UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA DE LISBOA

GENETIC, MOLECULAR AND CELLULAR DETERMINANTS OF THE CAUSAL ASSOCIATION BETWEEN OBESITY AND HODGKIN LYMPHOMA

Andreia Isabel Lucas de Matos

Orientador: Professor Doutor Manuel Diamantino Pires Bicho

Co-Orientador: Prof. Doutor Ricardo Jorge Teixeira Ribeiro

Dissertação especialmente elaborada para obtenção do grau de Mestre em Doenças Metabólicas e Comportamento Alimentar

2015

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Orientador

Professor Doutor Manuel Diamantino Pires Bicho
Professor Catedrático
Laboratório de Genética
Faculdade de Medicina da Universidade de Lisboa

Co – Orientador

Prof. Doutor Ricardo Jorge Teixeira Ribeiro
Investigador
Instituto Português de Oncologia, Porto

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You cannot hope to build a better world without improving the individuals. To that end each of us must work for its own improvement and at the same time-share a general responsibility for all humanity, our particular duty being to aid those to whom we think we can be most useful.

Marie Curie

Physicist & Chemist

A todos os que acreditaram que era possível.

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Um lugar pro coração pousar
Um endereço que frequente sem morar
Ali na esquina do sonho com a razão
No centro do peito, no largo da ilusão
Marisa Monte
Cantinho Escondido


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CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ABVD	Adriamycin/Bleomycin/Vinblastine/Dacarbazine
AdipoQ	Adiponectin
AKT	Akt kinase
ALS	Acid labile subunit
BM	Bone marrow
BMI	Body mass index
C/EBPβ	CCAAT/enhancer binding protein beta
CCL17	Chemokine (C-C motif) ligand 17
CCL22	Chemokine (C-C motif) ligand 22
CCL3	Chemokine (C-C motif) ligand 3
CCL5	Chemokine (C-C motif) ligand 5
CD163	Cluster of Differentiation 163
CD30	Cluster of Differentiation 30
CD40	Cluster of Differentiation 40
CD68+	Cluster of Differentiation 68
CD95L	Fas Ligand
CD95	Fas receptor
CD99	Cluster of Differentiation 99
CRP	C-reactive protein
CSS	Cause-specific survival
CT	Control
CTLs	Cytotoxic T lymphocytes
CTSK	Cathepsin K
DEXA	Dual-energy X-ray absorptiometry
DKK-1	Dickkopf-related protein 1
EBV	Epstein-Barr Virus
ECM	Extracellular matrix
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
FABP4	Fatty acid biding protein 4

Fas	Fatty acids
FFA	Free-fatty acids
FGF	Fibroblast growth factor
FGF-6	Fibroblast growth factor 6
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia inducible factor 1 alfa
HL	Hodgkin Lymphoma
Hp	Haptoglobin
HRP-Streptavidin	Horseradish peroxidase-Streptavidin
HRS	Hodgkin Reed-Sternberg cell
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin growth factor receptor 1
IGFBP-1	Insulin-like growth factor-binding protein 1
IGFBP-2	Insulin-like growth factor-binding protein 2
IGFBP-3	Insulin-like growth factor-binding protein 3
IL-1	Interleukin 1
IL-10	Interleukin 10
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-1α	Interleukin 1 alfa
IL-1β	Interleukin 1 beta
IL-23	Interleukin 23
IL-2R	Interleukin 2 receptor
IL-3	Interleukin 3
IL-3	Interleukin 3
IL-3R	Interleukin 3 receptor
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-6R	Interleukin 6 receptor
IL-7	Interleukin 7
IL-8	Interleukin 8
IL-9	Interleukin 9

IL-9R	Interleukin 9 receptor
IMF	Interstitial marrow fluid
iNOS	Inducible nitric oxide
IPS	International prognostic score
IQR	Interquartile range
JAK	Janus kinase
JAK/STAT	Janus Kinase/signal transducer and activator of transcription
LepR	Leptin receptor
LOX	Lysyl oxidase
M	Median age
M-CSF	Macrophage colony stimulating
M-CSFR	Macrophage colony-stimulating factor receptor
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MDS	Macrophage-derived chemokine
MHCI/II	Major histocompatibility complex class I/II
MIP-1a/CCL3	Macrophage Inflammatory Protein 1alpha
MIP-1b/CCL4	Macrophage Inflammatory Protein 1beta
MMP	Matrix metalloproteinase
MMP9	Matrix metalloproteinase 9
MSCs	Mesenchymal stem cells
mTOR	Mammalian target of rapamycin
NF-IL6	A nuclear factor for IL-6 expression
NF-kB	Nuclear factor kappa B
NGF	Nerve growth factor
NK cells	Natural Killer cells
NW	Normal weight
OB	Obese
OPG	Osteoprotegerin
OPN	Osteopontin
OW	Overweight
PAI-1	Plasminogen activator inhibitor-1
PGE2	Prostaglandin E2

PI3K	Phosphatidylinositol 3'-kinase
PIGF	Placental growth factor
PPARγ	Peroxisome proliferator-activated receptor gamma
PPARγ2	Peroxisome proliferator-activated receptor gamma 2
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RANTES (CCL5)	Regulated on activation, normal T cell expressed and secreted
ROS	Reactive oxygen species;
SDF-1	Stromal derived factor 1
SHBG	Sex hormone binding globulin
SPARC	Osteonectin
STAT	Signal transducer and activator of transcription
STAT3	Signal transducer and activator of transcription 3
TARC	Thymus and activation-regulated chemokine
TGFβ	Transforming growth factor beta
Th2	T helper cells
TNF-a	Tumor necrosis factor alpha
TNF-β	Tumor necrosis factor beta
TNFR1	Tumor necrosis factor receptor 1
TNFR2	Tumor necrosis factor receptor 2
Treg	Regulatory T cell
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
WHO	World Heath Organization
YKL-40	Chitinase-3-like protein 1

RESUMO

A obesidade tem sido descrita como uma causa de morbidade e mortalidade e estima-se que o número de mortes provocadas por cancro duplique até 2030, como resultado da epidemia de obesidade. A associação entre obesidade e vários tipos de cancro tem sido consistentemente estabelecida. No Linfoma de Hodgkin (LH), apesar de uma associação aparentemente variável para excesso de peso e obesidade (de acordo com a classificação da Organização Mundial de Saúde (OMS), baseado no índice de massa corporal), uma meta-análise contemporânea em estudos prospectivos estabeleceu uma associação significativa entre o índice de massa corporal (IMC) e risco para LH. Concordantemente, um recente estudo prospectivo em mais de 1 milhão de indivíduos demonstraram maior risco para LH em cada 10 $kg.m^{-2}$ unidades de aumento do IMC. Mesmo que a obesidade parece estar epidemiologicamente associada ao LH, a lógica biológica e mecanismos por de trás dessa relação causal permanecem em grande parte inexplicável.

Cerca de 20% dos pacientes com LH morrem após recidiva ou progressão da doença. O LH é uma neoplasia linfóide derivada das células B, caracterizada pela presença de células malignas Hodgkin / Reed-Sternberg (HRS), em gânglios linfáticos, que ocasionalmente metastizam para a medula óssea. O LH apresenta a peculiaridade de que é o único entre vários tipos de cancro em que as células malignas são muito ultrapassados por células reativas e do estroma, e portanto, compostas por um microambiente tumoral altamente modulado pela interação intercelular.

O excesso de adiposidade correlaciona-se com o aumento da produção de pró-adipoquinas tumorais e pró-angiogénicos e é caracterizado por um estado inflamatório crónico moderado. A nossa hipótese é que uma vez que a inflamação é um pilar na fisiopatologia do LH, o papel do tecido adiposo, seja através do sistema endócrino ou mecanismos parácrinos e tanto no seu local primário como na medula óssea, deve ser considerado. Para este efeito, foram medidas adipoquinas na medula óssea em casos com LH e controlos de forma a tentar revelar o efeito resultante da interação do microambiente da medula, que inclui as células HRS.

RESUMO

Durante 12 meses, 16 novos casos de LH elegíveis para biopsia de medula óssea e 11 controlos normais compatíveis para a idade, género e IMC, foram incluídos neste estudo. Concomitantemente, também foram obtidas amostras de sangue periférico. Uma membrana de matriz de adipocinas (Raybiotech) foi utilizada para determinar simultaneamente 62 moléculas desreguladas no líquido intersticial medular (LIM) em indivíduos com LH e com excesso de peso / obesidade (EP / OB). Em seguida, as conclusões do LIM foram validadas utilizando ELISA (Raybiotech), e correlacionado com os níveis circulantes de adipocinas.

A osteoprotegerina esteve significativamente sobre-expressa no LIM de indivíduos com EP / OB comparativamente a indivíduos com IMC normal. No LIM de LH, diversas adipoquinas estiveram significativamente sub-expressas, nomeadamente, as proteínas do eixo do factor de crescimento da insulina (IGF), fatores de crescimento, proteínas envolvidas em redes reguladoras de imuno-inflamação e vias de remodelação óssea. De realçar, as proteínas de ligação ao IGF, a IGFBP-3 (6.4- vezes) e IGFBP-1 (13.2- vezes), que estiveram sub-expressas em pacientes com LH independentemente do estado de obesidade. A expressão de adipoquinas inflamatórias, interleucina (IL) -8, IL-1 alfa (a), IL-12 e IL-1 beta (b), esteve diminuída (4.5- a 7.8- vezes) no LIM de LH, tal como, fatores de crescimento e hormonas, factor de crescimento transformante beta (TGF-β1) (5.2 vezes), o factor de crescimento de fibroblastos (FGF)-6 (7.6 vezes) e a leptina (12.6 vezes). A comparação de subgrupos extremos, LH simultaneamente EP / OB (n = 4) versus controlos com IMC normal (n = 4), corroborou o envolvimento potencial do eixo IGF na associação obesidade-LH. IGFBP-1, IGFBP-3 e de IGFBP-2 estiveram significativamente reduzidos (3.2- a 14.6- vezes) no LIM de pacientes com LH que tinham EP / OB. As adipoquinas inflamatórias IL-8, IL-1b e IL-12, estiveram correspondentemente reduzidas (3.9- a 8,0 vezes) nesse grupo.

Embora o IMC esteja bem correlacionado com perímetro abdominal, somente o perímetro abdominal esteve inversamente associado com IGFBP-3 e com os níveis de IGFBP-1 no LIM ($r = -0.416$, $P = 0.039$ e $r = -0.473$, $P = 0.017$, respectivamente).

Relativamente, aos parâmetros clínicos, uma forte tendência foi encontrada para baixos valores de IL-8 no LIM de pacientes com LH e

sintomas B (com sintomas, média 0.02±0.004 pg/mL/μg, e sem sintomas, media 0.05±0.01 pg/mL/μg, P=0.052)

Uma boa correlação foi encontrada para os níveis de adipoquinas entre LIM e soro.

Este estudo abordou a associação causal da obesidade e do LH a partir da perspectiva do microambiente da medula óssea. A sua natureza exploratória em uma amostra biológica rara solicitou a utilização de uma matriz para identificar a desregulação de proteínas no LIM.

Os resultados deste estudo piloto sugerem que as vias de adipocinas podem estar envolvidas na fisiopatologia do LH (nomeadamente o eixo IGF, alguns fatores de crescimento e hormonas, e marcadores inflamatórios), melhorando ainda mais a nossa compreensão da associação biológica da obesidade e o LH. O potencial efeito protetor da obesidade sobre microambiente da medula óssea do Linfoma de Hodgkin aplica-se exclusivamente ao prognóstico, tornando a associação obesidade-Linfoma de Hodgkin outro caso paradoxal da influência protetora da obesidade no cancro.

