenchymal Stem Cells. *STEM CELLS* **30**, 965–974 (2012).
200. Spalding, K. L. *et al.* Dynamics of fat cell turnover in humans. *Nature* **453**, 783–787 (2008).
201. Strawford, A., Antelo, F., Christiansen, M. & Hellerstein, M. K. Adipose tissue triglyceride turnover, de novo lipogenesis, and cell proliferation in humans measured with <sup>2</sup>H<sub>2</sub>O. *Am. J. Physiol. Endocrinol. Metab.* **286**, E577–588 (2004).
202. Rigamonti, A., Brennand, K., Lau, F. & Cowan, C. A. Rapid Cellular Turnover in Adipose Tissue. *PLoS ONE* **6**, e17637 (2011).

203. Wiseman BS, Werb Z. Stromal effects on mammary gland development and breast cancer. *Science* **296**, 1046–9 (2002).
204. Rossmeislová, L. *et al.* Weight loss improves the adipogenic capacity of human preadipocytes and modulates their secretory profile. *Diabetes* **62**, 1990–1995 (2013).
205. Cartwright, M. J., Tchkonina, T. & Kirkland, J. L. Aging in adipocytes: potential impact of inherent, depot-specific mechanisms. *Exp. Gerontol.* **42**, 463–471 (2007).
206. Coppack, S. W., Jensen, M. D. & Miles, J. M. In vivo regulation of lipolysis in humans. *J. Lipid Res.* **35**, 177–193 (1994).
207. Large, V., Peroni, O., Letexier, D., Ray, H. & Beylot, M. Metabolism of lipids in human white adipocyte. *Diabetes Metab.* **30**, 294–309 (2004).
208. Walther, T. C. & Farese, R. V., Jr. The life of lipid droplets. *Biochim. Biophys. Acta* **1791**, 459–466 (2009).
209. Engfeldt, P. & Arner, P. Lipolysis in human adipocytes, effects of cell size, age and of regional differences. *Horm. Metab. Res. Suppl. Ser.* **19**, 26–29 (1988).
210. Fried, S. K., Bunkin, D. A. & Greenberg, A. S. Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. *J. Clin. Endocrinol. Metab.* **83**, 847–850 (1998).
211. Kershaw, E. E. & Flier, J. S. Adipose tissue as an endocrine organ. *J. Clin. Endocrinol. Metab.* **89**, 2548–2556 (2004).
212. Galic, S., Oakhill, J. S. & Steinberg, G. R. Adipose tissue as an endocrine organ. *Mol. Cell. Endocrinol.* **316**, 129–139 (2010).
213. Fain, J. N., Madan, A. K., Hiler, M. L., Cheema, P. & Bahouth, S. W. Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. *Endocrinology* **145**, 2273–2282 (2004).
214. Zhang, Y. *et al.* Positional cloning of the mouse obese gene and its human homologue. *Nature* **372**, 425–432 (1994).
215. Bado, A. *et al.* The stomach is a source of leptin. *Nature* **394**, 790–793 (1998).
216. Smith-Kirwin, S. M. *et al.* Leptin expression in human mammary epithelial cells and breast milk. *J. Clin. Endocrinol. Metab.* **83**, 1810–1813 (1998).
217. Wang, J., Liu, R., Hawkins, M., Barzilai, N. & Rossetti, L. A nutrient-sensing pathway regulates leptin gene expression in muscle and fat. *Nature* **393**, 684–688 (1998).
218. Señaris, R. *et al.* Synthesis of leptin in human placenta. *Endocrinology* **138**, 4501–4504 (1997).
219. Wang, B., Wood, I. S. & Trayhurn, P. Hypoxia induces leptin gene expression and secretion in human preadipocytes: differential effects of hypoxia on adipokine expression by preadipocytes. *J. Endocrinol.* **198**, 127–134 (2008).
220. Montague, C. T., Prins, J. B., Sanders, L., Digby, J. E. & O’Rahilly, S. Depot- and sex-specific differences in human leptin mRNA expression: implications for the control of regional fat distribution. *Diabetes* **46**, 342–347 (1997).
221. Laharrague, P. *et al.* High expression of leptin by human bone marrow adipocytes in primary culture. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* **12**, 747–752 (1998).
222. Friedman, J. M. & Halaas, J. L. Leptin and the regulation of body weight in mammals. *Nature* **395**, 763–770 (1998).
223. Viengchareun, S., Zennaro, M.-C., Pascual-Le Tallec, L. & Lombes, M. Brown adipocytes are novel sites of expression and regulation of adiponectin and resistin. *FEBS Lett.* **532**, 345–350 (2002).
224. Dalamaga, M., Diakopoulos, K. N. & Mantzoros, C. S. The role of adiponectin in cancer: a review of current evidence. *Endocr. Rev.* **33**, 547–594 (2012).
225. Yamauchi, T. *et al.* The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat. Med.* **7**, 941–946 (2001).
226. Price, T. *et al.* Determination of aromatase cytochrome P450 messenger ribonucleic acid in human breast tissue by competitive polymerase chain reaction amplification. *J. Clin. Endocrinol. Metab.* **74**, 1247–1252 (1992).

227. Cawthorn, W. P. & Sethi, J. K. TNF-alpha and adipocyte biology. *FEBS Lett.* **582**, 117–131 (2008).
228. Pricola, K. L., Kuhn, N. Z., Haleem-Smith, H., Song, Y. & Tuan, R. S. Interleukin-6 maintains bone marrow-derived mesenchymal stem cell stemness by an ERK1/2-dependent mechanism. *J. Cell. Biochem.* **108**, 577–588 (2009).
229. Mohamed-Ali, V. *et al.* Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. *J. Clin. Endocrinol. Metab.* **82**, 4196–4200 (1997).
230. Fernández-Real, J. M. & Ricart, W. Insulin resistance and chronic cardiovascular inflammatory syndrome. *Endocr. Rev.* **24**, 278–301 (2003).
231. Rahimi, N., Tremblay, E., McAdam, L., Roberts, A. & Elliott, B. Autocrine secretion of TGF-beta 1 and TGF-beta 2 by pre-adipocytes and adipocytes: a potent negative regulator of adipocyte differentiation and proliferation of mammary carcinoma cells. *In Vitro Cell. Dev. Biol. Anim.* **34**, 412–420 (1998).
232. Massagué, J. TGFβ signalling in context. *Nat. Rev. Mol. Cell Biol.* **13**, 616–630 (2012).
233. Li, M. O., Wan, Y. Y., Sanjabi, S., Robertson, A.-K. L. & Flavell, R. A. Transforming growth factor-beta regulation of immune responses. *Annu. Rev. Immunol.* **24**, 99–146 (2006).
234. Wozniak SE, Gee LL, Wachtel MS, Frezza EE. Adipose tissue: the new endocrine organ? A review article. *Dig Sci* **54**, 1847–56 (2008).
235. Eric Vanderweyer and Dina Hertens. Quantification of glands and fat in breast tissue\_An experimental determination. *Ann. Anat.* **184**, 181–184 (2002).
236. DT Ramsay, JC Kent, RA Hartmann, and PE Hartman. Anatomy of the lactating human breast redefined with ultrasound imaging. *J Anat* **206**, 525–534 (2005).
237. Macias, H. & Hinck, L. Mammary gland development. *Wiley Interdiscip. Rev. Dev. Biol.* **1**, 533–557 (2012).
238. Hovey, R. C., McFadden, T. B. & Akers, R. M. Regulation of mammary gland growth and morphogenesis by the mammary fat pad: a species comparison. *J. Mammary Gland Biol. Neoplasia* **4**, 53–68 (1999).
239. Adding fat to the fire. *Nat. Med.* **19**, 947–947 (2013).
240. Caballero, B. The Global Epidemic of Obesity: An Overview. *Epidemiol. Rev.* **29**, 1–5 (2007).
241. Sun, K., Kusminski, C. M. & Scherer, P. E. Adipose tissue remodeling and obesity. *J. Clin. Invest.* **121**, 2094–2101 (2011).
242. Leal, V. de O. & Mafra, D. Adipokines in obesity. *Clin. Chim. Acta Int. J. Clin. Chem.* **419**, 87–94 (2013).
243. Kahn, S. E., Hull, R. L. & Utzschneider, K. M. Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* **444**, 840–846 (2006).
244. Van Gaal, L. F., Mertens, I. L. & De Block, C. E. Mechanisms linking obesity with cardiovascular disease. *Nature* **444**, 875–880 (2006).
245. Poirier, P. *et al.* Obesity and Cardiovascular Disease: Pathophysiology, Evaluation, and Effect of Weight Loss An Update of the 1997 American Heart Association Scientific Statement on Obesity and Heart Disease From the Obesity Committee of the Council on Nutrition, Physical Activity, and Metabolism. *Circulation* **113**, 898–918 (2006).
246. Lorincz, A. M. & Sukumar, S. Molecular links between obesity and breast cancer. *Endocr. Relat. Cancer* **13**, 279–292 (2006).
247. Calle, E. E. & Kaaks, R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. *Nat. Rev. Cancer* **4**, 579–591 (2004).
248. Lafontan, M. Historical perspectives in fat cell biology: the fat cell as a model for the investigation of hormonal and metabolic pathways. *Am. J. Physiol. Cell Physiol.* **302**, C327–359 (2012).
249. Green, H. & Meuth, M. An established pre-adipose cell line and its differentiation in culture. *Cell* **3**, 127–133 (1974).
250. Green, H. & Kehinde, O. An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. *Cell* **5**, 19–27 (1975).
251. Green, H. & Kehinde, O. Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. *Cell* **7**, 105–113 (1976).

252. Green, H. & Kehinde, O. Formation of normally differentiated subcutaneous fat pads by an established preadipose cell line. *J. Cell. Physiol.* **101**, 169–171 (1979).
253. Mandrup, S., Loftus, T. M., MacDougald, O. A., Kuhajda, F. P. & Lane, M. D. Obese gene expression at in vivo levels by fat pads derived from s.c. implanted 3T3-F442A preadipocytes. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 4300–4305 (1997).
254. Rodriguez, A.-M. *et al.* Adipocyte differentiation of multipotent cells established from human adipose tissue. *Biochem. Biophys. Res. Commun.* **315**, 255–263 (2004).
255. Rodriguez, A.-M. *et al.* Transplantation of a multipotent cell population from human adipose tissue induces dystrophin expression in the immunocompetent mdx mouse. *J. Exp. Med.* **201**, 1397–1405 (2005).
256. Cawthorn, W. P., Scheller, E. L. & MacDougald, O. A. Adipose tissue stem cells meet preadipocyte commitment: going back to the future. *J. Lipid Res.* **53**, 227–246 (2011).
257. Mizuno, H., Tobita, M. & Uysal, A. C. Concise review: Adipose-derived stem cells as a novel tool for future regenerative medicine. *Stem Cells Dayt. Ohio* **30**, 804–810 (2012).
258. Hass, R., Kasper, C., Böhm, S. & Jacobs, R. Different populations and sources of human mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC. *Cell Commun. Signal.* **9**, 12 (2011).
259. Jansen, B. J. H. *et al.* Functional differences between mesenchymal stem cell populations are reflected by their transcriptome. *Stem Cells Dev.* **19**, 481–490 (2010).
260. Al-Nbaheen, M. *et al.* Human Stromal (Mesenchymal) Stem Cells from Bone Marrow, Adipose Tissue and Skin Exhibit Differences in Molecular Phenotype and Differentiation Potential. *Stem Cell Rev. Rep.* **9**, 32–43 (2013).
261. Marshall, S. Kinetics of insulin receptor biosynthesis and membrane insertion. Relationship to cellular function. *Diabetes* **32**, 319–325 (1983).
262. Herberman, R. B., Nunn, M. E. & Lavrin, D. H. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. I. Distribution of reactivity and specificity. *Int. J. Cancer J. Int. Cancer* **16**, 216–229 (1975).
263. Herberman, R. B., Nunn, M. E., Holden, H. T. & Lavrin, D. H. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. *Int. J. Cancer J. Int. Cancer* **16**, 230–239 (1975).
264. Kiessling, R., Klein, E. & Wigzell, H. 'Natural' killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *Eur. J. Immunol.* **5**, 112–117 (1975).
265. Kiessling, R., Klein, E., Pross, H. & Wigzell, H. 'Natural' killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur. J. Immunol.* **5**, 117–121 (1975).
266. Pross, H. F. & Jondal, M. Cytotoxic lymphocytes from normal donors. A functional marker of human non-T lymphocytes. *Clin. Exp. Immunol.* **21**, 226–235 (1975).
267. Barlozzari, T., Reynolds, C. W. & Herberman, R. B. In vivo role of natural killer cells: involvement of large granular lymphocytes in the clearance of tumor cells in anti-asialo GM1-treated rats. *J. Immunol. Baltim. Md 1950* **131**, 1024–1027 (1983).
268. Smyth, M. J., Crowe, N. Y. & Godfrey, D. I. NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma. *Int. Immunol.* **13**, 459–463 (2001).
269. Smyth, M. J. *et al.* Differential tumor surveillance by natural killer (NK) and NKT cells. *J. Exp. Med.* **191**, 661–668 (2000).
270. Kim, S., Iizuka, K., Aguila, H. L., Weissman, I. L. & Yokoyama, W. M. In vivo natural killer cell activities revealed by natural killer cell-deficient mice. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 2731–2736 (2000).
271. Orange, J. S. Human natural killer cell deficiencies. *Curr. Opin. Allergy Clin. Immunol.* **6**, 399–409 (2006).
272. Imai, K., Matsuyama, S., Miyake, S., Suga, K. & Nakachi, K. Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. *Lancet* **356**, 1795–1799 (2000).



273. Liu, R. B. *et al.* Densely granulated murine NK cells eradicate large solid tumors. *Cancer Res.* **72**, 1964–1974 (2012).
274. Coca, S. *et al.* The prognostic significance of intratumoral natural killer cells in patients with colorectal carcinoma. *Cancer* **79**, 2320–2328 (1997).
275. Ishigami, S. *et al.* Prognostic value of intratumoral natural killer cells in gastric carcinoma. *Cancer* **88**, 577–583 (2000).
276. Takanami, I., Takeuchi, K. & Giga, M. The prognostic value of natural killer cell infiltration in resected pulmonary adenocarcinoma. *J. Thorac. Cardiovasc. Surg.* **121**, 1058–1063 (2001).
277. Mamessier E, Sylvain A, Thibault ML, Houvenaeghel G, Jacquemier J, Castellano R, Gonçalves A, André P, Romagné F, Thibault G, Viens P, Birnbaum D, Bertucci F, Moretta A, Olive D. Human breast cancer cells enhance self tolerance by promoting evasion from NK cell antitumor immunity. *J Clin Invest* **121**, 3609–22 (2011).
278. Grégoire, C. *et al.* The trafficking of natural killer cells. *Immunol. Rev.* **220**, 169–182 (2007).
279. Walser, T. C. *et al.* Immune-mediated modulation of breast cancer growth and metastasis by the chemokine Mig (CXCL9) in a murine model. *J. Immunother. Hagerstown Md 1997* **30**, 490–498 (2007).
280. Lavergne, E. *et al.* Fractalkine mediates natural killer-dependent antitumor responses in vivo. *Cancer Res.* **63**, 7468–7474 (2003).
281. Wendel, M., Galani, I. E., Suri-Payer, E. & Cerwenka, A. Natural killer cell accumulation in tumors is dependent on IFN-gamma and CXCR3 ligands. *Cancer Res.* **68**, 8437–8445 (2008).
282. Bronger, H. *et al.* Modulation of CXCR3 ligand secretion by prostaglandin E2 and cyclooxygenase inhibitors in human breast cancer. *Breast Cancer Res. BCR* **14**, R30 (2012).
283. Lanier, L. L., Le, A. M., Civin, C. I., Loken, M. R. & Phillips, J. H. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. *J. Immunol. Baltim. Md 1950* **136**, 4480–4486 (1986).
284. Lanier, L. L. *et al.* Molecular and functional analysis of human natural killer cell-associated neural cell adhesion molecule (N-CAM/CD56). *J. Immunol. Baltim. Md 1950* **146**, 4421–4426 (1991).
285. Farag, S. S. & Caligiuri, M. A. Human natural killer cell development and biology. *Blood Rev.* **20**, 123–137 (2006).
286. Mamessier, E. *et al.* Human breast cancer cells enhance self tolerance by promoting evasion from NK cell antitumor immunity. *J. Clin. Invest.* **121**, 3609–3622 (2011).
287. Schleypen, J. S. *et al.* Cytotoxic markers and frequency predict functional capacity of natural killer cells infiltrating renal cell carcinoma. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **12**, 718–725 (2006).
288. Eisenhardt, M. *et al.* The CXCR3(+)CD56Bright phenotype characterizes a distinct NK cell subset with anti-fibrotic potential that shows dys-regulated activity in hepatitis C. *PLoS One* **7**, e38846 (2012).
289. Mamessier E, Sylvain A, Bertucci F, Castellano R, Finetti P, Houvenaeghel G, Charaffe-Jaufret E, Birnbaum D, Moretta A, Olive D. Human breast tumor cells induce self-tolerance mechanisms to avoid NKG2D-mediated and DNAM-mediated NK cell recognition. *Cancer Res* **71**, 6621–32 (2011).
290. Hagensars, M. *et al.* Characteristics of tumor infiltration by adoptively transferred and endogenous natural-killer cells in a syngeneic rat model: implications for the mechanism behind anti-tumor responses. *Int. J. Cancer J. Int. Cancer* **78**, 783–789 (1998).
291. Textor, S. *et al.* Activating NK cell receptor ligands are differentially expressed during progression to cervical cancer. *Int. J. Cancer J. Int. Cancer* **123**, 2343–2353 (2008).
292. Edsparr, K., Basse, P. H., Goldfarb, R. H. & Albertsson, P. Matrix metalloproteinases in cytotoxic lymphocytes impact on tumour infiltration and immunomodulation. *Cancer Microenviron. Off. J. Int. Cancer Microenviron. Soc.* **4**, 351–360 (2011).
293. Kim, M. H. *et al.* Secreted and membrane-associated matrix metalloproteinases of IL-2-activated NK cells and their inhibitors. *J. Immunol. Baltim. Md 1950* **164**, 5883–5889 (2000).

294. Garrod, K. R., Wei, S. H., Parker, I. & Cahalan, M. D. Natural killer cells actively patrol peripheral lymph nodes forming stable conjugates to eliminate MHC-mismatched targets. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 12081–12086 (2007).
295. Deguine, J., Breart, B., Lemaître, F., Di Santo, J. P. & Bousso, P. Intravital imaging reveals distinct dynamics for natural killer and CD8(+) T cells during tumor regression. *Immunity* **33**, 632–644 (2010).
296. Arnould, L. *et al.* Trastuzumab-based treatment of HER2-positive breast cancer: an antibody-dependent cellular cytotoxicity mechanism? *Br. J. Cancer* **94**, 259–267 (2006).
297. Deguine, J., Breart, B., Lemaître, F. & Bousso, P. Cutting Edge: Tumor-Targeting Antibodies Enhance NKG2D-Mediated NK Cell Cytotoxicity by Stabilizing NK Cell-Tumor Cell Interactions. *J. Immunol. Baltim. Md 1950* (2012). doi:10.4049/jimmunol.1202065
298. Lanier, L. L. NK cell recognition. *Annu. Rev. Immunol.* **23**, 225–274 (2005).
299. Moretta, A. *et al.* Receptors for HLA class-I molecules in human natural killer cells. *Annu. Rev. Immunol.* **14**, 619–648 (1996).
300. Algarra, I., Collado, A. & Garrido, F. Altered MHC class I antigens in tumors. *Int. J. Clin. Lab. Res.* **27**, 95–102 (1997).
301. Cabrera, T. *et al.* High frequency of altered HLA class I phenotypes in invasive breast carcinomas. *Hum. Immunol.* **50**, 127–134 (1996).
302. Kijima, M., Gardiol, N. & Held, W. Natural killer cell mediated missing-self recognition can protect mice from primary chronic myeloid leukemia in vivo. *PLoS One* **6**, e27639 (2011).
303. Liao, N. S., Bix, M., Zijlstra, M., Jaenisch, R. & Raulet, D. MHC class I deficiency: susceptibility to natural killer (NK) cells and impaired NK activity. *Science* **253**, 199–202 (1991).
304. Garcia-Lora, A., Algarra, I., Gaforio, J. J., Ruiz-Cabello, F. & Garrido, F. Immunoselection by T lymphocytes generates repeated MHC class I-deficient metastatic tumor variants. *Int. J. Cancer J. Int. Cancer* **91**, 109–119 (2001).
305. Garcia-Lora, A., Algarra, I. & Garrido, F. MHC class I antigens, immune surveillance, and tumor immune escape. *J. Cell. Physiol.* **195**, 346–355 (2003).
306. Lanier, L. L. On guard--activating NK cell receptors. *Nat. Immunol.* **2**, 23–27 (2001).
307. Raulet, D. H. Roles of the NKG2D immunoreceptor and its ligands. *Nat. Rev. Immunol.* **3**, 781–790 (2003).
308. Bauer, S. *et al.* Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* **285**, 727–729 (1999).
309. Cosman, D. *et al.* ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity* **14**, 123–133 (2001).
310. Cerwenka, A. *et al.* Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. *Immunity* **12**, 721–727 (2000).
311. Carayannopoulos, L. N., Naidenko, O. V., Fremont, D. H. & Yokoyama, W. M. Cutting edge: murine UL16-binding protein-like transcript 1: a newly described transcript encoding a high-affinity ligand for murine NKG2D. *J. Immunol. Baltim. Md 1950* **169**, 4079–4083 (2002).
312. Sivori, S. *et al.* p46, a novel natural killer cell-specific surface molecule that mediates cell activation. *J. Exp. Med.* **186**, 1129–1136 (1997).
313. Vitale, M. *et al.* NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. *J. Exp. Med.* **187**, 2065–2072 (1998).
314. Pende, D. *et al.* Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. *J. Exp. Med.* **190**, 1505–1516 (1999).
315. Brandt, C. S. *et al.* The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans. *J. Exp. Med.* **206**, 1495–1503 (2009).
316. Pogge von Strandmann, E. *et al.* Human leukocyte antigen-B-associated transcript 3 is released from tumor cells and engages the NKp30 receptor on natural killer cells. *Immunity* **27**, 965–974 (2007).

317. Lanier, L. L., Le, A. M., Phillips, J. H., Warner, N. L. & Babcock, G. F. Subpopulations of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu-11 (NK-15) antigens. *J. Immunol. Baltim. Md 1950* **131**, 1789–1796 (1983).
318. Kohl, S., Springer, T. A., Schmalstieg, F. C., Loo, L. S. & Anderson, D. C. Defective natural killer cytotoxicity and polymorphonuclear leukocyte antibody-dependent cellular cytotoxicity in patients with LFA-1/OKM-1 deficiency. *J. Immunol. Baltim. Md 1950* **133**, 2972–2978 (1984).
319. Bolhuis, R. L., Roozmond, R. C. & van de Griend, R. J. Induction and blocking of cytolysis in CD2+, CD3- NK and CD2+, CD3+ cytotoxic T lymphocytes via CD2 50 KD sheep erythrocyte receptor. *J. Immunol. Baltim. Md 1950* **136**, 3939–3944 (1986).
320. Orange, J. S. *et al.* The mature activating natural killer cell immunologic synapse is formed in distinct stages. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 14151–14156 (2003).
321. Liu, D. *et al.* Integrin-dependent organization and bidirectional vesicular traffic at cytotoxic immune synapses. *Immunity* **31**, 99–109 (2009).
322. Davis, D. M. *et al.* The human natural killer cell immune synapse. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 15062–15067 (1999).
323. Vyas, Y. M. *et al.* Spatial organization of signal transduction molecules in the NK cell immune synapses during MHC class I-regulated noncytolytic and cytolytic interactions. *J. Immunol. Baltim. Md 1950* **167**, 4358–4367 (2001).
324. Radoja, S. *et al.* CD8(+) tumor-infiltrating T cells are deficient in perforin-mediated cytolytic activity due to defective microtubule-organizing center mobilization and lytic granule exocytosis. *J. Immunol. Baltim. Md 1950* **167**, 5042–5051 (2001).
325. Krzewski, K. & Coligan, J. E. Human NK cell lytic granules and regulation of their exocytosis. *Front. Immunol.* **3**, 335 (2012).
326. Vyas, Y. M., Maniar, H. & Dupont, B. Visualization of signaling pathways and cortical cytoskeleton in cytolytic and noncytolytic natural killer cell immune synapses. *Immunol. Rev.* **189**, 161–178 (2002).
327. Bhat, R. & Watzl, C. Serial killing of tumor cells by human natural killer cells--enhancement by therapeutic antibodies. *PLoS One* **2**, e326 (2007).
328. Bouwens, L. & Wisse, E. Immuno-electron microscopic characterization of large granular lymphocytes (natural killer cells) from rat liver. *Eur. J. Immunol.* **17**, 1423–1428 (1987).
329. Smyth, M. J. *et al.* Activation of NK cell cytotoxicity. *Mol. Immunol.* **42**, 501–510 (2005).
330. Thiery, J. *et al.* Perforin pores in the endosomal membrane trigger the release of endocytosed granzyme B into the cytosol of target cells. *Nat. Immunol.* **12**, 770–777 (2011).
331. Smyth, M. J. & Trapani, J. A. Granzymes: exogenous proteinases that induce target cell apoptosis. *Immunol. Today* **16**, 202–206 (1995).
332. Russell, J. H. & Ley, T. J. Lymphocyte-mediated cytotoxicity. *Annu. Rev. Immunol.* **20**, 323–370 (2002).
333. Kägi, D. *et al.* Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature* **369**, 31–37 (1994).
334. Van den Broek, M. F., Kägi, D., Zinkernagel, R. M. & Hengartner, H. Perforin dependence of natural killer cell-mediated tumor control in vivo. *Eur. J. Immunol.* **25**, 3514–3516 (1995).
335. Kodama, T. *et al.* Perforin-dependent NK cell cytotoxicity is sufficient for anti-metastatic effect of IL-12. *Eur. J. Immunol.* **29**, 1390–1396 (1999).
336. Stepp, S. E. *et al.* Perforin gene defects in familial hemophagocytic lymphohistiocytosis. *Science* **286**, 1957–1959 (1999).
337. Chia, J. *et al.* Temperature sensitivity of human perforin mutants unmasks subtotal loss of cytotoxicity, delayed FHL, and a predisposition to cancer. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 9809–9814 (2009).
338. Pardo, J., Balkow, S., Anel, A. & Simon, M. M. Granzymes are essential for natural killer cell-mediated and perf-facilitated tumor control. *Eur. J. Immunol.* **32**, 2881–2887 (2002).
339. Davis, J. E., Smyth, M. J. & Trapani, J. A. Granzyme A and B-deficient killer lymphocytes are defective in eliciting DNA fragmentation but retain potent in vivo anti-tumor capacity. *Eur. J. Immunol.* **31**, 39–47 (2001).

340. Smyth, M. J., Street, S. E. A. & Trapani, J. A. Cutting edge: granzymes A and B are not essential for perforin-mediated tumor rejection. *J. Immunol. Baltim. Md 1950* **171**, 515–518 (2003).
341. Houchins, J. P., Yabe, T., McSherry, C., Miyokawa, N. & Bach, F. H. Isolation and characterization of NK cell or NK/T cell-specific cDNA clones. *J. Mol. Cell. Immunol. JMCI* **4**, 295–304; discussion 305–306 (1990).
342. Obata-Onai, A. *et al.* Comprehensive gene expression analysis of human NK cells and CD8(+) T lymphocytes. *Int. Immunol.* **14**, 1085–1098 (2002).
343. Clayberger, C. & Krensky, A. M. Granulysin. *Curr. Opin. Immunol.* **15**, 560–565 (2003).
344. Peña, S. V., Hanson, D. A., Carr, B. A., Goralski, T. J. & Krensky, A. M. Processing, subcellular localization, and function of 519 (granulysin), a human late T cell activation molecule with homology to small, lytic, granule proteins. *J. Immunol. Baltim. Md 1950* **158**, 2680–2688 (1997).
345. Gamen, S. *et al.* Granulysin-induced apoptosis. I. Involvement of at least two distinct pathways. *J. Immunol. Baltim. Md 1950* **161**, 1758–1764 (1998).
346. Kaspar, A. A. *et al.* A distinct pathway of cell-mediated apoptosis initiated by granulysin. *J. Immunol. Baltim. Md 1950* **167**, 350–356 (2001).
347. Zhang, H., Zhong, C., Shi, L., Guo, Y. & Fan, Z. Granulysin induces cathepsin B release from lysosomes of target tumor cells to attack mitochondria through processing of bid leading to Necroptosis. *J. Immunol. Baltim. Md 1950* **182**, 6993–7000 (2009).
348. Sekiya, M. *et al.* Adenovirus vector-mediated transfer of 9 kDa granulysin induces DNA fragmentation in HuD antigen-expressing small cell lung cancer murine model cells. *Respirol. Carlton Vic* **7**, 29–35 (2002).
349. Kishi, A. *et al.* Differential expression of granulysin and perforin by NK cells in cancer patients and correlation of impaired granulysin expression with progression of cancer. *Cancer Immunol. Immunother. CII* **50**, 604–614 (2002).
350. Huang, L. P., Lyu, S.-C., Clayberger, C. & Krensky, A. M. Granulysin-mediated tumor rejection in transgenic mice. *J. Immunol. Baltim. Md 1950* **178**, 77–84 (2007).
351. Kozlowski, M., Schorey, J., Portis, T., Grigoriev, V. & Kornbluth, J. NK Lytic-Associated Molecule: A Novel Gene Selectively Expressed in Cells with Cytolytic Function. *J. Immunol.* **163**, 1775–1785 (1999).
352. Portis, T., Anderson, J., Esposito, A. & Kornbluth, J. Gene structure of human and mouse NKLAM, a gene associated with cellular cytotoxicity. *Immunogenetics* **51**, 546–555 (2000).
353. Hoover, R. G., Gullickson, G. & Kornbluth, J. Impaired NK cytolytic activity and enhanced tumor growth in NK lytic-associated molecule-deficient mice. *J. Immunol. Baltim. Md 1950* **183**, 6913–6921 (2009).
354. Hoover, R. G., Gullickson, G. & Kornbluth, J. Natural killer lytic-associated molecule plays a role in controlling tumor dissemination and metastasis. *Front. Immunol.* **3**, 393 (2012).
355. Kashii, Y., Giorda, R., Herberman, R. B., Whiteside, T. L. & Vujanovic, N. L. Constitutive expression and role of the TNF family ligands in apoptotic killing of tumor cells by human NK cells. *J. Immunol. Baltim. Md 1950* **163**, 5358–5366 (1999).
356. Bossi, G. & Griffiths, G. M. Degranulation plays an essential part in regulating cell surface expression of Fas ligand in T cells and natural killer cells. *Nat. Med.* **5**, 90–96 (1999).
357. Kojima, Y. *et al.* Localization of Fas ligand in cytoplasmic granules of CD8+ cytotoxic T lymphocytes and natural killer cells: participation of Fas ligand in granule exocytosis model of cytotoxicity. *Biochem. Biophys. Res. Commun.* **296**, 328–336 (2002).
358. Thorburn, A. Death receptor-induced cell killing. *Cell. Signal.* **16**, 139–144 (2004).
359. Oshimi, Y., Oda, S., Honda, Y., Nagata, S. & Miyazaki, S. Involvement of Fas ligand and Fas-mediated pathway in the cytotoxicity of human natural killer cells. *J. Immunol. Baltim. Md 1950* **157**, 2909–2915 (1996).
360. Arase, H., Arase, N. & Saito, T. Fas-mediated cytotoxicity by freshly isolated natural killer cells. *J. Exp. Med.* **181**, 1235–1238 (1995).
361. Screpanti, V., Wallin, R. P., Ljunggren, H. G. & Grandien, A. A central role for death receptor-mediated apoptosis in the rejection of tumors by NK cells. *J. Immunol. Baltim. Md 1950* **167**, 2068–2073 (2001).