Por outro lado, no presente estudo e tal como a maioria dos estudos dependem de medidas de IMC e classificação de obesidade segundo a OMS, no entanto, são estimativas imperfeitas de adiposidade e risco de doença, respectivamente. Estes métodos usados para estratificar o estado de obesidade do indivíduo não abrangem determinados depósitos de gordura localizados (por exemplo, tecido adiposo visceral), tendo estes, perfis de expressão de adipocinas específicos, contribuindo para tumores mais agressivos. Estudos epidemiológicos futuros devem abordar a composição corporal através de métodos mais precisos (por exemplo determinações de gordura visceral e subcutânea por tomografia computadorizada, gordura de todo o corpo por bioimpedância tetrapolar ou medições de gordura corporal locais mais específicos por meio de ressonância magnética, entre outros) para avaliar o distribuição de gordura num todo e a local em associação com LH.

Em geral, os resultados deste estudo estão de acordo com um número muito limitado de relatórios sobre a obesidade como um fator de bom prognóstico para LH, embora em contraste com a evidência estabelecida de obesidade como fator de risco para o desenvolvimento de LH. Tomados em conjunto, um baixo perfil pró-inflamatório, juntamente com fatores de

crescimento diminuídos e leptina, e com o eixo IGF sobre-activado, encontramos na medula óssea de pacientes obesos HL são as principais características de um ambiente desfavorável para o início da metastização. Na obesidade, esperamos revelar que essas adipocinas são produzidos pelos adipócitos na medula óssea e para demonstrar que pode interferir com microambiente metastático no LH.

Desta forma, os resultados aqui apresentados são esperados para promover novos esforços de pesquisa.

Palavras – chave: Obesidade, adipócitos da medula, Linfoma de Hodgkin, microambiente

ABSTRACT

Inflammation is a cornerstone in Hodgkin's Lymphoma (HL) pathophysiology, so, the role of adipose tissue, either through endocrine or paracrine mechanisms and both at its primary site and in bone marrow, must be of importance.

During 12 months, 16 new cases of HL eligible for bone marrow biopsy and 11 age-, gender- and body mass index (BMI)-matched normal controls were included in this study; matched samples of peripheral blood were also obtained. An adipokine antibody array was used to determine deregulated molecules in interstitial marrow fluid (IMF) of HLs and overweigth/obese (OW/OB) subjects. Findings in IMF were validated using ELISA, and correlated with circulating adipokine levels. Osteoprotegerin was significantly overexpressed in the IMF of OW/OB subjects compared with normal BMI. IGFBP-3 (6.4-fold) and IGFBP-1 (13.2-fold) were underexpressed in HL patients independent of obesity status. The expression of interleukin (IL)-8, IL-1 alpha (a), IL-12, and IL-1 beta (b), was depressed (4.5- to 7.8-fold) in the IMF of HLs; and the transforming growth factor (TGF)-β1 (5.2-fold), fibroblast growth factor (FGF)-6 (7.6-fold) and leptin (12.6-fold). The comparison of utmost subgroups, HLs simultaneously OW/OB (n=4) versus controls with normal BMI (n=4), corroborated the potential involvement of IGF axis in the association obesity-HL. IGFBP-1, IGFBP-3 and IGFBP-2 were significantly reduced (3.2- to 14.6-fold) in the IMF of HL patients who were OW/OB. The inflammatory adipokines IL-8, IL-1b and IL-12, were correspondingly reduced (3.9- to 8.0-fold) in this group. The abdominal perimeter was only inversely associated with IGFBP-3 and with IGFBP-1 levels in IMF (r=-0.416, P=0.039 and r=-0.473, P=0.017, respectively). A strong trend was found towards lower IL-8 levels in the IMF of HL patients with B symptoms. A good correlation was found for adipokine levels between IMF and serum.

Findings from this pilot study suggest that adipokine pathways might be involved in HL pathophysiology.

Key - words: Obesity, bone marrow adipocytes, Hodgkin´s lymphoma, microenvironment

LITERATURE REVIEW

1. LITERATURE REVIEW

1.1. Introduction overview

Obesity has been described as a cause of morbidity and mortality and it is estimated that the number of deaths caused by cancer will double by 2030, as a result of the obesity epidemic [1]. The association between obesity and many cancer types has been consistently established [2]. In Hodgkin's Lymphoma (HL), despite an apparently variable association with overweight and obesity (according to the body mass index-based World Health Organization classification) [3-7], a contemporary meta-analysis on prospective studies established a significant association between body mass index (BMI) and risk for HL [8]. Concordantly, a recent prospective study in over 1 million individuals demonstrated increased risk for HL each 10 $Kg.m^{-2}$ units of increase in BMI [9]. Even though obesity seems to be epidemiologically associated with HL, the biological rationale and mechanisms behind this causal relationship remain largely unexplained.

HL is characterized by an inflammatory microenvironment at the tumor site in lymph nodes [10, 11]. The distinctive HL's malignant Hodgkin & Reed-Sternberg (HRS) cells reciprocally interact with the inflammatory milieu resulting in tumors with survival and evasion advantages [10]. Besides age, inflammation and Epstein-Barr virus (EBV), little is known concerning predictive factors of HL aggressiveness [12, 13]. Noteworthy, despite the high cure rate, in advanced stage disease approximately 25% to 30% of patients are not cured with standard therapeutic regimens alone, and about 20% of patients still die after relapse or disease progression [12, 14]. In advanced stages, where bone marrow infiltration is common, the international prognostic score and other risk stratification instruments remain only modestly effective to predict disease progression and therapeutic response [15].

Excess adiposity is regarded as a chronic inflammatory state, hence it is postulated that excess adiposity-mediated inflammation may play a role in promoting HL growth, survival and tumor immune evasion. We hypothesize that this accumulation of immune cells in HL, including T and B cells, neutrophils, eosinophils, and mast cells might be disturbed by excess adiposity, which is

known to modulate the immune system function [16, 17]. In addition to immunoinflammatory derangements, there is a systemic metabolic dysfunction in obesity that prompts altered circulating levels of pro-tumoral adipokines. Indeed, some of these adipokines have been associated with HL and advanced stages of disease [18-20]. Albeit the mechanisms of the causally invoked association between obesity and HL remain undetermined, a reasonable biological rationale supports plausibility for the relation of adipose tissue-inflammation-HRS axis with HL aggressiveness. Taken together these obesity-linked endocrine and paracrine mechanisms may contribute towards a favorable microenvironment for growth and spread of HRS malignant cells. Moreover, albeit still experimentally unproven, it is expected that the known paracrine action of adipocytes in the bone marrow microenvironment [21, 22] might facilitate HRS cells to metastasize and grow in the bone marrow.

Taken together, these considerations suggest that research should establish definite biological mechanisms involved in the association between excess adiposity and HL, which will foster the development of new molecular markers based on adipokine's pathways as indicators of clinical outcome and incorporated into prediction models. If confirmed, the effects of bone marrow adipocytes in metastatic HL cells might represent new potential therapeutic targets in this advanced stage of disease.

1.2. Mechanisms promoting Hodgkin Lymphoma's

HRS cells, despite their small representativeness in HLs (about 1%), are modulated by a mixed inflammatory microenvironment that influences tumor development [10, 23]. These unique features of HL are far outweighed by reactive and stromal cells [10]. Inflammation, among others, has been recently considered as a cancer hallmark [24]. In fact, survival, proliferation and immunoinflammatory mechanisms seem to have an important role in HL pathophysiology [10].

The HL tumor microenvironment, in particular, the cytokine/chemokine pattern secreted by HRS cells and non-neoplastic circulating cells may influence the proliferation and survival of malignant cells [25, 26]. Among

others, HRS cells secrete several interleukins (ILs) (e.g., IL-1α, IL-4, IL-6, IL-7, IL-8, IL-9, IL-10, IL-13), chemokines (thymus- and activation-regulated chemokine CCL17, TARC, and CCL22, MDC), macrophage colony stimulating factor (M-CSF), tumor necrosis factor- α (TNF-α) and β (TNF-β), transforming growth factor beta (TGF-β), soluble CD30 and chitinase-3-like protein 1 (YKL-40), with autocrine tumor growth effect [25]. Among these, thymus and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC), produced by HRS cells, are partially responsible for whittling HL microenvironment by inducing Th2 and Treg cells [25], which have been associated with reduced tumor immunosurveillance [27]. Moreover, paracrine actions were uncovered through upregulated pathways mediated by cytokine receptors (IL-2R, IL-6R, IL-9R, IL3R), macrophage colony-stimulating factor receptor (M-CSFR), tumor necrosis factor receptors (TNFR1, TNFR2), CD30 and CD40 [28]. Downstream signaling through the IL-3/IL3R pathway induces growth and extends survival in HL cells [29]. Paracrine signals may arise from non-malignant tumor-infiltrating cells in HL microenvironment (e.g., eosinophils, mast cells, neutrophils and macrophages) [10]. The nuclear factor-kappa B (NF-kB) and Janus Kinase-signal transducer and activator of transcription (JAK-STAT) pathways have been identified as important modulators in HL [26]. Activation of the NF-kB pathway, which is involved in the expression of multiple anti-apoptotic factors and pro-inflammatory cytokines, reduces the expression of CD99, a marker associated with HRS cells phenotype

Some authors identified circulating proteins as candidate biomarkers for HL, which demonstrated prognostic value and accuracy to predict the response to therapy. These molecules act at HL site and modulate the outcome [30-32]. A recent report showed increased levels of serum interleukin 6 (IL-6) and YKL-40 at diagnosis, which were correlated with stage of disease [18].

1.3. Obesity and Hodgkin Lymphoma

Obesity is currently considered epidemic worldwide [33]. Overweight and obesity are defined as excessive accumulation of adipose tissue and are associated with increased risk of morbidity and premature mortality. Besides other downstream health consequences, epidemiological studies reported

obesity as a risk factor for cancer [2, 34, 35]. In the United States of America the estimated risk of death from cancer in morbidly obese (BMI ≥ 40 kg/m^2) was 1.5 in men and 1.6 in women [34], whereas in Europe, obese have 1.5 to 3.5 higher risk of having cancer [1]. Alongside, it has been estimated that approximately 30% of cancer deaths might be related with dietary and behavioural factors, namely, high BMI, low intake of fruits and vegetables, lack of exercise, smoking and alcohol abuse. A recent meta-analysis based on prospective studies reported that obesity (BMI ≥ 30 kg/m^2) was positively correlated with risk of HL [8]. Indeed, a number of studies reported a positive relation of obesity with hematologic malignancies, including Hodgkin's lymphoma [3-7, 9, 35], even though others have not found such an association [36-42] (Table 1). Noteworthy, 2 studies found a protective role for obesity in the development of HL. Despite the batch of positive association studies between obesity and HL, it is important to highlight the time when the anthropometric assessments were made and the outcomes considered. Indeed, some authors consider that this potential protective effect of lower BMI in cancer may be due to the effects of cancer-related cachexia, a more deleterious than the potential adverse events related to a higher BMI [43].

Obesity may increase the risk for developing HL, even though its effects in survivors might be paradoxal, thus influencing the natural history of disease.

Table 1: Association of obesity (defined by BMI) with risk for Hodgkin's Lymphoma.