362. Kayagaki, N. *et al.* Expression and function of TNF-related apoptosis-inducing ligand on murine activated NK cells. *J. Immunol. Baltim. Md 1950* **163**, 1906–1913 (1999).
363. Takeda, K. *et al.* Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. *Nat. Med.* **7**, 94–100 (2001).
364. Takeda, K. *et al.* Critical role for tumor necrosis factor-related apoptosis-inducing ligand in immune surveillance against tumor development. *J. Exp. Med.* **195**, 161–169 (2002).
365. Cretney, E. *et al.* Increased susceptibility to tumor initiation and metastasis in TNF-related apoptosis-inducing ligand-deficient mice. *J. Immunol. Baltim. Md 1950* **168**, 1356–1361 (2002).
366. Trinchieri, G. Natural killer cells wear different hats: effector cells of innate resistance and regulatory cells of adaptive immunity and of hematopoiesis. *Semin. Immunol.* **7**, 83–88 (1995).
367. Chin, Y. E. *et al.* Cell growth arrest and induction of cyclin-dependent kinase inhibitor p21 WAF1/CIP1 mediated by STAT1. *Science* **272**, 719–722 (1996).
368. Kaplan, D. H. *et al.* Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 7556–7561 (1998).
369. Street, S. E., Cretney, E. & Smyth, M. J. Perforin and interferon-gamma activities independently control tumor initiation, growth, and metastasis. *Blood* **97**, 192–197 (2001).
370. Boehm, U., Klamp, T., Groot, M. & Howard, J. C. Cellular responses to interferon-gamma. *Annu. Rev. Immunol.* **15**, 749–795 (1997).
371. Snijders, A., Kalinski, P., Hilkens, C. M. & Kapsenberg, M. L. High-level IL-12 production by human dendritic cells requires two signals. *Int. Immunol.* **10**, 1593–1598 (1998).
372. Eisele, G. *et al.* TGF-beta and metalloproteinases differentially suppress NKG2D ligand surface expression on malignant glioma cells. *Brain J. Neurol.* **129**, 2416–2425 (2006).
373. Friese, M. A. *et al.* RNA interference targeting transforming growth factor-beta enhances NKG2D-mediated antiglioma immune response, inhibits glioma cell migration and invasiveness, and abrogates tumorigenicity in vivo. *Cancer Res.* **64**, 7596–7603 (2004).
374. Stern-Ginossar, N. *et al.* Human microRNAs regulate stress-induced immune responses mediated by the receptor NKG2D. *Nat. Immunol.* **9**, 1065–1073 (2008).
375. Salih, H. R., Rammensee, H.-G. & Steinle, A. Cutting edge: down-regulation of MICA on human tumors by proteolytic shedding. *J. Immunol. Baltim. Md 1950* **169**, 4098–4102 (2002).
376. Doubrovina, E. S. *et al.* Evasion from NK cell immunity by MHC class I chain-related molecules expressing colon adenocarcinoma. *J. Immunol. Baltim. Md 1950* **171**, 6891–6899 (2003).
377. Raffaghello, L. *et al.* Downregulation and/or release of NKG2D ligands as immune evasion strategy of human neuroblastoma. *Neoplasia N. Y. N* **6**, 558–568 (2004).
378. Wu, J. D. *et al.* Prevalent expression of the immunostimulatory MHC class I chain-related molecule is counteracted by shedding in prostate cancer. *J. Clin. Invest.* **114**, 560–568 (2004).
379. Holdenrieder, S. *et al.* Soluble MICA in malignant diseases. *Int. J. Cancer J. Int. Cancer* **118**, 684–687 (2006).
380. Tsuboi, S. *et al.* A novel strategy for evasion of NK cell immunity by tumours expressing core2 O-glycans. *EMBO J.* **30**, 3173–3185 (2011).
381. Hiraga, J. *et al.* Down-regulation of CD20 expression in B-cell lymphoma cells after treatment with rituximab-containing combination chemotherapies: its prevalence and clinical significance. *Blood* **113**, 4885–4893 (2009).
382. Tsai, P.-C., Hernandez-Ilizaliturri, F. J., Bangia, N., Olejniczak, S. H. & Czuczman, M. S. Regulation of CD20 in rituximab-resistant cell lines and B-cell non-Hodgkin lymphoma. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **18**, 1039–1050 (2012).
383. Lehmann, C., Zeis, M., Schmitz, N. & Uharek, L. Impaired binding of perforin on the surface of tumor cells is a cause of target cell resistance against cytotoxic effector cells. *Blood* **96**, 594–600 (2000).
384. Berthou, C. *et al.* Interferon-gamma-induced membrane PAF-receptor expression confers tumor cell susceptibility to NK perforin-dependent lysis. *Blood* **95**, 2329–2336 (2000).
385. Kaiserman, D. & Bird, P. I. Control of granzymes by serpins. *Cell Death Differ.* **17**, 586–595 (2010).

386. Bird, C. H. *et al.* Selective regulation of apoptosis: the cytotoxic lymphocyte serpin proteinase inhibitor 9 protects against granzyme B-mediated apoptosis without perturbing the Fas cell death pathway. *Mol. Cell. Biol.* **18**, 6387–6398 (1998).
387. Bladergroen, B. A. *et al.* Expression of the granzyme B inhibitor, protease inhibitor 9, by tumor cells in patients with non-Hodgkin and Hodgkin lymphoma: a novel protective mechanism for tumor cells to circumvent the immune system? *Blood* **99**, 232–237 (2002).
388. Van Houdt, I. S. *et al.* Expression of the apoptosis inhibitor protease inhibitor 9 predicts clinical outcome in vaccinated patients with stage III and IV melanoma. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **11**, 6400–6407 (2005).
389. Rousalova, I., Krepela, E., Prochazka, J., Cermak, J. & Benkova, K. Expression of proteinase inhibitor-9/serpinB9 in non-small cell lung carcinoma cells and tissues. *Int. J. Oncol.* **36**, 275–283 (2010).
390. Jiang, X., Ellison, S. J., Alarid, E. T. & Shapiro, D. J. Interplay between the levels of estrogen and estrogen receptor controls the level of the granzyme inhibitor, proteinase inhibitor 9 and susceptibility to immune surveillance by natural killer cells. *Oncogene* **26**, 4106–4114 (2007).
391. Sipione, S. *et al.* Identification of a novel human granzyme B inhibitor secreted by cultured sertoli cells. *J. Immunol. Baltim. Md 1950* **177**, 5051–5058 (2006).
392. Dimberg, J. *et al.* Expression of the serine protease inhibitor serpinA3 in human colorectal adenocarcinomas. *Oncol. Lett.* **2**, 413–418 (2011).
393. Shin, M. S. *et al.* Mutations of tumor necrosis factor-related apoptosis-inducing ligand receptor 1 (TRAIL-R1) and receptor 2 (TRAIL-R2) genes in metastatic breast cancers. *Cancer Res.* **61**, 4942–4946 (2001).
394. Takahashi, H. *et al.* FAS death domain deletions and cellular FADD-like interleukin 1beta converting enzyme inhibitory protein (long) overexpression: alternative mechanisms for deregulating the extrinsic apoptotic pathway in diffuse large B-cell lymphoma subtypes. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **12**, 3265–3271 (2006).
395. Saito, H., Osaki, T. & Ikeguchi, M. Decreased NKG2D expression on NK cells correlates with impaired NK cell function in patients with gastric cancer. *Gastric Cancer* **15**, 27–33 (2012).
396. Costello, R. T. *et al.* Defective expression and function of natural killer cell-triggering receptors in patients with acute myeloid leukemia. *Blood* **99**, 3661–3667 (2002).
397. Coudert, J. D. *et al.* Altered NKG2D function in NK cells induced by chronic exposure to NKG2D ligand-expressing tumor cells. *Blood* **106**, 1711–1717 (2005).
398. Oppenheim, D. E. *et al.* Sustained localized expression of ligand for the activating NKG2D receptor impairs natural cytotoxicity in vivo and reduces tumor immunosurveillance. *Nat. Immunol.* **6**, 928–937 (2005).
399. Clayton, A., Mitchell, J. P., Court, J., Mason, M. D. & Tabi, Z. Human tumor-derived exosomes selectively impair lymphocyte responses to interleukin-2. *Cancer Res.* **67**, 7458–7466 (2007).
400. Clayton, A. *et al.* Human tumor-derived exosomes down-modulate NKG2D expression. *J. Immunol. Baltim. Md 1950* **180**, 7249–7258 (2008).
401. Veuillen, C. *et al.* Primary B-CLL resistance to NK cell cytotoxicity can be overcome in vitro and in vivo by priming NK cells and monoclonal antibody therapy. *J. Clin. Immunol.* **32**, 632–646 (2012).
402. Reiners, K. S. *et al.* Soluble ligands for NK cell receptors promote evasion of chronic lymphocytic leukemia cells from NK cell anti-tumor activity. *Blood* **121**, 3658–3665 (2013).
403. Rosental, B. *et al.* Proliferating cell nuclear antigen is a novel inhibitory ligand for the natural cytotoxicity receptor NKp44. *J. Immunol. Baltim. Md 1950* **187**, 5693–5702 (2011).
404. Zhou, Q., Gil-Krzewska, A., Peruzzi, G. & Borrego, F. Matrix metalloproteinases inhibition promotes the polyfunctionality of human natural killer cells in therapeutic antibody-based anti-tumour immunotherapy. *Clin. Exp. Immunol.* **173**, 131–139 (2013).
405. Peruzzi, G. *et al.* Membrane-Type 6 Matrix Metalloproteinase Regulates the Activation-Induced Downmodulation of CD16 in Human Primary NK Cells. *J. Immunol. Baltim. Md 1950* (2013). doi:10.4049/jimmunol.1300313

406. Bukur, J., Jasinski, S. & Seliger, B. The role of classical and non-classical HLA class I antigens in human tumors. *Semin. Cancer Biol.* **22**, 350–358 (2012).
407. Kleinberg, L. *et al.* Expression of HLA-G in malignant mesothelioma and clinically aggressive breast carcinoma. *Virchows Arch. Int. J. Pathol.* **449**, 31–39 (2006).
408. Paul, P. *et al.* HLA-G expression in melanoma: a way for tumor cells to escape from immunosurveillance. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 4510–4515 (1998).
409. Lin, A. *et al.* HLA-G expression in human ovarian carcinoma counteracts NK cell function. *Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. ESMO* **18**, 1804–1809 (2007).
410. Riteau, B. *et al.* Exosomes bearing HLA-G are released by melanoma cells. *Hum. Immunol.* **64**, 1064–1072 (2003).
411. Rajagopalan, S. & Long, E. O. A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells. *J. Exp. Med.* **189**, 1093–1100 (1999).
412. Rouas-Freiss, N., Marchal, R. E., Kirszenbaum, M., Dausset, J. & Carosella, E. D. The alpha1 domain of HLA-G1 and HLA-G2 inhibits cytotoxicity induced by natural killer cells: is HLA-G the public ligand for natural killer cell inhibitory receptors? *Proc. Natl. Acad. Sci. U. S. A.* **94**, 5249–5254 (1997).
413. Riteau, B. *et al.* HLA-G2, -G3, and -G4 isoforms expressed as nonmature cell surface glycoproteins inhibit NK and antigen-specific CTL cytotoxicity. *J. Immunol. Baltim. Md 1950* **166**, 5018–5026 (2001).
414. Rouas-Freiss, N., Moreau, P., Menier, C., LeMaoult, J. & Carosella, E. D. Expression of tolerogenic HLA-G molecules in cancer prevents antitumor responses. *Semin. Cancer Biol.* **17**, 413–421 (2007).
415. Bertout, J. A., Patel, S. A. & Simon, M. C. The impact of O<sub>2</sub> availability on human cancer. *Nat. Rev. Cancer* **8**, 967–975 (2008).
416. Yamada, N. *et al.* Hypoxia downregulates the expression of cell surface MICA without increasing soluble MICA in osteosarcoma cells in a HIF-1 $\alpha$ -dependent manner. *Int. J. Oncol.* **41**, 2005–2012 (2012).
417. Siemens, D. R. *et al.* Hypoxia increases tumor cell shedding of MHC class I chain-related molecule: role of nitric oxide. *Cancer Res.* **68**, 4746–4753 (2008).
418. Sarkar, S. *et al.* Hypoxia Induced Impairment of NK Cell Cytotoxicity against Multiple Myeloma Can Be Overcome by IL-2 Activation of the NK Cells. *PloS One* **8**, e64835 (2013).
419. Gazit, E. *et al.* HLA-G expression is induced in Epstein-Barr virus-transformed B-cell lines by culture conditions. *Hum. Immunol.* **68**, 463–468 (2007).
420. Loeffler, D. A., Juneau, P. L. & Heppner, G. H. Natural killer-cell activity under conditions reflective of tumor micro-environment. *Int. J. Cancer J. Int. Cancer* **48**, 895–899 (1991).
421. Ivanović, V. *et al.* Elevated plasma levels of transforming growth factor-beta 1 (TGF-beta 1) in patients with advanced breast cancer: association with disease progression. *Eur. J. Cancer Oxf. Engl.* **1990** **39**, 454–461 (2003).
422. Lee, J.-C., Lee, K.-M., Kim, D.-W. & Heo, D. S. Elevated TGF-beta1 secretion and down-modulation of NKG2D underlies impaired NK cytotoxicity in cancer patients. *J. Immunol. Baltim. Md 1950* **172**, 7335–7340 (2004).
423. Massagué, J. TGFbeta in Cancer. *Cell* **134**, 215–230 (2008).
424. Castriconi, R. *et al.* Transforming growth factor beta 1 inhibits expression of NKp30 and NKG2D receptors: consequences for the NK-mediated killing of dendritic cells. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 4120–4125 (2003).
425. Rook, A. H. *et al.* Effects of transforming growth factor beta on the functions of natural killer cells: depressed cytolytic activity and blunting of interferon responsiveness. *J. Immunol. Baltim. Md 1950* **136**, 3916–3920 (1986).
426. Trotta, R. *et al.* TGF-beta utilizes SMAD3 to inhibit CD16-mediated IFN-gamma production and antibody-dependent cellular cytotoxicity in human NK cells. *J. Immunol. Baltim. Md 1950* **181**, 3784–3792 (2008).
427. Blay, J., White, T. D. & Hoskin, D. W. The extracellular fluid of solid carcinomas contains immunosuppressive concentrations of adenosine. *Cancer Res.* **57**, 2602–2605 (1997).

428. Williams, B. A., Manzer, A., Blay, J. & Hoskin, D. W. Adenosine acts through a novel extracellular receptor to inhibit granule exocytosis by natural killer cells. *Biochem. Biophys. Res. Commun.* **231**, 264–269 (1997).
429. Lokshin, A. *et al.* Adenosine-mediated inhibition of the cytotoxic activity and cytokine production by activated natural killer cells. *Cancer Res.* **66**, 7758–7765 (2006).
430. Raskovalova, T. *et al.* Gs protein-coupled adenosine receptor signaling and lytic function of activated NK cells. *J. Immunol. Baltim. Md 1950* **175**, 4383–4391 (2005).
431. Mujoomdar, M., Hoskin, D. & Blay, J. Adenosine stimulation of the proliferation of colorectal carcinoma cell lines. Roles of cell density and adenosine metabolism. *Biochem. Pharmacol.* **66**, 1737–1747 (2003).
432. Synnestvedt, K. *et al.* Ecto-5'-nucleotidase (CD73) regulation by hypoxia-inducible factor-1 mediates permeability changes in intestinal epithelia. *J. Clin. Invest.* **110**, 993–1002 (2002).
433. Regateiro, F. S. *et al.* Generation of anti-inflammatory adenosine by leukocytes is regulated by TGF- $\beta$ . *Eur. J. Immunol.* **41**, 2955–2965 (2011).
434. Savic, V., Stefanovic, V., Ardaillou, N. & Ardaillou, R. Induction of ecto-5'-nucleotidase of rat cultured mesangial cells by interleukin-1 beta and tumour necrosis factor-alpha. *Immunology* **70**, 321–326 (1990).
435. Niemelä, J. *et al.* IFN-alpha induced adenosine production on the endothelium: a mechanism mediated by CD73 (ecto-5'-nucleotidase) up-regulation. *J. Immunol. Baltim. Md 1950* **172**, 1646–1653 (2004).
436. Hoechst, B. *et al.* Myeloid derived suppressor cells inhibit natural killer cells in patients with hepatocellular carcinoma via the NKp30 receptor. *Hepatol. Baltim. Md* **50**, 799–807 (2009).
437. Liu, C. *et al.* Expansion of spleen myeloid suppressor cells represses NK cell cytotoxicity in tumor-bearing host. *Blood* **109**, 4336–4342 (2007).
438. Li, H., Han, Y., Guo, Q., Zhang, M. & Cao, X. Cancer-expanded myeloid-derived suppressor cells induce anergy of NK cells through membrane-bound TGF-beta 1. *J. Immunol. Baltim. Md 1950* **182**, 240–249 (2009).
439. Trzonkowski, P., Szmit, E., Myśliwska, J., Dobyszuk, A. & Myśliwski, A. CD4+CD25+ T regulatory cells inhibit cytotoxic activity of T CD8+ and NK lymphocytes in the direct cell-to-cell interaction. *Clin. Immunol. Orlando Fla* **112**, 258–267 (2004).
440. Ghiringhelli, F. *et al.* CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner. *J. Exp. Med.* **202**, 1075–1085 (2005).
441. Grossman, W. J. *et al.* Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. *Blood* **104**, 2840–2848 (2004).
442. Cao, X. *et al.* Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity* **27**, 635–646 (2007).
443. He, K. M. *et al.* Donor double-negative Treg promote allogeneic mixed chimerism and tolerance. *Eur. J. Immunol.* **37**, 3455–3466 (2007).
444. Zimmer, J., Andrès, E. & Hentges, F. NK cells and Treg cells: a fascinating dance cheek to cheek. *Eur. J. Immunol.* **38**, 2942–2945 (2008).
445. Lindau, D., Gielen, P., Kroesen, M., Wesseling, P. & Adema, G. J. The immunosuppressive tumour network: myeloid-derived suppressor cells, regulatory T cells and natural killer T cells. *Immunology* **138**, 105–115 (2013).
446. Reeves, G. K. *et al.* Cancer incidence and mortality in relation to body mass index in the Million Women Study: cohort study. *BMJ* **335**, 1134 (2007).
447. Renehan, A. G., Tyson, M., Egger, M., Heller, R. F. & Zwahlen, M. Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. *Lancet* **371**, 569–578 (2008).
448. Park, J., Euhus, D. M. & Scherer, P. E. Paracrine and endocrine effects of adipose tissue on cancer development and progression. *Endocr. Rev.* **32**, 550–570 (2011).
449. Adams, T. D. *et al.* Cancer incidence and mortality after gastric bypass surgery. *Obes. Silver Spring Md* **17**, 796–802 (2009).



450. Nieman, K. M., Romero, I. L., Van Houten, B. & Lengyel, E. Adipose tissue and adipocytes support tumorigenesis and metastasis. *Biochim. Biophys. Acta* (2013). doi:10.1016/j.bbali.2013.02.010
451. Dirat B, Bochet L, Dabek M, Daviaud D, Dauvillier S, Majed B, Wang YY, Meulle A, Salles B, Le Gonidec S, Garrido I, Escourrou G, Valet P, Muller C. Cancer-Associated Adipocytes Exhibit an Activated Phenotype and Contribute to Breast Cancer Invasion. *Cancer Res* **71**, 2455–2465 (2011).
452. Elliott, B. E., Tam, S. P., Dexter, D. & Chen ZQ. Capacity of adipose tissue to promote growth and metastasis of a murine mammary carcinoma: effect of estrogen and progesterone. *Int. J. Cancer J. Int. Cancer* **51**, 416–424 (1992).
453. Manabe Y, Toda S, Miyazaki K, Sugihara H. Mature adipocytes, but not preadipocytes, promote the growth of breast carcinoma cells in collagen gel matrix culture through cancer-stromal cell interactions. *J Pathol* **201**, 221–8 (2003).
454. Zimmerlin, L. *et al.* Regenerative therapy and cancer: in vitro and in vivo studies of the interaction between adipose-derived stem cells and breast cancer cells from clinical isolates. *Tissue Eng. Part A* **17**, 93–106 (2011).
455. Nieman KM, Kenny HA, Penicka CV, Ladanyi A, Buell-Gutbrod R, Zillhardt MR, Romero IL, Carey MS, Mills GB, Hotamisligil GS, Yamada SD, Peter ME, Gwin K, Lengyel E. Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. *Nat Med* **17**, 1498–503 (2011).
456. WARBURG, O. On the origin of cancer cells. *Science* **123**, 309–314 (1956).
457. Sonveaux, P. *et al.* Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice. *J. Clin. Invest.* **118**, 3930–3942 (2008).
458. Yang, C. *et al.* Glioblastoma cells require glutamate dehydrogenase to survive impairments of glucose metabolism or Akt signaling. *Cancer Res.* **69**, 7986–7993 (2009).
459. Pavlides, S. *et al.* The reverse Warburg effect: aerobic glycolysis in cancer associated fibroblasts and the tumor stroma. *Cell Cycle Georget. Tex* **8**, 3984–4001 (2009).
460. Whitaker-Menezes, D. *et al.* Evidence for a stromal-epithelial ‘lactate shuttle’ in human tumors: MCT4 is a marker of oxidative stress in cancer-associated fibroblasts. *Cell Cycle Georget. Tex* **10**, 1772–1783 (2011).
461. Soares, A. F. *et al.* Effects of oxidative stress on adiponectin secretion and lactate production in 3T3-L1 adipocytes. *Free Radic. Biol. Med.* **38**, 882–889 (2005).
462. Pérez de Heredia, F., Wood, I. S. & Trayhurn, P. Hypoxia stimulates lactate release and modulates monocarboxylate transporter (MCT1, MCT2, and MCT4) expression in human adipocytes. *Pflüg. Arch. Eur. J. Physiol.* **459**, 509–518 (2010).
463. Mermier, P. & Baker, N. Flux of free fatty acids among host tissues, ascites fluid, and Ehrlich ascites carcinoma cells. *J. Lipid Res.* **15**, 339–351 (1974).
464. Das, S. K. *et al.* Adipose triglyceride lipase contributes to cancer-associated cachexia. *Science* **333**, 233–238 (2011).
465. Tisdale, M. J. Cachexia in cancer patients. *Nat. Rev. Cancer* **2**, 862–871 (2002).
466. Rashid, A. *et al.* Elevated expression of fatty acid synthase and fatty acid synthetic activity in colorectal neoplasia. *Am. J. Pathol.* **150**, 201–208 (1997).
467. Shurbaji, M. S., Kalbfleisch, J. H. & Thurmond, T. S. Immunohistochemical detection of a fatty acid synthase (OA-519) as a predictor of progression of prostate cancer. *Hum. Pathol.* **27**, 917–921 (1996).
468. Gansler, T. S., Hardman, W., 3rd, Hunt, D. A., Schaffel, S. & Hennigar, R. A. Increased expression of fatty acid synthase (OA-519) in ovarian neoplasms predicts shorter survival. *Hum. Pathol.* **28**, 686–692 (1997).
469. Pizer, E. S., Lax, S. F., Kuhajda, F. P., Pasternack, G. R. & Kurman, R. J. Fatty acid synthase expression in endometrial carcinoma: correlation with cell proliferation and hormone receptors. *Cancer* **83**, 528–537 (1998).
470. Alo', P. L. *et al.* Expression of fatty acid synthase (FAS) as a predictor of recurrence in stage I breast carcinoma patients. *Cancer* **77**, 474–482 (1996).

471. Milgraum, L. Z., Witters, L. A., Pasternack, G. R. & Kuhajda, F. P. Enzymes of the fatty acid synthesis pathway are highly expressed in in situ breast carcinoma. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **3**, 2115–2120 (1997).
472. Kuhajda, F. P. *et al.* Synthesis and antitumor activity of an inhibitor of fatty acid synthase. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 3450–3454 (2000).
473. Zhou, W. *et al.* Fatty acid synthase inhibition activates AMP-activated protein kinase in SKOV3 human ovarian cancer cells. *Cancer Res.* **67**, 2964–2971 (2007).
474. Ishikawa, M., Kitayama, J. & Nagawa, H. Enhanced expression of leptin and leptin receptor (OB-R) in human breast cancer. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **10**, 4325–4331 (2004).
475. Tsuchiya, T., Shimizu, H., Horie, T. & Mori, M. Expression of leptin receptor in lung: leptin as a growth factor. *Eur. J. Pharmacol.* **365**, 273–279 (1999).
476. Mix, H. *et al.* Expression of leptin and leptin receptor isoforms in the human stomach. *Gut* **47**, 481–486 (2000).
477. Hardwick, J. C., Van Den Brink, G. R., Offerhaus, G. J., Van Deventer, S. J. & Peppelenbosch, M. P. Leptin is a growth factor for colonic epithelial cells. *Gastroenterology* **121**, 79–90 (2001).
478. Uddin, S. *et al.* Overexpression of leptin receptor predicts an unfavorable outcome in Middle Eastern ovarian cancer. *Mol. Cancer* **8**, 74 (2009).
479. Hino, M. *et al.* Leptin receptor and leukemia. *Leuk. Lymphoma* **36**, 457–461 (2000).
480. Garofalo, C. & Surmacz, E. Leptin and cancer. *J. Cell. Physiol.* **207**, 12–22 (2006).
481. Soma D, Kitayama J, Yamashita H, Miyato H, Ishikawa M, Nagawa H. Leptin Augments Proliferation of Breast Cancer Cells via Transactivation of HER2. *J Surg Res* **149**, 9–14 (2008).
482. Fiorio, E. *et al.* Leptin/HER2 crosstalk in breast cancer: in vitro study and preliminary in vivo analysis. *BMC Cancer* **8**, 305 (2008).
483. Amemori, S. *et al.* Adipocytes and preadipocytes promote the proliferation of colon cancer cells in vitro. *Am. J. Physiol. Gastrointest. Liver Physiol.* **292**, G923–G929 (2007).
484. Endo, H. *et al.* Leptin acts as a growth factor for colorectal tumours at stages subsequent to tumour initiation in murine colon carcinogenesis. *Gut* **60**, 1363–1371 (2011).
485. Zheng, Q. *et al.* Leptin deficiency suppresses MMTV-Wnt-1 mammary tumor growth in obese mice and abrogates tumor initiating cell survival. *Endocr. Relat. Cancer* **18**, 491–503 (2011).
486. Ribeiro, A. M. *et al.* Prostate cancer cell proliferation and angiogenesis in different obese mice models. *Int. J. Exp. Pathol.* **91**, 374–386 (2010).
487. Aparicio, T. *et al.* Leptin stimulates the proliferation of human colon cancer cells in vitro but does not promote the growth of colon cancer xenografts in nude mice or intestinal tumorigenesis in Apc(Min/+) mice. *Gut* **54**, 1136–1145 (2005).
488. Koda, M., Sulkowska, M., Kanczuga-Koda, L., Surmacz, E. & Sulkowski, S. Overexpression of the obesity hormone leptin in human colorectal cancer. *J. Clin. Pathol.* **60**, 902–906 (2007).
489. Körner, A. *et al.* Total and high-molecular-weight adiponectin in breast cancer: in vitro and in vivo studies. *J. Clin. Endocrinol. Metab.* **92**, 1041–1048 (2007).
490. Petridou, E. T. *et al.* Circulating adiponectin levels and expression of adiponectin receptors in relation to lung cancer: two case-control studies. *Oncology* **73**, 261–269 (2007).
491. Takahata, C. *et al.* Demonstration of adiponectin receptors 1 and 2 mRNA expression in human breast cancer cells. *Cancer Lett.* **250**, 229–236 (2007).
492. Yoneda, K. *et al.* Expression of adiponectin receptors, AdipoR1 and AdipoR2, in normal colon epithelium and colon cancer tissue. *Oncol. Rep.* **20**, 479–483 (2008).
493. Dalamaga, M. *et al.* Pancreatic cancer expresses adiponectin receptors and is associated with hypoleptinemia and hyperadiponectinemia: a case-control study. *Cancer Causes Control CCC* **20**, 625–633 (2009).
494. Kang, J. H. *et al.* Adiponectin induces growth arrest and apoptosis of MDA-MB-231 breast cancer cell. *Arch. Pharm. Res.* **28**, 1263–1269 (2005).
495. Dieudonne MN, Bussiere M, Dos Santos E, Leneuve MC, Giudicelli Y, Pecquery R. Adiponectin mediates antiproliferative and apoptotic responses in human MCF7 breast cancer cells. *Biochem Biophys Res Commun* **345**, 271–9 (2006).

496. Saxena, N. K. *et al.* Adiponectin modulates C-jun N-terminal kinase and mammalian target of rapamycin and inhibits hepatocellular carcinoma. *Gastroenterology* **139**, 1762–1773, 1773.e1–5 (2010).
497. Ishikawa, M. *et al.* Adiponectin inhibits the growth and peritoneal metastasis of gastric cancer through its specific membrane receptors AdipoR1 and AdipoR2. *Cancer Sci.* **98**, 1120–1127 (2007).
498. Cong, L. *et al.* Human adiponectin inhibits cell growth and induces apoptosis in human endometrial carcinoma cells, HEC-1-A and RL95 2. *Endocr. Relat. Cancer* **14**, 713–720 (2007).
499. Wang, Y. *et al.* Adiponectin modulates the glycogen synthase kinase-3beta/beta-catenin signaling pathway and attenuates mammary tumorigenesis of MDA-MB-231 cells in nude mice. *Cancer Res.* **66**, 11462–11470 (2006).
500. Otani, K. *et al.* Adiponectin suppresses tumorigenesis in Apc(Min)(/+) mice. *Cancer Lett.* **288**, 177–182 (2010).
501. Sun Y, Lodish HF. Adiponectin deficiency promotes tumor growth in mice by reducing macrophage infiltration. *PLoS One* **5**, e11987. (2010).
502. Nishihara, T. *et al.* Adiponectin deficiency enhances colorectal carcinogenesis and liver tumor formation induced by azoxymethane in mice. *World J. Gastroenterol. WJG* **14**, 6473–6480 (2008).
503. Wang, Y., Lam, K. S. L. & Xu, A. Adiponectin as a negative regulator in obesity-related mammary carcinogenesis. *Cell Res.* **17**, 280–282 (2007).
504. Nkhata KJ, Ray A, Schuster TF, Grossmann ME, Cleary MP. Effects of adiponectin and leptin co-treatment on human breast cancer cell growth. *Oncol Rep* **21**, 1611–9 (2009).
505. Ollberding, N. J. *et al.* Prediagnostic leptin, adiponectin, C-reactive protein, and the risk of postmenopausal breast cancer. *Cancer Prev. Res. Phila. Pa* **6**, 188–195 (2013).
506. Chen, D.-C. *et al.* Serum adiponectin and leptin levels in Taiwanese breast cancer patients. *Cancer Lett.* **237**, 109–114 (2006).
507. Stocks, T. *et al.* Components of the metabolic syndrome and colorectal cancer risk; a prospective study. *Int. J. Obes. 2005* **32**, 304–314 (2008).
508. Ashizawa, N. *et al.* Serum leptin-adiponectin ratio and endometrial cancer risk in postmenopausal female subjects. *Gynecol. Oncol.* **119**, 65–69 (2010).
509. Simpson, E. R. *et al.* Estrogen--the good, the bad, and the unexpected. *Endocr. Rev.* **26**, 322–330 (2005).
510. Zhao, Y., Agarwal, V. R., Mendelson, C. R. & Simpson, E. R. Estrogen biosynthesis proximal to a breast tumor is stimulated by PGE2 via cyclic AMP, leading to activation of promoter II of the CYP19 (aromatase) gene. *Endocrinology* **137**, 5739–5742 (1996).
511. Chan, J. M. *et al.* Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. *Science* **279**, 563–566 (1998).
512. Yu, H. *et al.* Plasma levels of insulin-like growth factor-I and lung cancer risk: a case-control analysis. *J. Natl. Cancer Inst.* **91**, 151–156 (1999).
513. Ma, J. *et al.* Prospective study of colorectal cancer risk in men and plasma levels of insulin-like growth factor (IGF)-I and IGF-binding protein-3. *J. Natl. Cancer Inst.* **91**, 620–625 (1999).
514. Giovannucci, E. *et al.* A prospective study of plasma insulin-like growth factor-1 and binding protein-3 and risk of colorectal neoplasia in women. *Cancer Epidemiol. Biomark. Prev. Publ. Am. Assoc. Cancer Res. Cosponsored Am. Soc. Prev. Oncol.* **9**, 345–349 (2000).
515. Palmqvist, R. *et al.* Plasma insulin-like growth factor 1, insulin-like growth factor binding protein 3, and risk of colorectal cancer: a prospective study in northern Sweden. *Gut* **50**, 642–646 (2002).
516. D'Esposito, V. *et al.* Adipocyte-released insulin-like growth factor-1 is regulated by glucose and fatty acids and controls breast cancer cell growth in vitro. *Diabetologia* **55**, 2811–2822 (2012).
517. Rehman, J. *et al.* Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation* **109**, 1292–1298 (2004).
518. Bell, L. N. *et al.* Adipose tissue production of hepatocyte growth factor contributes to elevated serum HGF in obesity. *Am. J. Physiol. Endocrinol. Metab.* **291**, E843–848 (2006).