Year	Ref.	Population	Obesity and HL risk	Outcome	Risk Factors
2001	Wolk et al.,	Sweden (n=28129 HL)	YES	RISK	BMI ≥30 Kg/m^2 and especially in Men.
2006	Willet et al.,	United Kingdom (n=216 HL/216 CT)	YES	RISK	BMI ≥30 Kg/, especially in Men and among older.
2007	Engeland et al.,	Norway (n=1224 HL)	YES	RISK	BMI ≥30 Kg/m^2.
2013	Murphy et al.,	United Kingdom (n=9162 HL)	YES	RISK	↑10 Kg/m^2 in BMI.
2013	Li et al.,	USA (n=567 HLs/679 C)	YES	RISK	Women with BMI 25.0-29.9 Kg/m^2 and <35 years old.
2005	Landgren et al.,	Stockholm (n=301 HL)	NO	PROTECTIVE	↑BMI: Better prognosis risk profile and improved CSS.
2014	Hong et al.,	USA (n=794HL)	NO	PROTECTIVE	↑ BMI: Favorable prognosis.
2005	Oh SW et al.,	Koreans (n=31 HLs: 21NW, n=10 OW)	NO	NO ASSOCIATION	-
2005	Chang E et al.,	Scandinavian Men and Women (n=413HL Younger; n=205 HL Older)	NO	NO ASSOCIATION	Younger HL patients had lower BMI than controls.
2006	Samanic et al.,	Swedish Men (n=201HL: n=134NW, n=61OW, n=16OB)	NO	NO ASSOCIATION	-
2007	Lim et al.	USA (n=57HL: n=20NW, n=23OW, n=14OB)	NO	NO ASSOCIATION	-
2009	Söderbeg KC et al.,	Swedish and Finnish (n=32HL: n=21NW, n=11OW)	NO	NO ASSOCIATION	An indication in the younger cohort with OW.

HL, Hodgkin Lymphoma; CT, Control; BMI, body mass index; NW, normal weight; OB, obese; OW, overweight; CSS, cause-specific survival.

Most studies rely on body mass index measures and World Health Organization classification of obesity, although BMI and WHO cutoffs are imperfect estimates of adiposity and disease risk, respectively [44-46]. Additionally, these methods used to stratify subject's obesity status do not account for local fat depots (e.g., visceral adipose tissue), which were shown to have adverse specific adipokine expression profiles, contributing towards more aggressive tumors [47-49]. Future epidemiological studies should address these issues by using more precise methods (e.g. visceral and subcutaneous fat determinations by computed tomography scan, whole body fatness by tetrapolar bioimpedance, or local and whole body fat measurements through magnetic resonance imaging, among others) to evaluate whole and local body fatness in association with HL.

At present, the adipocyte is no longer considered a passive component of human metabolism. It is known as an endocrine/paracrine organ that exerts many biological effects through production of growth factors, cytokines and hormones [50]. These biologically active molecules secreted primarily, partially or exclusively by adipocytes, known as adipokines, have a significant role in regulating angiogenesis and tumor growth [51]. In fact, the importance of the interaction between cancer cells and surrounding stroma cells has been increasingly accepted. These interactions are particularly prominent in environments rich in adipocytes [52]. The excess body fatness is characterized by a chronic low-grade inflammatory state with altered circulating levels of adipokines, including IL-6, IL-8, leptin, adiponectin, TNF-α, vascular endothelial growth factor (VEGF), osteopontin (OPN), haptoglobin (Hp), YKL-40, among others [53-55] (Figure 1). These molecules impact cancer-related mechanisms such as cell proliferation, apoptosis and migration [56, 57]. The altered secretion of adipokines in obesity deranges metabolic homeostasis, together with influences in immunological status [58] (Figure 1). In this context, the evaluation of markers related to obesity and immune response in HL, might reveal new opportunities for understanding the mechanisms responsible by the association between obesity and HL. Various adipokines have already been shown to be linked with HL risk and with advanced stage of disease, namely IL-6 and interleukin 7 (IL-7) [18, 19, 59], while others endure as promising targets for future studies (e.g., leptin, adiponectin, resistin, HGF, visfatin, …). Thus, the

chronic inflammation sustained by expanding adipose tissue may modulate host immunosurveillance [16] and exert a direct effect at both local tumor microenvironment and distant tumor cells through the systemic effects of endocrine signals (Figure 1).

Angiogenesis is a well-established hallmark of tumor development both in solid tumors and hematological malignancies (including HL). Reasonable data, mostly supported by retrospective immunohistochemistry evaluations, stands for a relevant role of angiogenesis in HL, where a shift towards an angiogenic phenotype is observed as result of unbalanced angiogenic versus anti-angiogenic stimulus [60]. Interestingly, many of the adipokines overexpressed by adipose tissue in obesity are well-known for their potent pro-angiogenic effects [53]; therefore, it seems plausible that these adipokines might mediate the causally invoked association between excess adiposity and HL through a modulatory effect in angiogenesis (Figure 1).

Besides adipokine secretion, the expanding adipose tissue is also infiltrated by macrophages with M2-to-M1 differentiation, further contributing towards the obesity-associated systemic chronic inflammation and insulin resistance [58, 61] (Figure 1). Leptin and adiponectin, hormones exclusively produced by adipocytes and with opposing effects in obesity and cancer, contribute to the polarization of macrophages [62, 63], respectively. Interestingly, in HLs the presence of $CD68^+$ tumor-associated macrophages is indicative of poor prognosis [23, 64], whereas paediatric HL patients have higher adiponectin levels [65]. Recently, the soluble circulating CD163 and TARC were identified as possible biomarkers of HL [30]. CD163 is a known marker for M2 macrophages polarization and a receptor for Hp, which is a major acute phase protein overexpressed in conditions such as obesity and HL [66, 67]. Nonetheless, Hp is induced not only by IL-6 downstream transcription factor STAT3, but also by hypoxia-inducible factor-1α (HIF-1α) that are overexpressed in HL [68]. Therefore, excess adiposity may interfere with malignant cell signaling pathways and to modulate macrophage differentiation, both of which can impact the tumor. This obesity-driven inflammatory environment exerts tumor-promoting effects, due to alter inflammation pathways implicated in cell proliferation, survival, angiogenesis, and metastasis associated with cancer (Figure 1). These potentially unrevealed links should

foster experimental research to uncover the impact of these obesity-associated molecules in HL.

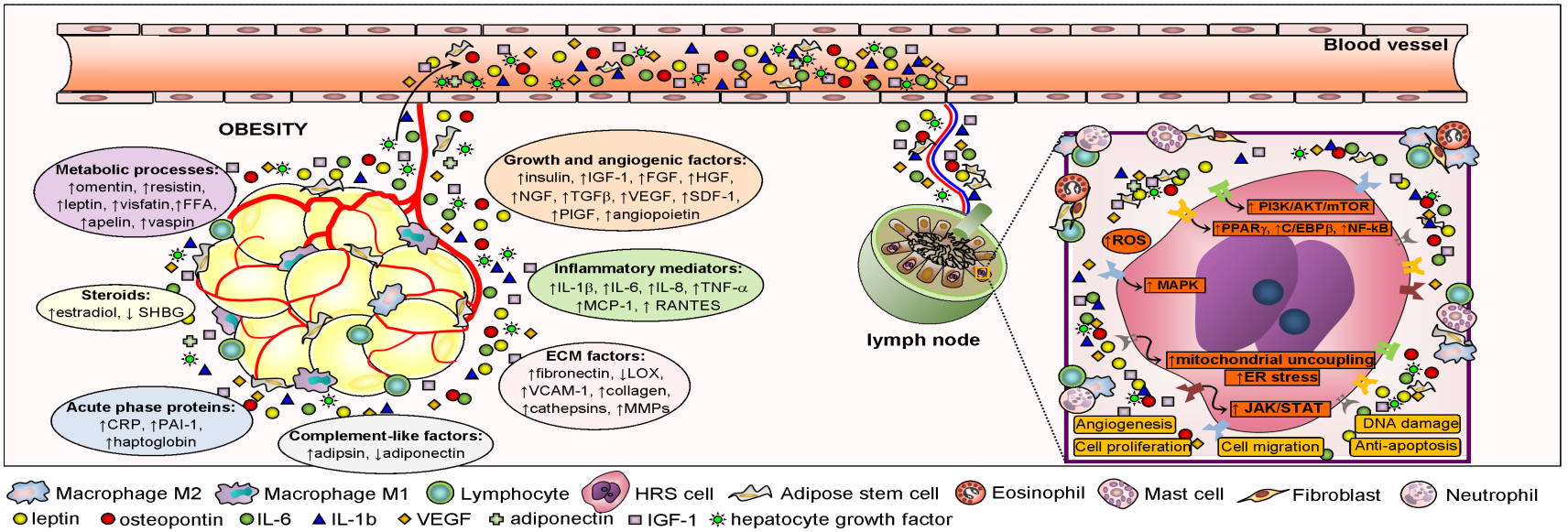


Figure 1: Endocrine effects of obesity impact Hodgkin Reed-Sternberg lymphoma cells.
Excess adiposity modulates HRS aggressiveness in lymph nodes through a systemic effect mediated by adipokines and migrating adipose stem cells. In obesity states the adipose tissue acquires the following characteristics: hypertrophied adipocytes, neoangiogenesis with increased vessel density, infiltration with M1 type macrophages, increased amount of adipose stem cells and upregulated secretion of pro-tumoral adipokines whereas anti-tumoral adipokines are downexpressed (e.g. adiponectin, SHBG and LOX). The full black arrow represents adipokines and adipose stem cells entering peripheral blood. The circulation levels of these adipokines are significantly increased in obese (as opposed to adiponectin levels), reaching lymph nodes, where they may induce either direct effects to HRS cells through direct binding to cell receptors or indirect actions by interaction with cells in microenvironment modulating their crosstalk with HRS cells. Ultimately, adipokines may induce intracellular signalling pathways (represented by solid black arrows within HRS cell) and mechanisms that will lead to angiogenesis, and to cell proliferation, cell migration, DNA damage or anti-apoptosis of HRS cells. AKT, Akt kinase; C/EBPβ, CCAAT/enhancer binding protein beta; CRP, C-reactive protein; ER, endoplasmic reticulum; ECM, extracellular matrix; FGF, fibroblast growth factor; FFA, free-fatty acids; HGF, hepatocyte growth factor; HRS, Hodgkin Reed-Sternberg cell; IGF-1, insulin-like growth factor-1; IL-1β, interleukin 1 beta; IL-6, interleukin 6; IL-8, interleukin 8; JAK, Janus kinase; LOX, lysyl oxidase; mTOR, mammalian target of rapamycin; MMP, matrix metalloproteinase; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein 1; NGF, nerve growth factor; NF-kB, nuclear factor kappa B; PAI-1, plasminogen activator inhibitor–1; PI3K, phosphatidylinositol 3'–kinase; PIGF, placental growth factor; PPARγ, peroxisome proliferator-activated receptor gamma; RANTES (CCL5), regulated on activation, normal T cell expressed and secreted; ROS, reactive oxygen species; SHBG, sex hormone binding globulin; STAT, signal transducer and activator of transcription; SDF-1, stromal derived factor 1; TGFβ, transforming growth factor beta; TNF-α, tumor necrosis factor alpha; VCAM-1, vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor.

1.4. Bone marrow adipocytes and Hodgkin Lymphoma

Adipocytes in the bone marrow have been implicated as regulators of marrow microenvironment [21, 69], and present a distinctive phenotype, which resembles both, white and brown adipose tissue [70]. Besides representativeness, bone marrow adipocytes present an unilocular lipid morphology similar to white adipose tissue, and are a unique adipose depot that overexpresses genes associated with cell differentiation and with inflammation [71, 72]. Amplified bone marrow adiposity due to diet-induced obesity in mice was recently implicated in altered bone metabolism and inflammation within the bone microenvironment [73]. Adiposity in bone marrow is modulated by high fat diet, diabetes, aging, dyslipidemia and obesity, through diverse pathways that comes together to regulate the expression and activity of a key pro-adipogenic transcription factor, the peroxisome proliferator-activated receptor γ2 (PPAR-γ2) [74].