519. Beviglia, L., Matsumoto, K., Lin, C. S., Ziober, B. L. & Kramer, R. H. Expression of the c-Met/HGF receptor in human breast carcinoma: correlation with tumor progression. *Int. J. Cancer J. Int. Cancer* **74**, 301–309 (1997).
520. Wong, A. S. *et al.* Coexpression of hepatocyte growth factor-Met: an early step in ovarian carcinogenesis? *Oncogene* **20**, 1318–1328 (2001).
521. Ichimura, E., Maeshima, A., Nakajima, T. & Nakamura, T. Expression of c-met/HGF receptor in human non-small cell lung carcinomas in vitro and in vivo and its prognostic significance. *Jpn. J. Cancer Res. Gann* **87**, 1063–1069 (1996).
522. Edakuni, G., Sasatomi, E., Satoh, T., Tokunaga, O. & Miyazaki, K. Expression of the hepatocyte growth factor/c-Met pathway is increased at the cancer front in breast carcinoma. *Pathol. Int.* **51**, 172–178 (2001).
523. Rahimi, N., Saulnier, R., Nakamura, T., Park, M. & Elliott, B. Role of hepatocyte growth factor in breast cancer: a novel mitogenic factor secreted by adipocytes. *DNA Cell Biol.* **13**, 1189–1197 (1994).
524. Takayama, H. *et al.* Diverse tumorigenesis associated with aberrant development in mice overexpressing hepatocyte growth factor/scatter factor. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 701–706 (1997).
525. Cao, Y. Adipose tissue angiogenesis as a therapeutic target for obesity and metabolic diseases. *Nat. Rev. Drug Discov.* **9**, 107–115 (2010).
526. Mick, G. J., Wang, X. & McCormick, K. White adipocyte vascular endothelial growth factor: regulation by insulin. *Endocrinology* **143**, 948–953 (2002).
527. Borges, J., Müller, M. C., Momeni, A., Stark, G. B. & Torio-Padron, N. In vitro analysis of the interactions between preadipocytes and endothelial cells in a 3D fibrin matrix. *Minim. Invasive Ther. Allied Technol. MITAT Off. J. Soc. Minim. Invasive Ther.* **16**, 141–148 (2007).
528. Sierra-Honigmann, M. R. *et al.* Biological action of leptin as an angiogenic factor. *Science* **281**, 1683–1686 (1998).
529. Denzel, M. S. *et al.* Adiponectin deficiency limits tumor vascularization in the MMTV-PyV-mT mouse model of mammary cancer. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **15**, 3256–3264 (2009).
530. Landskroner-Eiger, S. *et al.* Proangiogenic contribution of adiponectin toward mammary tumor growth in vivo. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **15**, 3265–3276 (2009).
531. Mu, H. *et al.* Adipokine resistin promotes in vitro angiogenesis of human endothelial cells. *Cardiovasc. Res.* **70**, 146–157 (2006).
532. Saiki, A., Watanabe, F., Murano, T., Miyashita, Y. & Shirai, K. Hepatocyte growth factor secreted by cultured adipocytes promotes tube formation of vascular endothelial cells in vitro. *Int. J. Obes. 2005* **30**, 1676–1684 (2006).
533. Dobson, D. E. *et al.* 1-Butyryl-glycerol: a novel angiogenesis factor secreted by differentiating adipocytes. *Cell* **61**, 223–230 (1990).
534. Meliga, E., Strem, B. M., Duckers, H. J. & Serruys, P. W. Adipose-derived cells. *Cell Transplant.* **16**, 963–970 (2007).
535. Tang, W. *et al.* White fat progenitor cells reside in the adipose vasculature. *Science* **322**, 583–586 (2008).
536. Zhang, Y. *et al.* Stromal progenitor cells from endogenous adipose tissue contribute to pericytes and adipocytes that populate the tumor microenvironment. *Cancer Res.* **72**, 5198–5208 (2012).
537. Valastyan, S. & Weinberg, R. A. Tumor metastasis: molecular insights and evolving paradigms. *Cell* **147**, 275–292 (2011).
538. Kimijima, I., Ohtake, T., Sagara, H., Watanabe, T. & Takenoshita, S. Scattered fat invasion: an indicator for poor prognosis in premenopausal, and for positive estrogen receptor in postmenopausal breast cancer patients. *Oncology* **59 Suppl 1**, 25–30 (2000).
539. Hasebe, T., Imoto, S., Sasaki, S. & Mukai, K. A proposal for a new histological classification scheme for predicting short-term tumor recurrence and death in patients with invasive ductal carcinoma of the breast. *Jpn. J. Cancer Res. Gann* **89**, 1358–1373 (1998).

540. Yamaguchi, J., Ohtani, H., Nakamura, K., Shimokawa, I. & Kanematsu, T. Prognostic impact of marginal adipose tissue invasion in ductal carcinoma of the breast. *Am. J. Clin. Pathol.* **130**, 382–388 (2008).
541. Walter, M., Liang, S., Ghosh, S., Hornsby, P. J. & Li, R. Interleukin 6 secreted from adipose stromal cells promotes migration and invasion of breast cancer cells. *Oncogene* **28**, 2745–2755 (2009).
542. Trusolino, L. & Comoglio, P. M. Scatter-factor and semaphorin receptors: cell signalling for invasive growth. *Nat. Rev. Cancer* **2**, 289–300 (2002).
543. Tannapfel, A., Yasui, W., Yokozaki, H., Wittekind, C. & Tahara, E. Effect of hepatocyte growth factor on the expression of E- and P-cadherin in gastric carcinoma cell lines. *Virchows Arch. Int. J. Pathol.* **425**, 139–144 (1994).
544. Miura, H. *et al.* Effects of hepatocyte growth factor on E-cadherin-mediated cell-cell adhesion in DU145 prostate cancer cells. *Urology* **58**, 1064–1069 (2001).
545. Comoglio, P. M., Giordano, S. & Trusolino, L. Drug development of MET inhibitors: targeting oncogene addiction and expedience. *Nat. Rev. Drug Discov.* **7**, 504–516 (2008).
546. Kubo, Y., Kaidzu, S., Nakajima, I., Takenouchi, K. & Nakamura, F. Organization of extracellular matrix components during differentiation of adipocytes in long-term culture. *In Vitro Cell. Dev. Biol. Anim.* **36**, 38–44 (2000).
547. Iyengar, P. *et al.* Adipocyte-secreted factors synergistically promote mammary tumorigenesis through induction of anti-apoptotic transcriptional programs and proto-oncogene stabilization. *Oncogene* **22**, 6408–6423 (2003).
548. Iyengar, P. *et al.* Adipocyte-derived collagen VI affects early mammary tumor progression in vivo, demonstrating a critical interaction in the tumor/stroma microenvironment. *J. Clin. Invest.* **115**, 1163–1176 (2005).
549. Park, J. & Scherer, P. E. Adipocyte-derived endotrophin promotes malignant tumor progression. *J. Clin. Invest.* **122**, 4243–4256 (2012).
550. Chandler, E. M., Saunders, M. P., Yoon, C. J., Gourdon, D. & Fischbach, C. Adipose progenitor cells increase fibronectin matrix strain and unfolding in breast tumors. *Phys. Biol.* **8**, 015008 (2011).
551. Balanis, N. *et al.* Epithelial to mesenchymal transition promotes breast cancer progression via a fibronectin-dependent STAT3 signaling pathway. *J. Biol. Chem.* **288**, 17954–17967 (2013).
552. Chavey, C. *et al.* Matrix metalloproteinases are differentially expressed in adipose tissue during obesity and modulate adipocyte differentiation. *J. Biol. Chem.* **278**, 11888–11896 (2003).
553. Kessenbrock, K., Plaks, V. & Werb, Z. Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell* **141**, 52–67 (2010).
554. Andarawewa, K. L. *et al.* Stromelysin-3 is a potent negative regulator of adipogenesis participating to cancer cell-adipocyte interaction/crosstalk at the tumor invasive front. *Cancer Res.* **65**, 10862–10871 (2005).
555. Matziari, M., Dive, V. & Yiotakis, A. Matrix metalloproteinase 11 (MMP-11; stromelysin-3) and synthetic inhibitors. *Med. Res. Rev.* **27**, 528–552 (2007).
556. Motrescu, E. R. *et al.* Matrix metalloproteinase-11/stromelysin-3 exhibits collagenolytic function against collagen VI under normal and malignant conditions. *Oncogene* **27**, 6347–6355 (2008).
557. Noël, A. *et al.* Demonstration in vivo that stromelysin-3 functions through its proteolytic activity. *Oncogene* **19**, 1605–1612 (2000).
558. Andarawewa, K. L. *et al.* Dual stromelysin-3 function during natural mouse mammary tumor virus-ras tumor progression. *Cancer Res.* **63**, 5844–5849 (2003).
559. Jia, L. *et al.* siRNA targeted against matrix metalloproteinase 11 inhibits the metastatic capability of murine hepatocarcinoma cell Hca-F to lymph nodes. *Int. J. Biochem. Cell Biol.* **39**, 2049–2062 (2007).
560. Cheng, C.-W. *et al.* The clinical implications of MMP-11 and CK-20 expression in human breast cancer. *Clin. Chim. Acta Int. J. Clin. Chem.* **411**, 234–241 (2010).

561. Jones, L. E., Humphreys, M. J., Campbell, F., Neoptolemos, J. P. & Boyd, M. T. Comprehensive analysis of matrix metalloproteinase and tissue inhibitor expression in pancreatic cancer: increased expression of matrix metalloproteinase-7 predicts poor survival. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **10**, 2832–2845 (2004).
562. Thewes, M. *et al.* Stromelysin-3 (ST-3) mRNA expression in colorectal carcinomas. Localization and clinicopathologic correlations. *Diagn. Mol. Pathol. Am. J. Surg. Pathol. Part B* **5**, 284–290 (1996).
563. Motrescu, E. R. & Rio, M.-C. Cancer cells, adipocytes and matrix metalloproteinase 11: a vicious tumor progression cycle. *Biol. Chem.* **389**, 1037–1041 (2008).
564. Yeh, W.-L., Lu, D.-Y., Lee, M.-J. & Fu, W.-M. Leptin induces migration and invasion of glioma cells through MMP-13 production. *Glia* **57**, 454–464 (2009).
565. Rosenthal, E. L. *et al.* Role of the plasminogen activator and matrix metalloproteinase systems in epidermal growth factor- and scatter factor-stimulated invasion of carcinoma cells. *Cancer Res.* **58**, 5221–5230 (1998).
566. Monvoisin, A. *et al.* Involvement of matrix metalloproteinase type-3 in hepatocyte growth factor-induced invasion of human hepatocellular carcinoma cells. *Int. J. Cancer J. Int. Cancer* **97**, 157–162 (2002).
567. Pramanik, R., Sheng, X., Ichihara, B., Heisterkamp, N. & Mittelman, S. D. Adipose tissue attracts and protects acute lymphoblastic leukemia cells from chemotherapy. *Leuk. Res.* **37**, 503–509 (2013).
568. Ishizaki, Y., Cheng, L., Mudge, A. W. & Raff, M. C. Programmed cell death by default in embryonic cells, fibroblasts, and cancer cells. *Mol. Biol. Cell* **6**, 1443–1458 (1995).
569. Giancotti, F. G. & Ruoslahti, E. Integrin signaling. *Science* **285**, 1028–1032 (1999).
570. Sherman-Baust, C. A. *et al.* Remodeling of the extracellular matrix through overexpression of collagen VI contributes to cisplatin resistance in ovarian cancer cells. *Cancer Cell* **3**, 377–386 (2003).
571. Xing, H. *et al.* Fibronectin-mediated activation of Akt2 protects human ovarian and breast cancer cells from docetaxel-induced apoptosis via inhibition of the p38 pathway. *Apoptosis Int. J. Program. Cell Death* **13**, 213–223 (2008).
572. Rafii, A. *et al.* Oncologic trogocytosis of an original stromal cells induces chemoresistance of ovarian tumours. *PloS One* **3**, e3894 (2008).
573. Behan, J. W. *et al.* Adipocytes impair leukemia treatment in mice. *Cancer Res.* **69**, 7867–7874 (2009).
574. Holland, W. L. *et al.* Receptor-mediated activation of ceramidase activity initiates the pleiotropic actions of adiponectin. *Nat. Med.* **17**, 55–63 (2011).
575. Reynolds, C. P., Maurer, B. J. & Kolesnick, R. N. Ceramide synthesis and metabolism as a target for cancer therapy. *Cancer Lett.* **206**, 169–180 (2004).
576. Chi, M. *et al.* Adipocytes Contribute to Resistance of Human Melanoma Cells to Chemotherapy and Targeted Therapy. *Curr. Med. Chem.* (2013).
577. Bochet L, Meulle A, Imbert S, Salles B, Valet P, Muller C. Cancer-associated adipocytes promotes breast tumor radioresistance. *Biochem Biophys Res Commun* [Epub ahead of print], (2011).
578. Hao, M. *et al.* Bone marrow stromal cells protect myeloma cells from bortezomib induced apoptosis by suppressing microRNA-15a expression. *Leuk. Lymphoma* **52**, 1787–1794 (2011).
579. Roodhart, J. M. L. *et al.* Mesenchymal stem cells induce resistance to chemotherapy through the release of platinum-induced fatty acids. *Cancer Cell* **20**, 370–383 (2011).
580. Kabon, B. *et al.* Obesity decreases perioperative tissue oxygenation. *Anesthesiology* **100**, 274–280 (2004).
581. Hosogai, N. *et al.* Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. *Diabetes* **56**, 901–911 (2007).
582. Chen, B. *et al.* Hypoxia dysregulates the production of adiponectin and plasminogen activator inhibitor-1 independent of reactive oxygen species in adipocytes. *Biochem. Biophys. Res. Commun.* **341**, 549–556 (2006).