Bone marrow adipocytes behave as energy suppliers to bone physiological functions, including bone remodeling [69, 75]. In addition to energy storage, these adipocytes secrete adipokines and fatty acids that impact significantly on metabolism and function of other neighboring cells in the bone microenvironment [72, 76]. From this interaction in bone marrow milieu, an inverse relationship has been described between osteoblastogenesis and adipogenesis, with a negative correlation of marrow adiposity with osteoblast number and bone mineral density [74]. In fact, several factors produced in the bone marrow may exert a regulatory role in local adipocytes, as well as adipokines secreted in marrow adipocytes might influence other cellular players through a paracrine effect [76] (Figure 2).

Hodgkin's lymphoma involving the bone marrow ranges between 2 to 32%, with an average incidence of 10% [77]. Although the chronic low-grade inflammation and the upregulated secretory profile associated with obesity may exert endocrine effects, we should not overlook paracrine actions of adipocytes in bone marrow microenvironment bearing metastasis from primary HL tumors [22]. The crosstalk between adipocytes and cancer cells has been demonstrated to support progression and aggressiveness of tumors in other

oncologic models [52, 78-80], as well as metastatic cell growth in the bone marrow [81]. Fat cells seem to be able of translocating stored lipids to metastatic tumor cells, ultimately driving cancer growth and motility [78, 82]. The complex interaction between components of bone marrow, including adipocytes and eventually tumor cells, is depicted in Figure 2.

In other oncologic models, the fatty acid binding protein 4 (FABP4) was shown to be implicated in adipocyte-tumor cell interactions [78, 81]. Notwithstanding FABP4 is transcriptionally regulated by PPARγ, this lipid chaperone also controls PPARγ [83, 84], while both seem to be involved in adipocyte-induced metabolic switching in cancer microenvironment. Interestingly, in B lymphoma cells the decreased PPARγ expression was related with increased proliferation and survival, and initiation of inflammatory pathways, specifically the activation of nuclear factor kappa B (NF-κB) [85], further underlining the link between inflammation and neoplastic progression.

Primary cancer-derived metastases that home to the bone are by themselves incapable of inducing bone resorption. However, these aggressive malignant cells interact with bone constituents and influence the function of bone-degrading cells (osteoclasts), inducing osteolytic lesions [86]. Bone metastases from HLs have been described as osteolytic [87, 88]. The complex interaction of tumor cells with bone marrow microenvironment, including adipocytes, exerts profound influence in proteolytic degradation and bone resorption, enabling metastasis allocation. Obesity and aging are known effectors of bone remodeling by forming adipocytes instead of osteoblasts, which will lead to increased osteoclast activity and osteoporosis [76, 89]. A key enzyme for osteoclastic bone resorption is cathepsin K (CTK) that degrades the bone matrix protein collagen I and other proteins of the bone matrix [90]. CTK expression within the bone marrow milieu is high in osteoclasts and adipocytes, and results in accelerated bone turnover [91] and in a potential contribution to the metastatic process. CTK production was also described in cancer cells that metastasize to bone [92]. CTK acts by upregulating the processing of its substracts extracellularly, including the secreted protein acidic and rich in cysteine (SPARC or osteonectin) that interacts with collagen I and other matrix proteins to attract and anchor malignant cells in the bone [91]. In addition,

VEGF, a growth factor known to be involved in tumor cell migration and in osteoclast differentiation and migration has been proposed to be modulated in the bone microenvironment by CTK cleavage [90, 93]. Besides the key role of CTK in degrading collagen I, it also seems to be relevant for adiponectin cleavage, which may be a mechanism to stimulate osteoclastogenesis via increased expression of receptor activator of NFκB ligand (RANKL), to modulate marrow fatness, or to influence adiponectin-mediated suppression of tumorigenesis [94, 95]. Bone marrow adipocytes also stimulate osteoclast differentiation and activity by directly secreting RANKL [96].

Bone marrow adipocytes are a significant secondary source of leptin and IL-6, whereas only trace amounts of IL-1β and TNF-α were found [97]. These molecules exert interactive regulatory mechanisms between them, in order to modulate the marrow environment, controlling the proliferation and differentiation of hematopoietic precursors as well as the maturation of stromal cells [98]. Given the importance of JAK/STAT signaling in HL malignant cells and that leptin and IL-6 downstream signals are mediated by this pathway [99, 100], we hypothesize these adipokines might influence HRS cell survival and proliferation both through an endocrine mechanism in lymph nodes and by a paracrine effect in the bone marrow. Since leptin and IL-6 are upregulated in the serum of obese subjects, they can partially explain the association between excess adiposity and HL. Only few unpowered studies have measured serum leptin and adiponectin levels in HL patients, mostly children, with inconclusive mixed results [65, 101, 102]. With respect to IL-6, several reports demonstrated that it was a relevant cytokine for HRS proliferation and survival and a useful biomarker of aggressiveness [103, 104]. It is largely unknown whether bone marrow adipocytes behave differently in presence of HL malignant cells. Thus, further investigation on the interactions of bone marrow adipocytes with HRS cells is required to clarify many unanswered questions and advance knowledge with potential clinical translation.

Here, we hypothesize that when HRS cells are metastasized to bone marrow, resident adipocytes may have a role in bone remodeling, yielding tumor cells with adipokines and fatty acids to boost growth and survival, concurring towards worst prognosis. Further clinical studies should follow disease behavior in HL obese patients and evaluate the impact of intervening in

obesity on HL prognosis. Therefore, obesity and its effect on bone marrow adipocytes may represent a potential therapeutic target in the future.

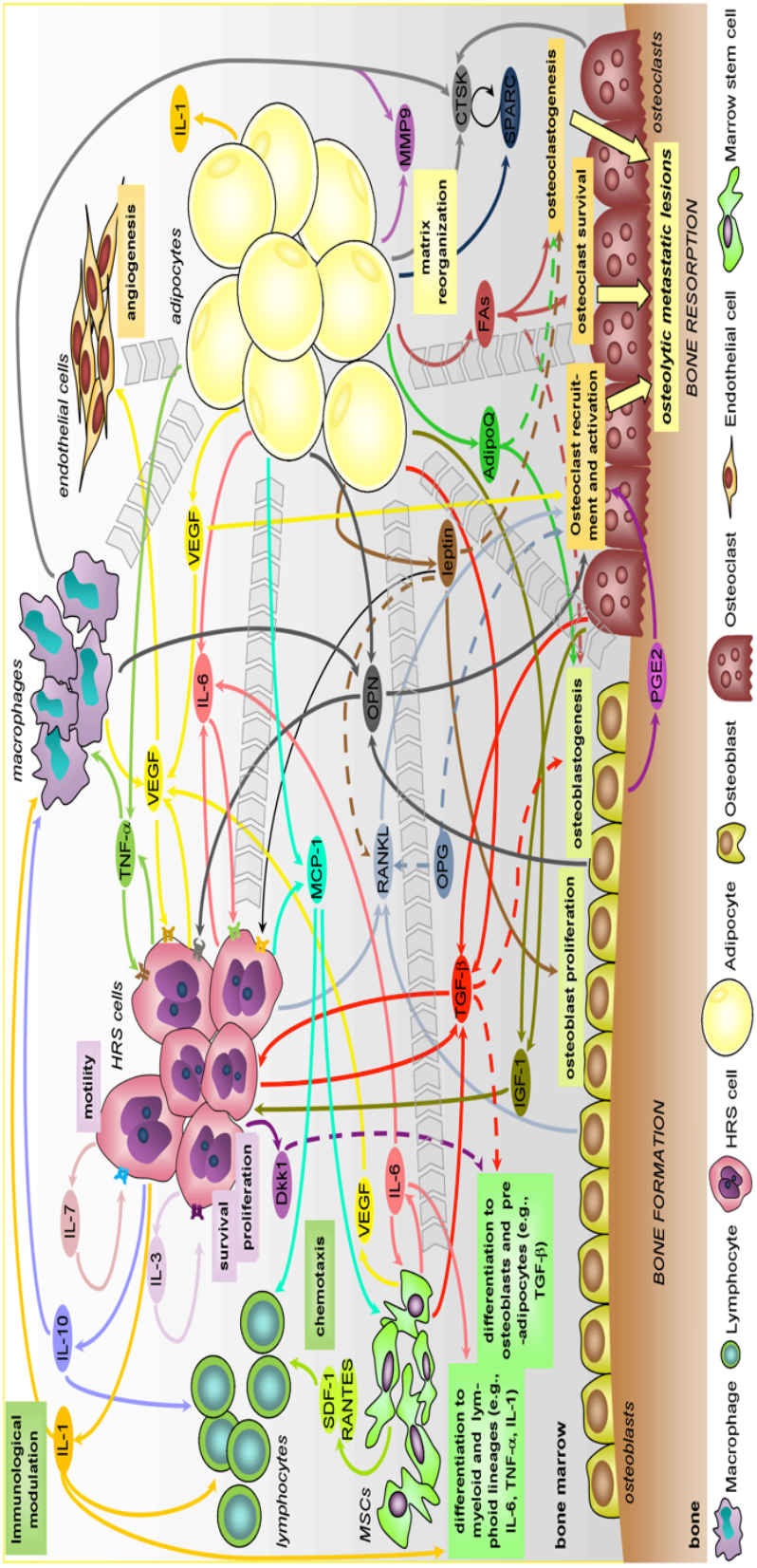


Figure 2: Hypothetical role of obesity and adipocytes in the bone marrow microenvironment invaded by HRS cells.
This figure depicts the complex interaction between cellular components in the bone marrow and its mediators, particularly the contribution of bone marrow adipocytes to bone homeostasis and metastatic progression. Most adipokines produced in adipocytes (leptin, VEGF, osteopontin, TNF-α, MMP9, IL-1, IL-6, TGF-β, IGF-1, MCP-1) may contribute either directly (through an effect in HRS tumor cells mediated by their specific receptor downstream signalling – impacting HRS cell motility, survival and proliferation) or indirectly (by influencing other cells in the microenvironment, including osteoblasts, osteoclasts, lymphocytes, macrophages, endothelial and mesenchymal stem cells, to acquire an pro-tumoral behaviour – immunological modulation, increased chemotaxis, cell differentiation, matrix reorganization, neoangiogenesis, increased osteoclast recruitment and activation, ultimately resulting in bone resorption and osteolytic metastatic lesions) to this stage aggressiveness and HL prognosis. Solid black lines with arrows represent secretion or the effect of a given adipokine, whereas dashed lines denote inhibitory action. Multiple light yellow arrows mean the reciprocal impact other cells might have in adipocytes. AdipoQ, adiponectin; CTSK, cathepsin K; DKK-1, dickkopf-related protein 1; FAs, fatty acids; IL-1, interleukin 1; IL-3, interleukin 3; IL-7, interleukin 7; IL-10, interleukin 10; MMP9, matrix metalloproteinase 9; MSCs, mesenchymal stem cells; OPG, osteoprotegerin

OBJECTIVES

2. OBJECTIVES

2.1. General objectives

The overall goal of this study is to examine the association between excess adiposity and Hodgkin's Lymphoma and contribute towards untangling the mechanistic clues behind. Ultimately, we aim at characterizing mechanistically the obesity-HL relationship from a molecular, cellular and genetic perspective.

2.2. Specific objectives

- Evaluate whether and which adipokine levels in the bone marrow are altered according to obesity, pathological status and disease aggressiveness, using a multiplexed array.
- Correlate the levels of altered adipokines on the bone marrow with peripheral blood circulating levels, to ascertain novel adipokines that may serve as HL serum biomarkers.