583. Lolmède, K., Durand de Saint Front, V., Galitzky, J., Lafontan, M. & Bouloumié, A. Effects of hypoxia on the expression of proangiogenic factors in differentiated 3T3-F442A adipocytes. *Int. J. Obes. Relat. Metab. Disord. J. Int. Assoc. Study Obes.* **27**, 1187–1195 (2003).
584. Wang, B., Wood, I. S. & Trayhurn, P. Dysregulation of the expression and secretion of inflammation-related adipokines by hypoxia in human adipocytes. *Pflüg. Arch. Eur. J. Physiol.* **455**, 479–492 (2007).
585. Ye, J., Gao, Z., Yin, J. & He, Q. Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of ob/ob and dietary obese mice. *Am. J. Physiol. Endocrinol. Metab.* **293**, E1118–1128 (2007).
586. Cao, Y. Angiogenesis modulates adipogenesis and obesity. *J. Clin. Invest.* **117**, 2362–2368 (2007).
587. Khan, T. *et al.* Metabolic dysregulation and adipose tissue fibrosis: role of collagen VI. *Mol. Cell. Biol.* **29**, 1575–1591 (2009).
588. Ouchi, N., Parker, J. L., Lugus, J. J. & Walsh, K. Adipokines in inflammation and metabolic disease. *Nat. Rev. Immunol.* **11**, 85–97 (2011).
589. Maffei, M. *et al.* Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat. Med.* **1**, 1155–1161 (1995).
590. Considine, R. V. *et al.* Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N. Engl. J. Med.* **334**, 292–295 (1996).
591. Owecki, M., Miczke, A., Nikisch, E., Pupek-Musialik, D. & Sowiński, J. Serum resistin concentrations are higher in human obesity but independent from insulin resistance. *Exp. Clin. Endocrinol. Diabetes Off. J. Ger. Soc. Endocrinol. Ger. Diabetes Assoc.* **119**, 117–121 (2011).
592. Roytblat, L. *et al.* Raised interleukin-6 levels in obese patients. *Obes. Res.* **8**, 673–675 (2000).
593. Fontana, L., Eagon, J. C., Trujillo, M. E., Scherer, P. E. & Klein, S. Visceral fat adipokine secretion is associated with systemic inflammation in obese humans. *Diabetes* **56**, 1010–1013 (2007).
594. Kern, P. A. *et al.* The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase. *J. Clin. Invest.* **95**, 2111–2119 (1995).
595. Ziccardi, P. *et al.* Reduction of inflammatory cytokine concentrations and improvement of endothelial functions in obese women after weight loss over one year. *Circulation* **105**, 804–809 (2002).
596. Arita, Y. *et al.* Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem. Biophys. Res. Commun.* **257**, 79–83 (1999).
597. Bastard, J. P. *et al.* Elevated levels of interleukin 6 are reduced in serum and subcutaneous adipose tissue of obese women after weight loss. *J. Clin. Endocrinol. Metab.* **85**, 3338–3342 (2000).
598. Bruun, J. M. *et al.* Regulation of adiponectin by adipose tissue-derived cytokines: in vivo and in vitro investigations in humans. *Am. J. Physiol. Endocrinol. Metab.* **285**, E527–533 (2003).
599. Wang, B., Jenkins, J. R. & Trayhurn, P. Expression and secretion of inflammation-related adipokines by human adipocytes differentiated in culture: integrated response to TNF-alpha. *Am. J. Physiol. Endocrinol. Metab.* **288**, E731–740 (2005).
600. Adams, E. F., Rafferty, B. & White, M. C. Interleukin 6 is secreted by breast fibroblasts and stimulates 17 beta-oestradiol oxidoreductase activity of MCF-7 cells: possible paracrine regulation of breast 17 beta-oestradiol levels. *Int. J. Cancer J. Int. Cancer* **49**, 118–121 (1991).
601. Duncan, L. J., Coldham, N. G. & Reed, M. J. The interaction of cytokines in regulating oestradiol 17 beta-hydroxysteroid dehydrogenase activity in MCF-7 cells. *J. Steroid Biochem. Mol. Biol.* **49**, 63–68 (1994).
602. Lukanova, A. *et al.* Body mass index, circulating levels of sex-steroid hormones, IGF-I and IGF-binding protein-3: a cross-sectional study in healthy women. *Eur. J. Endocrinol. Eur. Fed. Endocr. Soc.* **150**, 161–171 (2004).
603. Rehman, J. *et al.* Obesity is associated with increased levels of circulating hepatocyte growth factor. *J. Am. Coll. Cardiol.* **41**, 1408–1413 (2003).
604. Cinti, S. *et al.* Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J. Lipid Res.* **46**, 2347–2355 (2005).

605. Strissel, K. J. *et al.* Adipocyte death, adipose tissue remodeling, and obesity complications. *Diabetes* **56**, 2910–2918 (2007).
606. Murano, I. *et al.* Dead adipocytes, detected as crown-like structures, are prevalent in visceral fat depots of genetically obese mice. *J. Lipid Res.* **49**, 1562–1568 (2008).
607. Alkhoury, N. *et al.* Adipocyte apoptosis, a link between obesity, insulin resistance, and hepatic steatosis. *J. Biol. Chem.* **285**, 3428–3438 (2010).
608. Pajvani, U. B. *et al.* Fat apoptosis through targeted activation of caspase 8: a new mouse model of inducible and reversible lipodystrophy. *Nat. Med.* **11**, 797–803 (2005).
609. Weisberg, S. P. *et al.* Obesity is associated with macrophage accumulation in adipose tissue. *J. Clin. Invest.* **112**, 1796–1808 (2003).
610. Freud, A. G. & Caligiuri, M. A. Human natural killer cell development. *Immunol. Rev.* **214**, 56–72 (2006).
611. Shields, J. W. Lymph, lymph glands, and homeostasis. *Lymphology* **25**, 147–153 (1992).
612. Pond, C. M. Adipose tissue and the immune system. *Prostaglandins Leukot. Essent. Fatty Acids* **73**, 17–30 (2005).
613. Goralski, K. B. *et al.* Chemerin, a novel adipokine that regulates adipogenesis and adipocyte metabolism. *J. Biol. Chem.* **282**, 28175–28188 (2007).
614. Bozaoglu, K. *et al.* Chemerin is a novel adipokine associated with obesity and metabolic syndrome. *Endocrinology* **148**, 4687–4694 (2007).
615. Bondue, B., Wittamer, V. & Parmentier, M. Chemerin and its receptors in leukocyte trafficking, inflammation and metabolism. *Cytokine Growth Factor Rev.* **22**, 331–338 (2011).
616. Wittamer, V. *et al.* Specific recruitment of antigen-presenting cells by chemerin, a novel processed ligand from human inflammatory fluids. *J. Exp. Med.* **198**, 977–985 (2003).
617. Roh, S. *et al.* Chemerin—a new adipokine that modulates adipogenesis via its own receptor. *Biochem. Biophys. Res. Commun.* **362**, 1013–1018 (2007).
618. Muruganandan, S. *et al.* Chemerin, a novel peroxisome proliferator-activated receptor gamma (PPARgamma) target gene that promotes mesenchymal stem cell adipogenesis. *J. Biol. Chem.* **286**, 23982–23995 (2011).
619. Parlee, S. D., Ernst, M. C., Muruganandan, S., Sinal, C. J. & Goralski, K. B. Serum chemerin levels vary with time of day and are modified by obesity and tumor necrosis factor- $\alpha$ . *Endocrinology* **151**, 2590–2602 (2010).
620. Kralisch, S. *et al.* Interleukin-1 $\beta$  induces the novel adipokine chemerin in adipocytes in vitro. *Regul. Pept.* **154**, 102–106 (2009).
621. Bauer, S. *et al.* Sterol regulatory element-binding protein 2 (SREBP2) activation after excess triglyceride storage induces chemerin in hypertrophic adipocytes. *Endocrinology* **152**, 26–35 (2011).
622. Parolini, S. *et al.* The role of chemerin in the colocalization of NK and dendritic cell subsets into inflamed tissues. *Blood* **109**, 3625–3632 (2007).
623. Pachynski, R. K. *et al.* The chemoattractant chemerin suppresses melanoma by recruiting natural killer cell antitumor defenses. *J. Exp. Med.* **209**, 1427–1435 (2012).
624. Bruun, J. M. *et al.* Higher production of IL-8 in visceral vs. subcutaneous adipose tissue. Implication of nonadipose cells in adipose tissue. *Am. J. Physiol. Endocrinol. Metab.* **286**, E8–13 (2004).
625. Morohashi, H. *et al.* Expression of both types of human interleukin-8 receptors on mature neutrophils, monocytes, and natural killer cells. *J. Leukoc. Biol.* **57**, 180–187 (1995).
626. Vujanovic, L., Ballard, W., Thorne, S. H., Vujanovic, N. L. & Butterfield, L. H. Adenovirus-engineered human dendritic cells induce natural killer cell chemotaxis via CXCL8/IL-8 and CXCL10/IP-10. *Oncoimmunology* **1**, 448–457 (2012).
627. Sebok, K. *et al.* IL-8 induces the locomotion of human IL-2-activated natural killer cells. Involvement of a guanine nucleotide binding (Go) protein. *J. Immunol. Baltim. Md* **150**, 1524–1534 (1993).
628. Pagès, G. *et al.* LPA as a paracrine mediator of adipocyte growth and function. *Ann. N. Y. Acad. Sci.* **905**, 159–164 (2000).



629. Hashimoto, T., Igarashi, J. & Kosaka, H. Sphingosine kinase is induced in mouse 3T3-L1 cells and promotes adipogenesis. *J. Lipid Res.* **50**, 602–610 (2009).
630. Jin, Y., Knudsen, E., Wang, L. & Maghazachi, A. A. Lysophosphatidic acid induces human natural killer cell chemotaxis and intracellular calcium mobilization. *Eur. J. Immunol.* **33**, 2083–2089 (2003).
631. Kveberg, L., Bryceson, Y., Inngjerdigen, M., Rolstad, B. & Maghazachi, A. A. Sphingosine 1 phosphate induces the chemotaxis of human natural killer cells. Role for heterotrimeric G proteins and phosphoinositide 3 kinases. *Eur. J. Immunol.* **32**, 1856–1864 (2002).
632. Walzer, T. *et al.* Natural killer cell trafficking in vivo requires a dedicated sphingosine 1-phosphate receptor. *Nat. Immunol.* **8**, 1337–1344 (2007).
633. Zhao, Y., Sun, R., You, L., Gao, C. & Tian, Z. Expression of leptin receptors and response to leptin stimulation of human natural killer cell lines. *Biochem. Biophys. Res. Commun.* **300**, 247–252 (2003).
634. Tartaglia, L. A. *et al.* Identification and expression cloning of a leptin receptor, OB-R. *Cell* **83**, 1263–1271 (1995).
635. Baumann, H. *et al.* The full-length leptin receptor has signaling capabilities of interleukin 6-type cytokine receptors. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 8374–8378 (1996).
636. Tian, Z., Sun, R., Wei, H. & Gao, B. Impaired natural killer (NK) cell activity in leptin receptor deficient mice: leptin as a critical regulator in NK cell development and activation. *Biochem. Biophys. Res. Commun.* **298**, 297–302 (2002).
637. Mori, A. *et al.* Severe pulmonary metastasis in obese and diabetic mice. *Int. J. Cancer J. Int. Cancer* **119**, 2760–2767 (2006).
638. Lo, C. K. C. *et al.* Leptin signaling protects NK cells from apoptosis during development in mouse bone marrow. *Cell. Mol. Immunol.* **6**, 353–360 (2009).
639. Elinav, E. *et al.* Suppression of hepatocellular carcinoma growth in mice via leptin, is associated with inhibition of tumor cell growth and natural killer cell activation. *J. Hepatol.* **44**, 529–536 (2006).
640. Wrann, C. D. *et al.* Short-term and long-term leptin exposure differentially affect human natural killer cell immune functions. *Am. J. Physiol. Endocrinol. Metab.* **302**, E108–116 (2012).
641. Leptin: its pharmacokinetics and tissue distribution. *Publ. Online 30 July 1998*  
*Doi101038sjjo0800656* **22**, (1998).
642. Pang, T. T. L. & Narendran, P. The distribution of adiponectin receptors on human peripheral blood mononuclear cells. *Ann. N. Y. Acad. Sci.* **1150**, 143–145 (2008).
643. Wilk, S. *et al.* Adiponectin modulates NK-cell function. *Eur. J. Immunol.* **43**, 1024–1033 (2013).
644. Chan, A. *et al.* CD56bright human NK cells differentiate into CD56dim cells: role of contact with peripheral fibroblasts. *J. Immunol. Baltim. Md 1950* **179**, 89–94 (2007).
645. Romagnani, C. *et al.* CD56brightCD16- killer Ig-like receptor- NK cells display longer telomeres and acquire features of CD56dim NK cells upon activation. *J. Immunol. Baltim. Md 1950* **178**, 4947–4955 (2007).
646. Kim KY, Kim JK, Han SH, Lim JS, Kim KI, Cho DH, Lee MS, Lee JH, Yoon DY, Yoon SR, Chung JW, Choi I, Kim E, Yang Y. Adiponectin is a negative regulator of NK cell cytotoxicity. *J Immunol* **176**, 5958–64. (2006).
647. Han, S. *et al.* Adiponectin deficiency suppresses lymphoma growth in mice by modulating NK cells, CD8 T cells, and myeloid-derived suppressor cells. *J. Immunol. Baltim. Md 1950* **190**, 4877–4886 (2013).
648. Yamasaki, K. *et al.* Cloning and expression of the human interleukin-6 (BSF-2/IFN beta 2) receptor. *Science* **241**, 825–828 (1988).
649. Taga, T. *et al.* Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. *Cell* **58**, 573–581 (1989).
650. Hibi, M. *et al.* Molecular cloning and expression of an IL-6 signal transducer, gp130. *Cell* **63**, 1149–1157 (1990).
651. Passos, S. T. *et al.* IL-6 promotes NK cell production of IL-17 during toxoplasmosis. *J. Immunol. Baltim. Md 1950* **184**, 1776–1783 (2010).

652. Smyth, M. J. & Ortaldo, J. R. Comparison of the effect of IL-2 and IL-6 on the lytic activity of purified human peripheral blood large granular lymphocytes. *J. Immunol. Baltim. Md 1950* **146**, 1380–1384 (1991).
653. Wognum, A. W., van Gils, F. C. & Wagemaker, G. Flow cytometric detection of receptors for interleukin-6 on bone marrow and peripheral blood cells of humans and rhesus monkeys. *Blood* **81**, 2036–2043 (1993).
654. Gallagher, G., Stimson, W. H., Findlay, J. & al-Azzawi, F. Interleukin-6 enhances the induction of human lymphokine-activated killer cells. *Cancer Immunol. Immunother. CII* **31**, 49–52 (1990).
655. Klingemann, H. G. & Wong, E. Interleukin-6 does not support interleukin-2 induced generation of human lymphokine-activated killer cells. *Cancer Immunol. Immunother. CII* **33**, 395–397 (1991).
656. Kaufmann, Y. *et al.* Lymphokine-activated killer (LAK) cells: interferon-gamma synergizes with interleukin-2 to induce LAK cytotoxicity in homogeneous leukemic preparations. *Clin. Immunol. Immunopathol.* **58**, 278–288 (1991).
657. Lin, C.-Y. *et al.* Combined immunogene therapy of IL-6 and IL-15 enhances anti-tumor activity through augmented NK cytotoxicity. *Cancer Lett.* **272**, 285–295 (2008).
658. Luger, T. A. *et al.* IFN-beta 2/IL-6 augments the activity of human natural killer cells. *J. Immunol. Baltim. Md 1950* **143**, 1206–1209 (1989).
659. Smyth, M. J. *et al.* IL-2 and IL-6 synergize to augment the pore-forming protein gene expression and cytotoxic potential of human peripheral blood T cells. *J. Immunol. Baltim. Md 1950* **145**, 1159–1166 (1990).
660. Iho, S., Shau, H. Y. & Golub, S. H. Characteristics of interleukin-6-enhanced lymphokine-activated killer cell function. *Cell. Immunol.* **135**, 66–77 (1991).
661. Rabinowich, H., Sedlmayr, P., Herberman, R. B. & Whiteside, T. L. Response of human NK cells to IL-6 alterations of the cell surface phenotype, adhesion to fibronectin and laminin, and tumor necrosis factor-alpha/beta secretion. *J. Immunol. Baltim. Md 1950* **150**, 4844–4855 (1993).
662. Tanner, J. & Tosato, G. Impairment of natural killer functions by interleukin 6 increases lymphoblastoid cell tumorigenicity in athymic mice. *J. Clin. Invest.* **88**, 239–247 (1991).
663. Scheid, C. *et al.* Immune function of patients receiving recombinant human interleukin-6 (IL-6) in a phase I clinical study: induction of C-reactive protein and IgE and inhibition of natural killer and lymphokine-activated killer cell activity. *Cancer Immunol. Immunother. CII* **38**, 119–126 (1994).
664. Massagué, J. TGF-beta signal transduction. *Annu. Rev. Biochem.* **67**, 753–791 (1998).
665. Marcoe, J. P. *et al.* TGF- $\beta$  is responsible for NK cell immaturity during ontogeny and increased susceptibility to infection during mouse infancy. *Nat. Immunol.* **13**, 843–850 (2012).
666. Ortaldo, J. R. *et al.* Mechanistic studies of transforming growth factor-beta inhibition of IL-2-dependent activation of CD3- large granular lymphocyte functions. Regulation of IL-2R beta (p75) signal transduction. *J. Immunol. Baltim. Md 1950* **146**, 3791–3798 (1991).
667. Bellone, G., Aste-Amezaga, M., Trinchieri, G. & Rodeck, U. Regulation of NK cell functions by TGF-beta 1. *J. Immunol. Baltim. Md 1950* **155**, 1066–1073 (1995).
668. Hunter, C. A., Bermudez, L., Beernink, H., Waegell, W. & Remington, J. S. Transforming growth factor-beta inhibits interleukin-12-induced production of interferon-gamma by natural killer cells: a role for transforming growth factor-beta in the regulation of T cell-independent resistance to *Toxoplasma gondii*. *Eur. J. Immunol.* **25**, 994–1000 (1995).
669. Jewett, A., Cavalcanti, M. & Bonavida, B. Pivotal role of endogenous TNF-alpha in the induction of functional inactivation and apoptosis in NK cells. *J. Immunol. Baltim. Md 1950* **159**, 4815–4822 (1997).
670. Ross, M. E. & Caligiuri, M. A. Cytokine-induced apoptosis of human natural killer cells identifies a novel mechanism to regulate the innate immune response. *Blood* **89**, 910–918 (1997).
671. Baxevanis, C. N. *et al.* Elevated prostaglandin E2 production by monocytes is responsible for the depressed levels of natural killer and lymphokine-activated killer cell function in patients with breast cancer. *Cancer* **72**, 491–501 (1993).