MATERIAL AND METHODS

3. MATERIAL AND METHODS

3.1. Population

3.1.1. Patients and sample collection

Iliac crest or sternum bone marrow aspirates and matched peripheral blood samples were obtained at the same time of staging procedures from 16 patients (median age, M=31 years; interquartile range, IQR=15 years) diagnosed and treated at the Department of Onco-hematology of the Portuguese Institute of Oncology Porto Centre (IPO Porto) and from 11 marrow donors (M age=28 years; IQR=12 years), recruited from the Laboratory of Cellular Therapy at IPO Porto, and the Immunotherapy Unit and Clinical Pathology Department of Sta Maria Hospital, Lisbon. All individuals complied to participate by signing a written informed consent, whereas the study was approved by the ethics' committees of participating institutions. Research was conducted according to principles of the Declaration of Helsinki.

Participants and donnors were enrolled between May 2014 and May 2015. HL patients were included at the time of the first visit to confirm diagnosis in the Department of Onco-hematology of IPO Porto. One patient was excluded from final analyses since the final histological diagnosis was histiocytic sarcoma. Donors were selected to match HL's by age, gender and BMI. Bone marrow and peripheral blood samples were collected in the morning, and for HLs at the time of diagnosis and before starting treatment.

Subsequent studies on the relevance of adipokine pathways in HL will be conducted in primary disease location at malignant lymph nodes. Paraffin-embedded tumor samples from a retrospectively recruited cohort of HL patients (n=71) at the Pathology Department of Sta Maria Hospital will be used for studying the expression of altered adipokines and its receptors. This ensuing study includes 71 HL patients diagnosed between 2009 and 2013 and with clinical follow-up documented at the same hospital. Interestingly, at least 30 patients have matched paraffin-embedded samples of bone marrow infiltration and 11 mediastinal malignant tissue.

3.1.2. Anthropometric measurements

Anthropometric data included height, weight and abdominal perimeter, and followed standardized procedures: a digital scale and a wall-mounted stadiometer were used for weight and height determination; abdominal perimeter was measured, with precision up to 0.1 cm, at the midpoint between the lower rib margin and the iliac crest. Body mass index (BMI) was calculated using the algorithm [weight (kg)/height^2 (m)], and thereafter categorized according to WHO classification into underweight (BMI < 18.5 kg/m^2), normal BMI (BMI = 18.5 to 25 kg/m^2), overweight (BMI = 25 to 30 kg/m^2) and obese (BMI ≥ 30kg/m^2). In this study, we separated patients into only two groups: normal BMI (BMI < 25 kg/m^2) and overweight/obese (OW/OB) (BMI ≥ 25kg/m^2).

3.2. Isolation of biological specimens from the bone marrow and blood

3.2.1. Bone marrow and blood samples processing

The first sample during the bone marrow (BM) biopsy was collected to a heparinized tube with 200 uL citrate, and immediately inverted with care, to avoid hemolysis and clot formation. The BM was centrifuged in Ficoll gradient (Histopaque, Sigma) and centrifuged at 1500 rpm at room temperature for 30min. The upper layer with adipocytes was washed and the isolated adipocytes were processed to RNA and stored at -80ºC. The interstitial marrow fluid (IMF) was collected, centrifuged and stored at -80ºC.

Stromal cells were also collected from the gradient centrifugation, washed and ressuspended in erythrocyte lysis buffer (NH4Cl). Isolated stromal cells were partially fixed in slides with acetone and stored at -20ºC, while the remaining pellet was processed to RNA and stored at -80ºC.

Peripheral blood was collected into one EDTA tube and one-biochemistry tube, and plasma and serum removed, respectively. For subsequent genetic profiling, a sample of whole blood was stored at -80ºC.

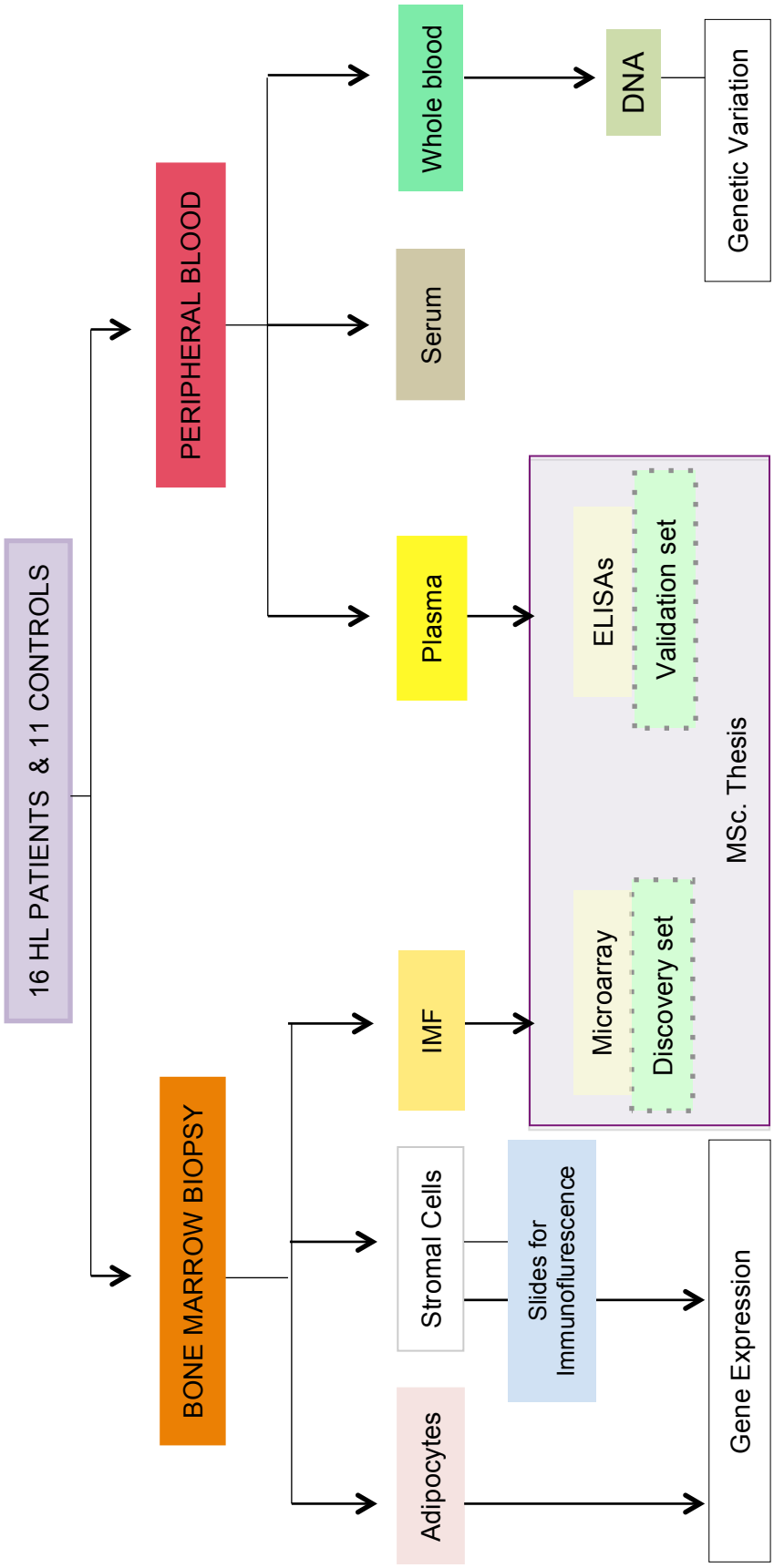


Figure 3: Flowchart resuming the full protocol for collection and processing of samples
In each participant, samples of bone marrow and peripheral blood were collected and properly processed. After centrifugation the bone marrow, the interstitial marrow fluid (IMF) collected was stored for, posteriorly, performed the multiplex array for simultaneous determination of 62 adipokines. From bone marrow were also isolated adipocytes and stromal cell for gene expression. 1,5 ml of whole blood was collected for DNA extraction, and the rest centrifuged to collect plasma and serum

3.3. Adipokine's protein array on interstitial marrow fluid

3.3.1. Adipokine profiling - Multiplex measurements

The IMFs were used to run a multiplex array (AAH-ADI-1-8 Obesity Adipokine antibody array C series 1, RayBio®) for simultaneous determination of 62 adipokines in matched HL patients group and a control group, by BMI, age and gender. The previously calculated sample size was 8 individuals in each group (with a α=0.05, β=0.8, rate=1.0 and assuming a difference of 25 intensity units between groups).

Briefly, membranes were initially incubated with blocking buffer under gentle shaking for 30 minutes, followed by overnight incubation with IMF at 4ºC. The membranes were then washed several times and incubated with a biotinylated antibody cocktail for 2 hours at RT. After another washing step, the membranes were labeled with HRP-Streptavidin for 2 hours. Signal intensity was detected by chemoluminescence after adding the substrate and revealed on X-ray film, using several exposure times.

3.3.2. Microarray data extraction and analysis

Signal intensities (intensity by milimeter squared) were calculated using 2-D densitometry software. Selection of films with appropriate exposure time followed the criteria of strong spot signals vs. low background response, strong positive control spot signals and similarity between positive control signals. We used the software ImageJ with protein array macros for quantification of signal intensity, while ensuring the same extraction circle dimensions (area, size, and shape).

Following raw numerical densitometric data extraction, the background was subtracted and the data normalized to the positive control signals, within each membrane. In agreement with manufacturer's intructions an algorithm was used to calculate the fold expression between analytes [X(Ny) = X(y) * P1/P(y)].

MCP-3 and TNF-α were excluded from the protein array data, once missing or negative values existed for more than 8 of the 16 samples. Then, adequate adjustments of measurements from array data were log2-transformed

to become normally distributed. There were imputed the missing values using R package. Statistical analyses were performed using LIMMAR R package, and the adjusted P-value was considered statistically significant when <0.1.

3.4. Validation of adipokine concentration in IMF by ELISA

Four of the most differentially expressed adipokines in IMF (IGFBP-1, IGFBP-3, IL-8 and OPG), were quantified in IMF and matched serum using pre-coated human ELISA kits (RayBiotech®). Briefly, samples were incubated for 2.5 and then washed. After incubation with biotinylated antibody for 1 hour and wash steps, streptavidin was added and incubated for 45 minutes. When the substrate was added and the color developed in proportion the reaction was stopped and optical densities determined in a microplate reader set to 450 nm. Log transformation of data was computed to allow curve fitting (nonlinear regression), interpolation of values, followed by conversion to concentration. GraphPad Prism 5 software was used to create the standard curve and interpolate adipokine concentrations. Total protein concentration in IMF and serum was measured using the Bradford method.

The intra- and inter- assay precisions were <10% and <12% for each assay, and the minimum detectable amounts were 5 pg/mL for IGFBP-1, 80 pg/mL for IGFBP-3, and 1 pg/mL for both IL-8 and OPG.

3.5. Statistical analysis

Departure from normality of continuous variables was tested using Shapiro-Wilk test. Then, parametric or non-parametric tests were accordingly used to compare means between groups. Independent samples t-test and Mann Whitney were used as appropriate, whenever variables were non-parametric. Determination of the Spearman correlation coefficient was applied to test the strength of association between continuous variables. Analyzes were performed using SPSS 21.0. P-values below $p<0.05$ were considered statistically significant.

RESULTS

4. RESULTS

4.1. Characterization of participants

During 15 months, 16 new cases of Hodgkin Lymphoma were recruited to participate in this study, together with 11 age-, gender- and BMI-matched controls. Age, gender and anthropometric information from each group are depicted in Table 2. No differences were found between groups in any of those variables. Anthropometrically, in this study we achieved a reasonable matching between HL and controls, once waist and BMI values were similar, as well as for obesity classification.

Table 2: Comparison of age, gender and anthropometric variables between Hodgkin's lymphomas and controls

	Controls (n= 11)	HL (n=16)	P-value **
Age (years)	29.6 ± 2.5	29.4 ± 1.9	0.951
Height (cm)	167.9 ± 3.3	170.4 ± 1.7	0.517
Weight (Kg)	72.0 ± 5.9	72.7 ± 3.2	0.906
Waist (cm)	84.8 ± 4.2	89.0 ± 12.0	0.402
BMI (Kg/m^2)	25.3 ± 1.5	25.1 ± 1.1	0.906
Gender			
Female	6 (54.5)	10 (62.5)	0.710 *
Male	5 (45.5)	6 (37.5)	
WHO classification			
Normal weight (18.5 - 25 kg/m^2)	6 (54.5)	11 (68.8)	0.453 *
Overweight/obese (≥ 25 kg/m^2)	5 (45.5)	5 (31.2)	

Data is presented as mean ± standard error of mean or as frequencies. * Chi-square test; ** Independent measures t-test. HL, Hodgkin Lymphoma; BMI, Body mass index; WHO, World Health Organization.

Starting from the whole recruited population from which we had collected bone marrow (n=16 HLs and n=11 donors), we undertook a selection of cases (by gender, ± 5 years of age, by obesity category) to more precisely match patients to allow the comparison adipokines in IMF through an adipokine protein array. Besides stratification by disease status (n=8 HLs and n=8 donnors), we were able to categorize further within each group for obesity status (for HLs, 4

obese/overweight and 4 normal weight patients, and for controls also 4 obese/overweight and 4 normal BMI subjects) (table 3).

Table 3: Confirmation of selection for stratified analysis by obesity status, for secondary analysis in protein array

	Controls (n=8)		HLs (n=8)		Overall (n=16)	
	Age	BMI	Age	BMI	Age	BMI
Normal BMI (n=4)	24.4±3.3	21.8±1.9	22.5±6.1	22.1±1.6	23.5±4.7	22.0±1.6
OW/OB (n=4)	33.5±10.2	27.5±3.1	33.5±6.2	29.9±4.7	33.5±7.8	28.7±3.9
P-value *	0.142	0.021	0.045	0.020	0.008	0.001

* Independent samples t-test comparing by BMI the normal BMI versus OW/OB groups. Normal BMI and OW/OB groups have n=8 subjects each on overall analysis. HLs, Hodgkin Lymphomas; BMI, body mass index; OW/OB, overweight/obese

For stratified secondary analysis in protein arrays, we were able to separate samples within each group (control and HL) by BMI, and confirmed that the normal weight group have significantly lower BMI than overweight/obese group, independent of the pathology group.

A descriptive clinicopathological characterization of participants with HL is depicted in Table 4 by obesity status. There are no clinical or pathological features associated with obesity, classified by WHO groups. Adding up to the HL's descriptive, 15.4% HL patients were at risk level 3, and over 50% were treated with Adriamycin/Bleomycin/Vinblastine/Dacarbazine (ABVD), followed by radiotherapy.

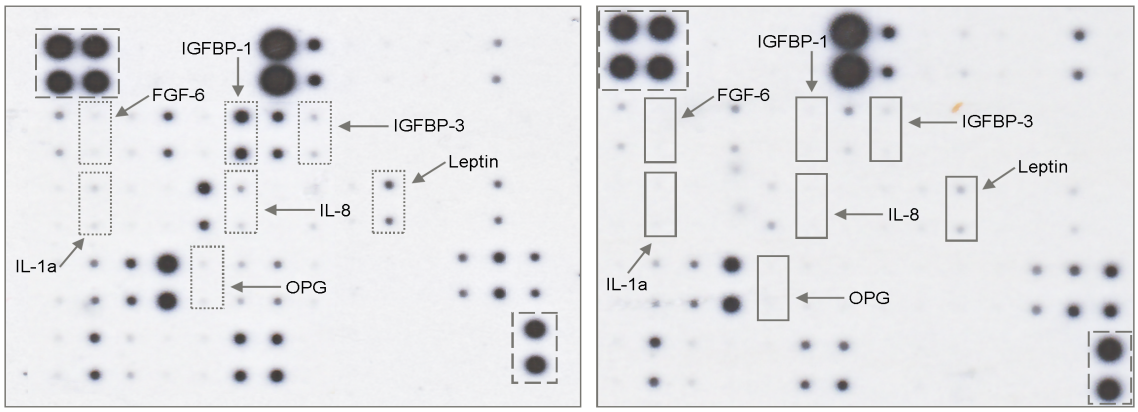
Table 4: Clinicopathological characteristics of Hodgkin's Lymphoma patients by body mass index group

	NW (n=10)	OW/OB (n=6)	Overall (n=16)	P-value *
Age (years)	26.7 ± 7.5	34.0 ± 4.9	29.4 ± 7.5	0.054
Gender				
Women	6 (60.0)	4 (66.7)	10 (62.5)	
Male	4 (40.0)	2 (33.3)	6 (37.5)	0.790
Clinical Stage [a]				
I	2 (20.0)	2 (33.3)	4 (26.7)	
II	5 (50.0)	3 (50.0)	8 (53.3)	
IV	3 (30.0)	1 (16.7)	4 (13.3)	0.766
Histopathology				
Nodular sclerosis	8 (88.9)	4 (66.7)	12 (80.0)	
Lymphocyte predominance	1 (11.1)	2 (33.3)	3 (20.0)	0.292
B-symptoms				
No	8 (80.0)	4 (66.7)	12 (75.0)	
Yes [b]	2 (20.0)	2 (33.3)	4 (25.0)	0.551
Risk level				
0	4 (44.4)	1 (25.0)	5 (38.5)	
1	3 (33.3)	2 (50.0)	5 (38.5)	
2	1 (11.1)	0 (0)	1 (7.7)	
3	1 (11.1)	1 (25.0)	2 (15.4)	0.738
Treatment				
ABVD	3 (27.3)	0 (0)	3 (18.8)	
ABVD + RT	8 (72.7)	5 (100.0)	13 (81.1)	0.195

[a] Ann Arbor staging. [b] Include fever, night sweats in the last month, and/or > 10% weight loss in the last 6 months. NW, normal weight; OW/OB, overweight/obese; ABVD, doxorubicin, bleomycin, vinblastine and dacarbazine; RT, radiotherapy. * Chi-square test, except for age, where independent measures t-test was used.

4.2. Adipokines in interstitial marrow fluid of HLs and OW/OB

The search for altered adipokine pathways in the bone marrow interstitial fluid was initially focused on the utilization of a protein array, in order to get insight about the most deregulated molecules (figure 4).



CONTROL HL

Figure 4: Representative blot membranes of adipokine expression in interstitial marrow fluid of one donor and one HL.
Comparison of the amount of adipokines in interstitial marrow fluid of controls and Hodgkin's Lymphoma using an adipokine membrane array. Representative membranes from one control and one HL (Two of sixteen arrays). IGFBP-3, insulin growth factor binding protein 3; IGFBP-1, insulin growth factor binding protein 1; FGF-6, Fibroblast growth factor 6; OPG, osteoprotegerin; IL-8, interleukin 8; IL-1a, interleukin 1 alpha; HL, Hodgkin's Lymphoma

Adipokine protein array analyses demonstrated that from 62 proteins only OPG was overexpressed in the marrow fluid of overweight/obese subjects compared with normal BMI, overall and within HL group of patients (table 5).

Table 5: Altered adipokines in interstitial marrow fluid of overweight/obese subjects.

Protein name	Description	Fold change	Unadjusted P
Overweight/obese versus normal weight			
All subjects			
OPG	*Osteoprotegerin*	+2.5	0.009
Controls			
none	---	---	---
Hodgkin Lymphoma			
OPG	*Osteoprotegerin*	+4.0	0.004

OPG, osteoprotegerin

In order to uncover whether adipokines were deregulated in bone marrow fluid of HLs, the amount of protein was compared against donors' marrow fluid. Table 6 presents the 10 most altered adipokines, overall, and within normal BMI and OW/OB groups. Notably, IGFBP-3 was down-expressed

independently of the obesity status, albeit other modulators of IGF axis were altered (IGFBP1, -13.2 fold). Circulating cytokines known to regulate inflammation, IL-8, IL-1a, IL-12 and IL-1b were all depressed in HLs' marrow fluid compared with controls (from -4.5 to -7.8 fold decrease). Leptin, a hormone almost exclusively secreted by adipocytes, was also significantly decreased by -12.6 fold. Other growth factors, TGF-β1 and FGF-6, together with osteoprotegerin were all lower in HLs when compared with donors' marrow fluid.

Table 6: Top-ranked 10 most altered proteins of interstitial marrow fluid of Hodgkin's Lymphoma patients in the array analyses

Protein name	Description	Fold change	Adjusted P
Hodgkin Lymphoma's versus Controls			
All subjects			
IGFBP-1	*IGF binding protein*	-13.2	0.0049
Leptin	*Leptin*	-12.6	0.0276
IL-8	*Interleukin 8*	-7.8	0.0029
FGF-6	*Fibroblast growth factor 6*	-7.6	0.0535
IL-1a	*Interleukin 1 alpha*	-6.6	0.0029
IGFBP-3	*IGF binding protein 3*	-6.4	0.0026
TGF- β1	*Transforming growth factor beta 1*	-5.2	0.0408
IL-12	*Interleukin 12*	-4.8	0.0029
IL-1b	*Interleukin 1 beta*	-4.5	0.0029
OPG	*Osteoprotegerin*	-4.2	0.0029
Normal weight			
FGF-6	*Fibroblast growth factor 6*	-21.3	0.0712
IGFBP-1	*IGF binding protein 1*	-10.6	0.0925
IL-8	*Interleukin 8*	-9.0	0.0329
OPG	*Osteoprotegerin*	-6.8	0.0186
IL-1a	*Interleukin 1 alpha*	-6.5	0.0540
IL-12	*Interleukin 12*	-6.2	0.0186
IGFBP-3	*IGF binding protein 3*	-5.0	0.0329
IL-1b	*Interleukin 1 beta*	-4.6	0.0329
IL-6 sR	*IL-6 soluble receptor*	-4.3	0.0186
Fas/CD95	*Fas ligand receptor*	-4.3	0.0186
Overweight/obese			
IGFBP-3	*IGF binding protein 3*	-8.2	0.0300

IGF, insulin growth factor;

The comparison of utmost subgroups, between HLs simultaneously OW/OB (n=4) and controls having normal BMI (n=4), yielded a reinforcement of the potential IGF axis involvement in the association obesity-HL (Table 7). IGFBP-1, IGFBP-3 and IGFBP-2 were all significantly reduced in the marrow fluid of OW/OB subjects with HL. Furthermore, cytokines involved in inflammatory regulation (IL-8, IL-1b, IL-12 and MIP-1b) were also diminished in this group.

Table 7: Altered proteins in interstitial marrow fluid of Hodgkin Lymphoma patients simultaneously overweight/obese compared with Controls that have normal BMI

Protein name	Description	Fold change	Unadjusted P
IGFBP-1	*IGF binding protein 1*	-14.6	0.0895
IGFBP-3	*IGF binding protein 3*	-8.0	0.0341
IL-8	*Interleukin 8*	-8.0	0.0895
IL-1b	*Interleukin 1 beta*	-4.4	0.0895
Fas/CD95	*Fas ligand receptor*	-4.0	0.0820
IL-12	*Interleukin 12*	-3.9	0.0895
IGFBP-2	*IGF binding protein 2*	-3.2	0.0895
MIP-1b/CCL4	*Macrophage inflammatory protein-1β*	-1.9	0.0895

Validation of four of the most consistently deregulated adipokines in bone marrow fluid was done using ELISA experiments. Data was further adjusted to the amount of protein in IMF. In table 8, from 4 adipokines deregulated in the array, only IGFBP-3 remained significantly lower in IMF of HL patients in ELISA.