672. Su, Y. *et al.* Cooperation of adenosine and prostaglandin E2 (PGE2) in amplification of cAMP-PKA signaling and immunosuppression. *Cancer Immunol. Immunother. CII* **57**, 1611–1623 (2008).
673. Holt, D., Ma, X., Kundu, N. & Fulton, A. Prostaglandin E(2) (PGE (2)) suppresses natural killer cell function primarily through the PGE(2) receptor EP4. *Cancer Immunol. Immunother. CII* **60**, 1577–1586 (2011).
674. Carson, W. E. *et al.* The functional characterization of interleukin-10 receptor expression on human natural killer cells. *Blood* **85**, 3577–3585 (1995).
675. Mocellin, S. *et al.* IL-10 stimulatory effects on human NK cells explored by gene profile analysis. *Genes Immun.* **5**, 621–630 (2004).
676. Cai, G., Kastelein, R. A. & Hunter, C. A. IL-10 enhances NK cell proliferation, cytotoxicity and production of IFN-gamma when combined with IL-18. *Eur. J. Immunol.* **29**, 2658–2665 (1999).
677. Lauw, F. N. *et al.* Proinflammatory effects of IL-10 during human endotoxemia. *J. Immunol. Baltim. Md 1950* **165**, 2783–2789 (2000).
678. Meyers, J. A. *et al.* Serum leptin concentrations and markers of immune function in overweight or obese postmenopausal women. *J. Endocrinol.* **199**, 51–60 (2008).
679. Samad, F., Yamamoto, K., Pandey, M. & Loskutoff, D. J. Elevated expression of transforming growth factor-beta in adipose tissue from obese mice. *Mol. Med. Camb. Mass* **3**, 37–48 (1997).
680. Alessi, M. C. *et al.* Plasminogen activator inhibitor 1, transforming growth factor-beta1, and BMI are closely associated in human adipose tissue during morbid obesity. *Diabetes* **49**, 1374–1380 (2000).
681. Porreca, E. *et al.* Transforming growth factor-beta1 levels in hypertensive patients: association with body mass index and leptin. *Am. J. Hypertens.* **15**, 759–765 (2002).
682. Fain, J. N., Tichansky, D. S. & Madan, A. K. Transforming growth factor beta1 release by human adipose tissue is enhanced in obesity. *Metabolism.* **54**, 1546–1551 (2005).
683. Curtis-Prior, P. B. Prostaglandins and obesity. *Lancet* **1**, 897–899 (1975).
684. Osborn, O., Gram, H., Zorrilla, E. P., Conti, B. & Bartfai, T. Insights into the roles of the inflammatory mediators IL-1, IL-18 and PGE2 in obesity and insulin resistance. *Swiss Med. Wkly.* **138**, 665–673 (2008).
685. Esposito, K. *et al.* Association of low interleukin-10 levels with the metabolic syndrome in obese women. *J. Clin. Endocrinol. Metab.* **88**, 1055–1058 (2003).
686. Nave, H. *et al.* Resistance of Janus kinase-2 dependent leptin signaling in natural killer (NK) cells: a novel mechanism of NK cell dysfunction in diet-induced obesity. *Endocrinology* **149**, 3370–3378 (2008).
687. Dovio, A. *et al.* Natural killer cell activity and sensitivity to positive and negative modulation in uncomplicated obese subjects: relationships to leptin and diet composition. *Int. J. Obes. Relat. Metab. Disord. J. Int. Assoc. Study Obes.* **28**, 894–901 (2004).
688. Nieman, D. C. *et al.* Influence of obesity on immune function. *J. Am. Diet. Assoc.* **99**, 294–299 (1999).
689. Lynch, L. A. *et al.* Are natural killer cells protecting the metabolically healthy obese patient? *Obes. Silver Spring Md* **17**, 601–605 (2009).
690. O’Shea D, Cawood TJ, O’Farrelly C, Lynch L. Natural killer cells in obesity: impaired function and increased susceptibility to the effects of cigarette smoke. *PLoS One* **5**, e8660 (2010).
691. Lautenbach, A., Breitmeier, D., Kuhlmann, S. & Nave, H. Human obesity reduces the number of hepatic leptin receptor (ob-R) expressing NK cells. *Endocr. Res.* **36**, 158–166 (2011).
692. Moriguchi, S., Oonishi, K., Kato, M. & Kishino, Y. Obesity is a risk factor for deteriorating cellular immune functions decreased with aging. *Nutr. Res.* **15**, 151–160 (1995).
693. Smith, A. G., Sheridan, P. A., Harp, J. B. & Beck, M. A. Diet-induced obese mice have increased mortality and altered immune responses when infected with influenza virus. *J. Nutr.* **137**, 1236–1243 (2007).
694. Lautenbach, A. *et al.* Altered phenotype of NK cells from obese rats can be normalized by transfer into lean animals. *Obes. Silver Spring Md* **17**, 1848–1855 (2009).

695. Kelley, D. S. *et al.* Energy restriction decreases number of circulating natural killer cells and serum levels of immunoglobulins in overweight women. *Eur. J. Clin. Nutr.* **48**, 9–18 (1994).
696. Scanga, C. B., Verde, T. J., Paolone, A. M., Andersen, R. E. & Wadden, T. A. Effects of weight loss and exercise training on natural killer cell activity in obese women. *Med. Sci. Sports Exerc.* **30**, 1666–1671 (1998).
697. Pekkarinen, T. & Mustajoki, P. Use of very low-calorie diet in preoperative weight loss: efficacy and safety. *Obes. Res.* **5**, 595–602 (1997).
698. Wing, E. J., Stanko, R. T., Winkelstein, A. & Adibi, S. A. Fasting-enhanced immune effector mechanisms in obese subjects. *Am. J. Med.* **75**, 91–96 (1983).
699. Tauro, B. J. *et al.* Comparison of ultracentrifugation, density gradient separation, and immunoaffinity capture methods for isolating human colon cancer cell line LIM1863-derived exosomes. *Methods San Diego Calif* **56**, 293–304 (2012).
700. Guan, H.-P. *et al.* A futile metabolic cycle activated in adipocytes by antidiabetic agents. *Nat. Med.* **8**, 1122–1128 (2002).
701. Müller, G., Jung, C., Straub, J., Wied, S. & Kramer, W. Induced release of membrane vesicles from rat adipocytes containing glycosylphosphatidylinositol-anchored microdomain and lipid droplet signalling proteins. *Cell. Signal.* **21**, 324–338 (2009).
702. Silha, J. V. *et al.* Plasma resistin, adiponectin and leptin levels in lean and obese subjects: correlations with insulin resistance. *Eur. J. Endocrinol. Eur. Fed. Endocr. Soc.* **149**, 331–335 (2003).
703. Brindley, D. N., Lin, F.-T. & Tigyi, G. J. Role of the autotaxin-lysophosphatidate axis in cancer resistance to chemotherapy and radiotherapy. *Biochim. Biophys. Acta* **1831**, 74–85 (2013).
704. Umezu-Goto, M. *et al.* Autotaxin has lysophospholipase D activity leading to tumor cell growth and motility by lysophosphatidic acid production. *J. Cell Biol.* **158**, 227–233 (2002).
705. Gesta, S. *et al.* Secretion of a lysophospholipase D activity by adipocytes: involvement in lysophosphatidic acid synthesis. *J. Lipid Res.* **43**, 904–910 (2002).
706. Hosogaya, S. *et al.* Measurement of plasma lysophosphatidic acid concentration in healthy subjects: strong correlation with lysophospholipase D activity. *Ann. Clin. Biochem.* **45**, 364–368 (2008).
707. Hla, T. Physiological and pathological actions of sphingosine 1-phosphate. *Semin. Cell Dev. Biol.* **15**, 513–520 (2004).
708. McCarty, M. F. & Whitaker, J. Manipulating tumor acidification as a cancer treatment strategy. *Altern. Med. Rev. J. Clin. Ther.* **15**, 264–272 (2010).
709. Beavis, P. A., Stagg, J., Darcy, P. K. & Smyth, M. J. CD73: a potent suppressor of antitumor immune responses. *Trends Immunol.* **33**, 231–237 (2012).
710. Potter, B. J., Sorrentino, D. & Berk, P. D. Mechanisms of Cellular Uptake of Free Fatty Acids. *Annu. Rev. Nutr.* **9**, 253–270 (1989).
711. Weisiger, R., Gollan, J. & Ockner, R. Receptor for albumin on the liver cell surface may mediate uptake of fatty acids and other albumin-bound substances. *Science* **211**, 1048–1051 (1981).
712. Lamas, B. *et al.* Leptin modulates dose-dependently the metabolic and cytolytic activities of NK-92 cells. *J. Cell. Physiol.* **228**, 1202–1209 (2013).
713. Leth-Larsen, R. *et al.* Metastasis-related plasma membrane proteins of human breast cancer cells identified by comparative quantitative mass spectrometry. *Mol. Cell. Proteomics MCP* **8**, 1436–1449 (2009).
714. Jin, D. *et al.* CD73 on tumor cells impairs antitumor T-cell responses: a novel mechanism of tumor-induced immune suppression. *Cancer Res.* **70**, 2245–2255 (2010).
715. Wang, L. *et al.* Ecto-5'-nucleotidase promotes invasion, migration and adhesion of human breast cancer cells. *J. Cancer Res. Clin. Oncol.* **134**, 365–372 (2008).
716. Loi, S. *et al.* CD73 promotes anthracycline resistance and poor prognosis in triple negative breast cancer. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 11091–11096 (2013).
717. Stagg, J. *et al.* CD73-deficient mice are resistant to carcinogenesis. *Cancer Res.* **72**, 2190–2196 (2012).

718. Yegutkin, G. G. *et al.* Altered purinergic signaling in CD73-deficient mice inhibits tumor progression. *Eur. J. Immunol.* **41**, 1231–1241 (2011).
719. Stagg, J. *et al.* CD73-deficient mice have increased antitumor immunity and are resistant to experimental metastasis. *Cancer Res.* **71**, 2892–2900 (2011).
720. Wang, L. *et al.* CD73 has distinct roles in nonhematopoietic and hematopoietic cells to promote tumor growth in mice. *J. Clin. Invest.* **121**, 2371–2382 (2011).
721. Priebe, T., Platsoucas, C. D. & Nelson, J. A. Adenosine receptors and modulation of natural killer cell activity by purine nucleosides. *Cancer Res.* **50**, 4328–4331 (1990).
722. Miller, J. S., Cervenka, T., Lund, J., Okazaki, I. J. & Moss, J. Purine metabolites suppress proliferation of human NK cells through a lineage-specific purine receptor. *J. Immunol. Baltim. Md 1950* **162**, 7376–7382 (1999).
723. Pellegatti, P. *et al.* Increased level of extracellular ATP at tumor sites: in vivo imaging with plasma membrane luciferase. *PLoS One* **3**, e2599 (2008).
724. Kim, B.-W., Choo, H.-J., Lee, J.-W., Kim, J.-H. & Ko, Y.-G. Extracellular ATP is generated by ATP synthase complex in adipocyte lipid rafts. *Exp. Mol. Med.* **36**, 476–485 (2004).
725. Paget, S. THE DISTRIBUTION OF SECONDARY GROWTHS IN CANCER OF THE BREAST. *The Lancet* **133**, 571–573 (1889).
726. Tan, J., Buache, E., Chenard, M.-P., Dali-Youcef, N. & Rio, M.-C. Adipocyte is a non-trivial, dynamic partner of breast cancer cells. *Int. J. Dev. Biol.* **55**, 851–859 (2011).
727. Hefetz-Sela, S. & Scherer, P. E. Adipocytes: Impact on tumor growth and potential sites for therapeutic intervention. *Pharmacol. Ther.* (2013). doi:10.1016/j.pharmthera.2013.01.008
728. Griner, S. E., Wang, K. J., Joshi, J. P. & Nahta, R. Mechanisms of Adipocytokine-Mediated Trastuzumab Resistance in HER2-Positive Breast Cancer Cell Lines. *Curr. Pharmacogenomics Pers. Med.* **11**, 31–41 (2013).
729. Ehsanipour, E. A. *et al.* Adipocytes cause leukemia cell resistance to L-asparaginase via release of glutamine. *Cancer Res.* **73**, 2998–3006 (2013).
730. Parekh, N., Chandran, U. & Bandera, E. V. Obesity in cancer survival. *Annu. Rev. Nutr.* **32**, 311–342 (2012).
731. Carmichael, A. R. Obesity as a risk factor for development and poor prognosis of breast cancer. *BJOG Int. J. Obstet. Gynaecol.* **113**, 1160–1166 (2006).
732. Gilbert, C. A. & Slingerland, J. M. Cytokines, obesity, and cancer: new insights on mechanisms linking obesity to cancer risk and progression. *Annu. Rev. Med.* **64**, 45–57 (2013).
733. Bochet, L. *et al.* Cancer-associated adipocytes promotes breast tumor radioresistance. *Biochem. Biophys. Res. Commun.* **411**, 102–106 (2011).
734. Sestak, I. *et al.* Effect of body mass index on recurrences in tamoxifen and anastrozole treated women: an exploratory analysis from the ATAC trial. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **28**, 3411–3415 (2010).
735. Pfeiler, G. *et al.* Impact of body mass index on the efficacy of endocrine therapy in premenopausal patients with breast cancer: an analysis of the prospective ABCSG-12 trial. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **29**, 2653–2659 (2011).
736. Gnant, M. *et al.* The predictive impact of body mass index on the efficacy of extended adjuvant endocrine treatment with anastrozole in postmenopausal patients with breast cancer: an analysis of the randomised ABCSG-6a trial. *Br. J. Cancer* **109**, 589–596 (2013).
737. Dignam, J. J. *et al.* Obesity, tamoxifen use, and outcomes in women with estrogen receptor-positive early-stage breast cancer. *J. Natl. Cancer Inst.* **95**, 1467–1476 (2003).
738. Sendur, M. A. N., Aksoy, S., Zengin, N. & Altundag, K. Efficacy of adjuvant aromatase inhibitor in hormone receptor-positive postmenopausal breast cancer patients according to the body mass index. *Br. J. Cancer* **107**, 1815–1819 (2012).
739. Crozier, J. A. *et al.* Effect of body mass index on tumor characteristics and disease-free survival in patients from the HER2-positive adjuvant trastuzumab trial N9831. *Cancer* **119**, 2447–2454 (2013).
740. Kim, J. H. *et al.* Adipocyte culture medium stimulates production of macrophage inhibitory cytokine 1 in MDA-MB-231 cells. *Cancer Lett.* **261**, 253–262 (2008).