Table 8: Concentrations of deregulated adipokines between pathology groups using ELISA

	Control (n=8)	HL (n=8)	P-value
Osteoprotegerin (pg/mL/μg)	0.10 ± 0.02	0.13 ± 0.04	0.600
IGF binding protein 1 (ng/mL/μg)	0.06 ± 0.03	0.01 ± 0.004	0.345
IGF binding protein 3 (ng/mL/μg)	0.18 ± 0.03	0.14 ± 0.06	0.046
Interleukin 8 (pg/mL/μg)	0.09 ± 0.05	0.07 ± 0.02	0.401

HL, Hodgkin's Lymphoma; IGF, insulin growth factor. Results are presented as adipokine concentration adjusted per μg of total protein concentration in IMF

When analyzed according to obesity groups (normal BMI versus

OB/OW), we observed that osteoprotegerin level in IMF, which was altered on the array, is not distinct between groups classified by BMI (Table 9).

Table 9: Concentrations of deregulated adipokines between obesity groups using ELISA.

	BMI ($Kg.m^{-2}$)		
	< 25 (n=8)	≥ 25 (n=8)	P-value
Osteoprotegerin (pg/mL/μg)	0.12 ± 0.04	0.10 ± 0.02	0.916
IGF binding protein 1 (ng/mL/μg)	0.05 ± 0.03	0.03 ± 0.01	0.401
IGF binding protein 3 (ng/mL/μg)	0.20 ± 0.06	0.10 ± 0.02	0.208
Interleukin 8 (pg/mL/μg)	0.10 ± 0.05	0.06 ± 0.02	0.753

HL, Hodgkin's Lymphoma; IGF, insulin growth factor. Results are presented as adipokine concentration adjusted per μg of protein

4.3. Correlative analyses of anthropometric measures and adipokine concentration in IMF and in circulation

Once the validation step was concluded on the same samples (n=16) used in the array, we extended ELISA measurements to the additional IMF samples, rendering a whole population of 11 controls and 16 HLs. In this set of subjects we questioned whether BMI was associated with abdominal perimeter, a specific measure of central obesity. Spearman correlation coefficient analysis demonstrated a good agreement between BMI and waist measurements ($r=0.859$, $P<0.0001$) (Figure 5).

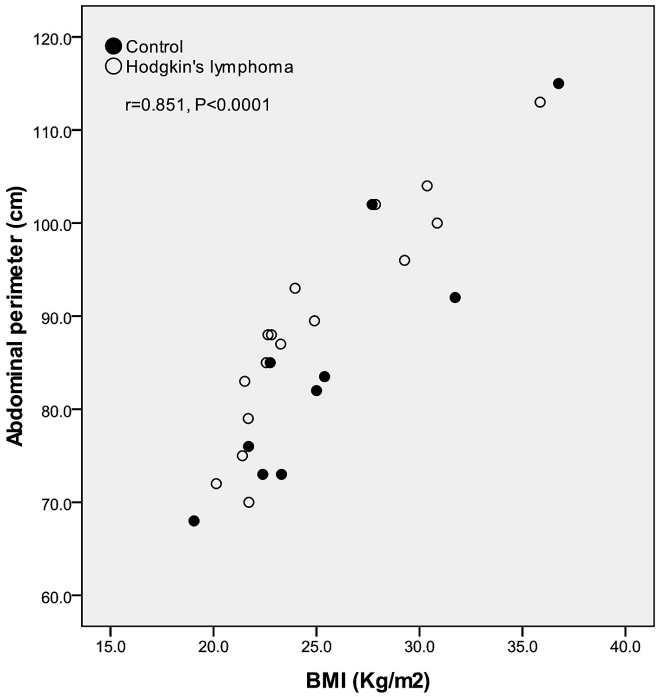


Figure 5: Association between BMI and abdominal perimeter.

BMI, body mass index. Spearman correlation coefficient.

Following this association between general and abdominal adiposity markers, we sought to examine the degree of correlation between central obesity and adipokines in IMF. We observed that the abdominal perimeter was inversely correlated with IGFBP-3 and with IGFBP-1 levels in IMF (r=-0.416, P=0.039 and r=-0.473, P=0.017, respectively), but no association was found for BMI (data not shown).

In HLs (n=16) we searched for an association between clinicopathological characteristics and IMF levels. The levels of adipokines were not related with histopathology subtypes, clinical staging and presence of B symptoms (data not shown), except for a strong trend towards lower IMF levels of IL-8 in patients with B symptoms (with symptoms, average 0.02±0.004 pg/mL/μg, and without symptoms average 0.05±0.01 pg/mL/μg, P=0.052).

The level of concordance between IMF adipokine and circulating serum concentrations was assessed using Spearman correlation coefficient. Table 10 depicts the degree of association of adipokine concentration between IMF and blood.

Table 10: Evidence of good representativeness of adipokine IMF concentrations in serum from peripheral blood

Conc. in IMF /μg protein		Conc. in serum/μg protein	r *	P-value
Osteoprotegerin	vs	Osteoprotegerin	0.156	0.456
IGF binding protein 1	vs	IGF binding protein 1	0.630	0.001
IGF binding protein 3	vs	IGF binding protein 3	0.467	0.021
Interleukin 8	vs	Interleukin 8	0.419	0.037

* Spearman correlation coefficient

A good correlation was found for adipokines levels in IMF versus serum, both using the concentrations adjusted for total protein (Table 10) and without adjustment (data not shown).

DISCUSSION

5. DISCUSSION

The marrow stroma is a cellularly rich portion of bone, in which a broad range of signals emanates from the crosstalk between adipocytes, fibroblasts, osteocytes, hematopoietic stem cells and their progeny, endothelial cells and macrophages. We hypothesized that distinctive environmental factors may influence absolute and relative cellular quantities and influence the marrow microenvironment. This unbalanced microenvironment not only can support the attraction of tumor cells towards the marrow but also the maintenance of a specific environment for tumor growth. In addition, bone resorption and bone formation are likely to become affected. The more aggressive phenotype of disease for many primary tumors, including Hodgkin's lymphoma (HL), is when malignant cells travel throughout the blood or lymphatic vessels until they are established and grow in the bone marrow [105]. In HL, an estimated 4 - 14% of patients will develop blastic bone metastasis, which represents a bad prognosis factor [106, 107].

It is expected that bone marrow characteristics would be noticeable in the fluid surrounding bone marrow cells, notably the assortment of locally- or systemically-produced adipokines in situations of excess adiposity with hypertrophied adipocytes. Current knowledge about the content of adipokines in the bone marrow fluid is practically unexplored in patients with cancer and/or with obesity.

Concerning the association of BMI with HL risk, there are few studies that have addressed this issue. From the twelve prospective studies, 5 identified risk for HL with an increase of 10kg/m^2 of BMI or obesity. However, 2 studies found a protective role for obesity in the development of HL. Despite the batch of positive association studies between obesity and HL, it is important to highlight the time when the anthropometric assessments were made and the outcomes considered. In fact, obesity may increase the risk for developing HL, even though its effects in survivors may be paradoxal, thus influencing the natural history of disease.

BMI is an imperfect estimate of adiposity, thus to uncover the obesity-cancer relationship, studies that assess adipokine profile may provide more accurate information. Using an adipokine membrane array, we sought to

characterize the bone marrow microenvironment concentrations of 62 adipokines. In the IMF from both HL patients (prior to diagnosis) and controls, we compared their expression using overweight/obesity as primary outcome and presence of HL as secondary outcome. Analysis of altered adipokines in the IMF of HLs revealed that several pathways were down-regulated, specifically in IGF axis (IGFBP-1, IGFBP-2, IGFBP-3), cytokines involved in immuno-inflammatory response (Leptin, IL-8, IL-12, IL-1β, IL-1α, IL-6sR, Fas/Cd95, MIP-1b/CCL4), growth factors (TGF-β, FGF-6) and bone remodeling (OPG).

When patients were stratified by BMI classification, we found that only osteoprotegerin (OPG) was significantly elevated in the IMF of OW/OB. Osteoprotegerin is a secreted glycoprotein member of TNF-alfa receptor superfamily, which acts in bone microenvironment as a decoy receptor, binding to RANKL-mediated osteoclast recruitment and activation. Therefore, the increased RANKL/OPG ratio is critical for osteoclastogenesis, by inhibiting osteoclasts differentiation and osteoclastic bone resorption [108]. Adipocytes, normal constituents at the bone marrow microenvironment, were reported to crosstalk with osteoblasts and increase RANKL while decreasing OPG secretion in the bone marrow, influencing RANKL/OPG ratio through a still unknown mechanism [109]. Previous data supports a reduced level of OPG and increased RANKL in the bone marrow of obese mice, which was correlated with higher marrow adiposity and upregulated osteoclastogenesis [69, 73]. In agreement, a report revealed higher OPG levels in the IMF of osteoporotic and leaner women [110]. Conversely, in our study OPG concentration was significantly higher in the IMF of OW/OB by 2.5-fold overall and by 4-fold in OW/OB with HL compared with lean HLs. When compared between HLs and controls the expression of OPG in IMF was significantly down regulated by 4- to 7-fold. These findings may reflect a systemic influence of the environment either modulated systemically by the tumor itself or through paracrine and/or endocrine mediators from adipocytes and adipose tissue. It is well established that OPG is secreted primarily by osteoblasts but also by adipocytes [111]. In conditions of excess adiposity, some reports mentioned increased marrow adipocyte count and hyperthrophy [112]. The mechanism involves PPARγ-mediated preferential mesenchymal stem cell differentiation into adipocytes

while simultaneously inhibiting osteogenesis [73]. This guidance towards adipogenesis induced by excess adiposity might also be responsible for the upregulation of OPG secretion in the bone marrow, particularly through leptin, which has been shown to influence OPG production. The increased disposal of OPG in OW/OB's marrow is likely to decrease RANKL bioavailability, and subsequently reduce osteoclastogenesis. This reduction may account for decreased bone resorption and release of growth factors and calcium [108, 113, 114], concurring towards a suppressive effect on metastasized tumor cell growth.

HL has a unique pathological feature characterized by a minority of malignant H-RS (0.1 - 1%) able to express receptors of a variety of cytokines/chemokines [115]. The cytokine network in HL-affected tissues has been described to be important not only for autocrine proliferative stimulus on HRS cells, but also for the maintenance of a favorable environment. ILs (e.g., IL-1α, IL-3, IL-4, IL-6, IL-7, IL-8, IL-9, IL-10, IL-13), chemokines (thymus- and activation-regulated chemokine CCL17, TARC, CCL22, MDC), M-CSF, TNF-α, TGF-β, soluble CD30 and chitinase-3-like protein 1 (YKL-40) are among the many cytokines that are expressed and influence the survival of HRS cells [18, 19, 29, 103].

Under this scenario, we found lower levels of IL-1a, IL-1b, IL-8 and IL-12 in interstitial marrow fluid of patients with HL. The mechanisms of immune escape of HRS cells are notable, preventing them from toxicity by inhibiting cytotoxic T and NK cells, as well as promoting Treg and Th2 cells [10, 115]. Other immune escape mechanisms include the overexpression of FAS ligand (CD95L), a member of the TNF receptor family, which plays a key role in promoting programmed cell death (apoptosis) and is activated by its ligand Fas-ligand (FasL), inducing apoptosis in cytotoxic T lymphocytes and the loss of expression of HLA class I and II molecules [116]. So, the down-expression of CD95 (both in HL normal weight and overweight / obese) allows an anti-apoptotic mechanism of the CD95+ cytotoxic T cells.