741. Breit, S. N. *et al.* The TGF- $\beta$  superfamily cytokine, MIC-1/GDF15: a pleiotropic cytokine with roles in inflammation, cancer and metabolism. *Growth Factors Chur Switz.* **29**, 187–195 (2011).
742. Welsh, J. B. *et al.* Large-scale delineation of secreted protein biomarkers overexpressed in cancer tissue and serum. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 3410–3415 (2003).
743. Lakhal, S. *et al.* Regulation of growth differentiation factor 15 expression by intracellular iron. *Blood* **113**, 1555–1563 (2009).
744. Eling, T. E., Baek, S. J., Shim, M. & Lee, C. H. NSAID activated gene (NAG-1), a modulator of tumorigenesis. *J. Biochem. Mol. Biol.* **39**, 649–655 (2006).
745. Karan, D. Inflammation-associated regulation of the macrophage inhibitory cytokine (MIC-1) gene in prostate cancer. *Oncol. Lett.* (2012). doi:10.3892/ol.2012.635
746. Wang, X., Baek, S. J. & Eling, T. E. The diverse roles of nonsteroidal anti-inflammatory drug activated gene (NAG-1/GDF15) in cancer. *Biochem. Pharmacol.* **85**, 597–606 (2013).
747. Bauskin, A. R. *et al.* The Propeptide Mediates Formation of Stromal Stores of PROMIC-1: Role in Determining Prostate Cancer Outcome. *Cancer Res.* **65**, 2330–2336 (2005).
748. Dostálová, I. *et al.* Increased serum concentrations of macrophage inhibitory cytokine-1 in patients with obesity and type 2 diabetes mellitus: the influence of very low calorie diet. *Eur. J. Endocrinol. Eur. Fed. Endocr. Soc.* **161**, 397–404 (2009).
749. Tan, M., Wang, Y., Guan, K. & Sun, Y. PTGF-beta, a type beta transforming growth factor (TGF-beta) superfamily member, is a p53 target gene that inhibits tumor cell growth via TGF-beta signaling pathway. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 109–114 (2000).
750. Johnen, H. *et al.* Tumor-induced anorexia and weight loss are mediated by the TGF-beta superfamily cytokine MIC-1. *Nat. Med.* **13**, 1333–1340 (2007).
751. Park, Y. J., Lee, H. & Lee, J.-H. Macrophage inhibitory cytokine-1 transactivates ErbB family receptors via the activation of Src in SK-BR-3 human breast cancer cells. *BMB Rep.* **43**, 91–96 (2010).
752. Kim, K.-K., Lee, J. J., Yang, Y., You, K.-H. & Lee, J.-H. Macrophage inhibitory cytokine-1 activates AKT and ERK-1/2 via the transactivation of ErbB2 in human breast and gastric cancer cells. *Carcinogenesis* **29**, 704–712 (2008).
753. Ding, Q. *et al.* Identification of macrophage inhibitory cytokine-1 in adipose tissue and its secretion as an adipokine by human adipocytes. *Endocrinology* **150**, 1688–1696 (2009).
754. Baek, S. J., Kim, K. S., Nixon, J. B., Wilson, L. C. & Eling, T. E. Cyclooxygenase inhibitors regulate the expression of a TGF-beta superfamily member that has proapoptotic and antitumorigenic activities. *Mol. Pharmacol.* **59**, 901–908 (2001).
755. Li, P. X. *et al.* Placental transforming growth factor-beta is a downstream mediator of the growth arrest and apoptotic response of tumor cells to DNA damage and p53 overexpression. *J. Biol. Chem.* **275**, 20127–20135 (2000).
756. Lee, D. H. *et al.* Macrophage inhibitory cytokine-1 induces the invasiveness of gastric cancer cells by up-regulating the urokinase-type plasminogen activator system. *Cancer Res.* **63**, 4648–4655 (2003).
757. Martinez, J. M. *et al.* Drug-induced expression of nonsteroidal anti-inflammatory drug-activated gene/macrophage inhibitory cytokine-1/prostate-derived factor, a putative tumor suppressor, inhibits tumor growth. *J. Pharmacol. Exp. Ther.* **318**, 899–906 (2006).
758. Lambert, J. R. *et al.* Prostate derived factor in human prostate cancer cells: gene induction by vitamin D via a p53-dependent mechanism and inhibition of prostate cancer cell growth. *J. Cell. Physiol.* **208**, 566–574 (2006).
759. Albertoni, M. *et al.* Anoxia induces macrophage inhibitory cytokine-1 (MIC-1) in glioblastoma cells independently of p53 and HIF-1. *Oncogene* **21**, 4212–4219 (2002).
760. Boyle, G. M. *et al.* Macrophage inhibitory cytokine-1 is overexpressed in malignant melanoma and is associated with tumorigenicity. *J. Invest. Dermatol.* **129**, 383–391 (2009).
761. Senapati, S. *et al.* Overexpression of macrophage inhibitory cytokine-1 induces metastasis of human prostate cancer cells through the FAK-RhoA signaling pathway. *Oncogene* **29**, 1293–1302 (2010).

762. Husaini, Y. *et al.* Macrophage inhibitory cytokine-1 (MIC-1/GDF15) slows cancer development but increases metastases in TRAMP prostate cancer prone mice. *PLoS One* **7**, e43833 (2012).
763. Roth, P. *et al.* GDF-15 contributes to proliferation and immune escape of malignant gliomas. *Clin. Cancer Res.* **16**, 3851–3859 (2010).
764. Joshi, J. P., Brown, N. E., Griner, S. E. & Nahta, R. Growth differentiation factor 15 (GDF15)-mediated HER2 phosphorylation reduces trastuzumab sensitivity of HER2-overexpressing breast cancer cells. *Biochem. Pharmacol.* **82**, 1090–1099 (2011).
765. Huang, C.-Y. *et al.* Molecular alterations in prostate carcinomas that associate with in vivo exposure to chemotherapy: identification of a cytoprotective mechanism involving growth differentiation factor 15. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **13**, 5825–5833 (2007).
766. Corre, J. *et al.* Bioactivity and prognostic significance of growth differentiation factor GDF15 secreted by bone marrow mesenchymal stem cells in multiple myeloma. *Cancer Res.* **72**, 1395–1406 (2012).
767. Meyer, N. & Penn, L. Z. Reflecting on 25 years with MYC. *Nat. Rev. Cancer* **8**, 976–990 (2008).
768. Chen, Y. & Olopade, O. I. MYC in breast tumor progression. *Expert Rev. Anticancer Ther.* **8**, 1689–1698 (2008).
769. Park, K., Kwak, K., Kim, J., Lim, S. & Han, S. c-myc amplification is associated with HER2 amplification and closely linked with cell proliferation in tissue microarray of nonselected breast cancers. *Hum. Pathol.* **36**, 634–639 (2005).
770. Meyer, N., Kim, S. S. & Penn, L. Z. The Oscar-worthy role of Myc in apoptosis. *Semin. Cancer Biol.* **16**, 275–287 (2006).
771. Zudaire, E., Martínez, A. & Cuttitta, F. Adrenomedullin and cancer. *Regul. Pept.* **112**, 175–183 (2003).
772. Oehler, M. K., Norbury, C., Hague, S., Rees, M. C. & Bicknell, R. Adrenomedullin inhibits hypoxic cell death by upregulation of Bcl-2 in endometrial cancer cells: a possible promotion mechanism for tumour growth. *Oncogene* **20**, 2937–2945 (2001).
773. Martínez, A. *et al.* The effects of adrenomedullin overexpression in breast tumor cells. *J. Natl. Cancer Inst.* **94**, 1226–1237 (2002).
774. Abasolo, I., Montuenga, L. M. & Calvo, A. Adrenomedullin prevents apoptosis in prostate cancer cells. *Regul. Pept.* **133**, 115–122 (2006).
775. Adamson, E. D. & Mercola, D. Egr1 transcription factor: multiple roles in prostate tumor cell growth and survival. *Tumour Biol. J. Int. Soc. Oncodevelopmental Biol. Med.* **23**, 93–102 (2002).
776. Huang, R. P., Liu, C., Fan, Y., Mercola, D. & Adamson, E. D. Egr-1 negatively regulates human tumor cell growth via the DNA-binding domain. *Cancer Res.* **55**, 5054–5062 (1995).
777. Huang, R. P. *et al.* Decreased Egr-1 expression in human, mouse and rat mammary cells and tissues correlates with tumor formation. *Int. J. Cancer J. Int. Cancer* **72**, 102–109 (1997).
778. Virolle, T. *et al.* Egr1 promotes growth and survival of prostate cancer cells. Identification of novel Egr1 target genes. *J. Biol. Chem.* **278**, 11802–11810 (2003).
779. DelaRosa, O. *et al.* Human adipose-derived stem cells impair natural killer cell function and exhibit low susceptibility to natural killer-mediated lysis. *Stem Cells Dev.* **21**, 1333–1343 (2012).
780. Sotiropoulou, P. A., Perez, S. A., Gritzapis, A. D., Baxevanis, C. N. & Papamichail, M. Interactions between human mesenchymal stem cells and natural killer cells. *Stem Cells Dayt. Ohio* **24**, 74–85 (2006).
781. Spaggiari, G. M. *et al.* Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. *Blood* **111**, 1327–1333 (2008).
782. Ljujic, B. *et al.* Human mesenchymal stem cells creating an immunosuppressive environment and promote breast cancer in mice. *Sci. Rep.* **3**, 2298 (2013).
783. Zvonic, S. *et al.* Secretome of primary cultures of human adipose-derived stem cells: modulation of serpins by adipogenesis. *Mol. Cell. Proteomics MCP* **6**, 18–28 (2007).
784. Blüher, M. Vaspin in obesity and diabetes: pathophysiological and clinical significance. *Endocrine* **41**, 176–182 (2012).

785. Spychala, J. *et al.* Role of estrogen receptor in the regulation of ecto-5'-nucleotidase and adenosine in breast cancer. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **10**, 708–717 (2004).
786. Krüger, K. H., Thompson, L. F., Kaufmann, M. & Möller, P. Expression of ecto-5'-nucleotidase (CD73) in normal mammary gland and in breast carcinoma. *Br. J. Cancer* **63**, 114–118 (1991).
787. Bochet, L. *et al.* Adipocyte-derived fibroblasts promote tumor progression and contribute to the desmoplastic reaction in breast cancer. *Cancer Res.* **73**, 5657–5668 (2013).
788. Clayton, A., Al-Taei, S., Webber, J., Mason, M. D. & Tabi, Z. Cancer exosomes express CD39 and CD73, which suppress T cells through adenosine production. *J. Immunol. Baltim. Md 1950* **187**, 676–683 (2011).
789. Müller, G., Jung, C., Wied, S., Biemer-Daub, G. & Frick, W. Transfer of the glycosylphosphatidylinositol-anchored 5'-nucleotidase CD73 from adiposomes into rat adipocytes stimulates lipid synthesis. *Br. J. Pharmacol.* **160**, 878–891 (2010).
790. Colgan, S. P., Eltzschig, H. K., Eckle, T. & Thompson, L. F. Physiological roles for ecto-5'-nucleotidase (CD73). *Purinergic Signal.* **2**, 351–360 (2006).
791. Terp, M. G. *et al.* Anti-Human CD73 Monoclonal Antibody Inhibits Metastasis Formation in Human Breast Cancer by Inducing Clustering and Internalization of CD73 Expressed on the Surface of Cancer Cells. *J. Immunol. Baltim. Md 1950* **191**, 4165–4173 (2013).



# ANNEX

**Preclinical activity of the type II CD20 antibody GA101 (obinutuzumab) compared with rituximab and ofatumumab in vitro and in xenograft models.**

Herter S, Herting F, Mundigl O, Waldhauer I, Weinzierl T, Fauti T, Muth G, Ziegler-Landesberger D, Van Puijenbroek E, Lang S, **Duong MN**, Reslan L, Gerdes CA, Friess T, Baer U, Burtscher H, Weidner M, Dumontet C, Umama P, Niederfellner G, Bacac M, Klein C.

**Abstract**

We report the first preclinical in vitro and in vivo comparison of GA101 (obinutuzumab), a novel glycoengineered type II CD20 monoclonal antibody, with rituximab and ofatumumab, the two currently approved type I CD20 antibodies. The three antibodies were compared in assays measuring direct cell death (AnnexinV/PI staining and time-lapse microscopy), complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP), and internalization. The models used for the comparison of their activity in vivo were SU-DHL4 and RL xenografts. GA101 was found to be superior to rituximab and ofatumumab in the induction of direct cell death (independent of mechanical manipulation required for cell aggregate disruption formed by antibody treatment), whereas it was 10 to 1,000 times less potent in mediating CDC. GA101 showed superior activity to rituximab and ofatumumab in ADCC and whole-blood B-cell depletion assays, and was comparable with these two in ADCP. GA101 also showed slower internalization rate upon binding to CD20 than rituximab and ofatumumab. In vivo, GA101 induced a strong antitumor effect, including complete tumor remission in the SU-DHL4 model and overall superior efficacy compared with both rituximab and ofatumumab. When rituximab-pretreated animals were used, second-line treatment with GA101 was still able to control tumor progression, whereas tumors escaped rituximab treatment. Taken together, the preclinical data show that the glycoengineered type II CD20 antibody GA101 is differentiated from the two approved type I CD20 antibodies rituximab and ofatumumab by its overall preclinical activity, further supporting its clinical investigation.

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## **BACKGROUND DESKTOP**

### ***1<sup>st</sup> year of PhD study:***

There will come a day when you'll feel like shit and you'll want to give up on this life and everything else.

When that day arrives, save yourself some trouble and don't do a thing.

Just take a nap.

### ***2<sup>nd</sup> year of PhD study:***

Do my best. Make the I of today different from the I of yesterday.

“Remembering that you are going to die is the best way I know to avoid the trap of thinking you have something to lose. You are already naked. There is no reason not to follow your heart.”

### ***3<sup>th</sup> year of PhD study:***

Take your time,

think a lot,

think of everything you've got.

For you will still be here tomorrow,

But your dream may not.

Here we are we've just begun

And after all this time our time has come

Ya here we are still going strong

Right here in the place where we belong!

I was here...

I lived, I loved

I was here...

I did, I've done, everything that I wanted

And it was more than I thought it would be

I will leave my mark so everyone will know

I was here.





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**TITLE****Roles of adipocytes in the resistance of breast cancer to trastuzumab-mediated antibody-dependent cellular cytotoxicity (ADCC)**

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**Abstract**

Trastuzumab is a monoclonal antibody already approved in the treatment of HER2-expressing breast cancer. Despite its efficacy, resistance often occurs. Here, we investigated the impact of adipocytes on antibody-dependent cellular cytotoxicity (ADCC), one of the main mechanisms of action of trastuzumab. We found that adipocytes, as well as preadipocytes, inhibited trastuzumab-mediated ADCC. We showed that adipocyte-derived factors, likely proteins or exosomes, mediated the inhibition of ADCC. No titration or degradation of the antibody was detected. Analysis of cell phenotype did not reveal any modification of NK cell receptors, nor of HER2 levels on cancer cells in the presence of adipocytes. Pre-incubation of cancer cells with adipocyte-conditioned medium reduced sensitivity of cancer cells to ADCC. We found that growth differentiation factor 15 (GDF15) was rapidly induced in cancer cells exposed to adipocyte-conditioned medium. Down-regulation of GDF15 by siRNA reversed the adipocyte-induced inhibition of ADCC. In conclusion, we demonstrated that adipocytes play a role in the resistance of breast cancer to trastuzumab-mediated ADCC, and suggested that targeting GDF15 or the crosstalk between adipocytes and cancer cells may sensitize cancer cells to monoclonal antibody treatment.

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**TITRE****Rôle des adipocytes dans la résistance des cellules de cancer du sein à la cytotoxicité cellulaire dépendante de l'anticorps (ADCC) médiée par le trastuzumab.**

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**Résumé**

Le trastuzumab est un anticorps monoclonal déjà utilisé dans le traitement du cancer du sein sur-exprimant la protéine HER2. Malgré son efficacité, la résistance est souvent apparue. Ici, nous avons étudié l'impact des cellules adipocytaires sur la cytotoxicité cellulaire dépendante de l'anticorps (ADCC), une des mécanismes d'action principaux du trastuzumab. Nous avons trouvé que les adipocytes, ainsi que les pré-adipocytes, inhibent l'ADCC médié par le trastuzumab. Nous avons montré que les facteurs dérivés d'adipocytes, comme des protéines ou des exosomes, causent l'inhibition d'ADCC. Aucune séquestration ou dégradation de l'anticorps a été observée. Des analyses phénotypiques n'ont pas révélé de modification des récepteurs des cellules NK, ni du niveau de HER2 sur les cellules cancéreuses en présence d'adipocytes. La pré-incubation des cellules cancéreuses avec le surnageant des adipocytes réduit la sensibilité tumorale à l'ADCC. Nous avons trouvé que le facteur de croissance et de différenciation GDF15 est rapidement induit dans les cellules cancéreuses exposées au surnageant d'adipocytes. Une diminution de l'expression de GDF15 par les siRNA réverse l'inhibition d'ADCC induite par les adipocytes. En conclusion, nous avons démontré que les adipocytes jouent un rôle dans la résistance des cellules de cancer du sein à l'ADCC médié par le trastuzumab, et nous suggérons que cibler GDF15 ou l'interaction entre les adipocytes et les cellules cancéreuses pourrait sensibiliser les cellules cancéreuses au traitement par l'anticorps monoclonal.

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DISCIPLINE: Cancérologie

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**KEYWORDS / MOTS-CLES**

Breast cancer, trastuzumab, adipocytes, GDF15, ADCC

Cancer du sein, trastuzumab, adipocytes, GDF15, ADCC

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**INTITULE ET ADRESSE DE L'U.F.R OU DU LABORATOIRE**

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