The low-grade chronic inflammation characteristic of obesity also represents a contribution to immunomodulation. Accumulated macrophages in adipose tissue are thought to be responsible for the obesity-induced inflammation and their infiltration is strongly correlated with body mass index

and total body fat. There, these recruited macrophages express high levels of inflammatory factors contributing to systemic inflammation and insulin resistance [117], including IL-12, IL-23, TNF-α, iNOS and MHCI/II. In inflammatory microenvironments, as in tumors, the macrophage inflammatory protein 1 alfa (MIP-1a) and 1 beta (MIP-1b) are potent chemoattractants for monocytes, contributing to the recruitment of this important component of of tumor tissue stroma [118]. Therefore, the reduced expression of MIP-1b/CCL4 in HL OW/OB subjects may influence the quality and quantity of immune-cell infiltration and, consequently, a non-proliferative milieu for HRS cells. Indeed, the production of CCR5 ligands (CCL5, CCL3 and CCL4) by stromal cells has been identified to contribute to H-RS cells proliferation [119].

Hitherto, the obesity-associated immunoinflammatory mechanisms suggest an unfavorable microenvironment for the growth of HRS cells in the bone marrow. Concordantly, we observed that pro-inflammatory adipokines, IL-8, IL-1β and IL-12 were also underexpressed independently of obesity status. Data from previous studies suggest that increased serum cytokines associate with disease relapse and inferior survival in HL [18, 19, 103], although the specificity of bone marrow microenvironment [120] may alter this profile, implicating adipocytes as negative regulators of the bone-marrow microenvironment, as previously reported [21]. IL-12 has anti-tumoral effects, such as differentiation of T helper cells type 1 (Th1) cells, stimulation of interferon (IFN)-gamma and tumor necrosis factor (TNF)-alpha production, enhancement of natural killer (NK) cell's cytotoxic activity, and anti-angiogenic activity. Furthermore, the macrophages associated with tumors produce IL-10, suppressing IL-12 expression, which favors tumor escape from immune surveillance [121]. Thus, the decreased expression of IL-12 in the IMF of HL patients is likely to represent a negative microenvironment for the settlement of HL metastatic cells. Another proinflammatory adipokine is IL-1β, which was observed here to be down-regulated, therefore contributing to attenuated metastasis via modulation of activator protein 1 (AP-1) and nuclear factor – IL6 (NF-IL6) [122].

Moreover, the bone modulation through the over-expression of OPG in HL overweight / obese, which induces a disruption of RANKL/RANK autocrine loop, influences the expression of IL-8 [113], committing the typical neutrophil

infiltration on HL microenvironment. Analyzing only in the association of HL clinicopathological characteristics with adipokines levels in the IMF, just IL-8 was border ($p = 0.052$) associated with B symptoms, contrary to previous reported in serum [103].

In the context of the association bone marrow-adipocytes, we found the hormone leptin was significantly lower in interstitial marrow fluid of HL patients. Leptin is one of the most important adipokines, with a well-described role in the regulation of obesity in the central nervous system, although its peripheral actions remain incompletely understood. In the bone marrow, its receptor (LepR)-expressing mesenchymal stromal cells are the major source of bone and adipocytes in adult bone marrow [123]. In addition, leptin favors osteogenesis [124]. Previous studies, reported that serum leptin levels were negatively correlated with international prognostic score (IPS) in HL [102], whereas in the present study bone marrow leptin levels significant decreased – by 12.6-fold in HLs. Taken together, these findings suggest that while increased circulating leptin correlates with better clinical prognosis, the decreased leptin in the bone marrow might reflect a protective effect for colonization by malignant cells. Indeed, the role of leptin in the promotion of hematologic tumorigenesis through activation of the PI3K/Akt, ERK and/or Jak2/STAT3 pathways, have been described [125]. Interestingly, the simultaneous downregulation of OPG and Leptin we observed, may underline the crosstalk between bone and fat, namely an activation of bone resorption.

Growth factors have been described as inducers of growth and survival of malignant cells, and as intervenients in tumor cell dissemination [126]. In HL, the Treg and H-RS cells produce IL-10 and TGF-β, exerting inhibitory effects on T-cell effector functions, especially on cytotoxic T lymphocytes (CTLs) [127]. Thus, as we noticed, and according to others, the underexpression of growth factors such as TGF- β and FGF-6 in HL may confer protection to establishment of metastasis [128].

The IGF axis has been one of the most extensively implicated pathways in obesity-cancer association. The onset of insulin resistance and compensatory hyperinsulinemia during obesity may explain cancer promotion by stimulating cell proliferation and inhibiting apoptosis through IGF-IR-mediated signaling mechanisms [129]. Elevated insulin levels lead to increased

bioavailable IGF-I, being synthesized and secreted by the liver and other organs, as well as, by tumors.

Besides its autocrine and paracrine actions, IGF enters the bloodstream and binds to specific proteins to form binary complexes with each of the six IGF binding proteins (IGFBPs).

IGF-I has been described to have a special relevance in shaping and sustaining the microenvironment of HL. IGF-1 expression has been demonstrated in HL, by the cells composing the microenvironment and by tumor cells, whereas its receptor (IGF-1R) immunoreactivity varied in H-RS cells [129]. Thus, while IGF-1 is produced by the tumor microenvironment and H-RS cells express the IGF-1R, we observed a down-regulation of IGBPs (IGFBP-1, IGFBP-2 and IGFBP-3). Together, these findings will likely result in increased free IGF-1 availability in the bone marrow, upregulating ligand-receptor activation in H-RS cells. Despite the activation of IGF-1/IGF-1R in HL cell lines was oncogenic, the upregulation of IGF-axis has been reported to be a significant predictor of a favorable outcome [130, 131]. While the explanation for these findings remains unknown, new research endeavours should focus on the resulting crosstalk between HL malignant cells and tumor microenvironment players, including adipocytes.

Interestingly, IGFBP-1 and IGFBP-3 in bone marrow were well correlated with serum, and were found to be inversely associated with abdominal perimeter, but not with BMI, even though BMI and abdominal perimeter were strongly correlated. Therefore, regional depots of adipose tissue (visceral adiposity) may have a stronger influence on distant microenvironment and be a better marker for obesity-associated HL.

Although we present novel data on the characterization of adipokine profile in IMF of obese and HL subjects, and identify the most relevant biological axes behind the relationship between obesity and HL, our results should be interpreted in the context of several potential limitations. The reduced sample size, particularly for validation of adipokines in IMF and for the correlation with peripheral blood levels, advises cautious interpretation of data, even though Hodgkin's lymphoma is a rare haematological malignancy. The estimated adipokine quantification has had an exploratory intent, without having the opportunity to validate our findings in a completely new and separate set of

patients and controls. Nevertheless, selection of patients and controls to include in the array was carefully age-, BMI-, and gender-matched, in order to minimize group heterogeneity. Therefore, further studies in IMF from independent populations and with utmost BMI phenotypes are required. Our OW/OB population was mostly composed of OW subjects (BMI, 25-30 Kg/m^2). Use of additional and more sensitive methods to evaluate adiposity (e.g. bioimpedance analysis, DEXA, visceral adiposity using CT scan) would yield a better stratification of excess adiposity for array analyses.

Once in our population no HL patients were positive for bone metastasis, we were unable to test further the hypothesis of bone marrow microenvironment response to the presence of tumor cells. The continued accrual of samples from patients and controls will likely result in added bone marrow samples from patients with bone metastasis. Then, a retrospective comparative analysis can be performed with present results in IMF from negative-bone metastasis HL patients.

Overall, the bone marrow microenvironment in HL may play a role allowing the H-RS cells to survive, by providing an environment that suppresses cytotoxic immune responses. This exploratory study, identified that underexpressed adipokine pathways in the bone marrow are likely modulators of immunoinflammatory (Fas/CD95, IL-12, IL-1 β, IL-8, MIP-1b/CCL4), hormonal (leptin), proliferative (TGF-β, FGF-6 and IGF-axis binding proteins, IGFBP-1, IGFBP-2, IGFBP-3) mechanisms, which ultimately convey protection to the establishment of metastatic disease in HL. Furthermore, the adipocyte-bone crosstalk, represented by leptin and OPG interaction, are likely to impact bone remodeling and consequently the metastatic phenotype.

CONCLUSIONS

6. CONCLUSIONS

This study addressed the causal association of obesity and Hodgkin's lymphoma (HL) from the perspective of bone marrow microenvironment. Its exploratory nature in a rare biological sample prompted the use of a protein screening array, which simultaneously analyzed 62 adipokines in a specific environment as the bone marrow fluid.

Overall, findings from this study agree with a very limited number of reports on obesity as a good prognostic factor for HL, albeit in contrast with the established evidence of obesity as risk factor for developing HL. Taken together, the low pro-inflammatory profile, together with decreased growth factors and leptin, and with upregulated IGF-axis activation, we found in the bone marrow of obese HL patients are key characteristics of an unfavorable environment for metastatic seeding. In obesity, we expect to unveil that these adipokines are produced by adipocytes in bone marrow and to demonstrate they may interfere with metastatic HL microenvironment. This potential protective effect of obesity on bone marrow microenvironment of HLs applies exclusively to prognosis, rendering obesity-Hodgkin Lymphoma association another paradoxical case of obesity protective influence in cancer.

In order to increase the precision of assessment of obesity-HL causal relationship, here we propose that stronger evidence would emanate from using more precise methods for evaluating both whole body and visceral fatness.

FUTURE PERSPECTIVES

7. FUTURE PERSPECTIVES

The exploratory nature of present work and the contour of results, urges for continuing the recruitment and accrual of bone marrow samples from HLs and donnors. This will allow, primarily an independent validation of results and then, because of higher probability to include metastatic patients, to study a group of patients with bone metastasis. Measurement of adipokines in the bone marrow of this group will contribute to uncovering the resulting effect of the crosstalk in marrow microenvironment that includes H-RS cells.

In order to understand with higher precision the effect of obesity in HL, future studies that include patients should use more sensitive and specific methods for measuring adiposity (DEXA, bioimpedance, CT scan, …), both from local and general perspectives. As for HL risk, the impact of obesity in HL prognosis warrants further research, requiring the need to follow-up patients using more adequate adiposity measurements.

Following the characterization of adipokine's profile in the bone marrow fluid of HL patients and/or obese, the ensuing step will be to ascertain which cell population, marrow adipocytes or stromal cells, is responsible for the altered expression of studied adipokines.

In parallel, *in vitro* studies using the HL cell line L428, should be undertaken to evaluate the impact of recombinant adipokines, which were previously shown to be deregulated, in cancer hallmarks (e.g., proliferation, apoptosis, invasion, motility, …). Furthermore, using dataset from a retrospective HLs' cohort that includes paraffin-embedded matched primary and metastatic HL tumors, will allow clinical validation of altered adipokine pathways.

In a larger population, we expect to determine a multilocus genetic risk score from altered adipokine pathways in HL, to improve risk prediction and prognosis in a continuously increasing population of obese subjects. This risk score should include besides genetic markers, other demographic and clinicopathological variables, such as Epstein Barr virus quantification.

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8. REFERENCES

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APPENDICES

9. APPENDICES

9.1. Paper I

MECHANISMS UNDERLYING THE ASSOCIATION BETWEEN OBESITY AND HODGKIN LYMPHOMA

Andreia Matos [1,2], Joana Marinho-Dias [3,4], Sofia Ramalheira [5], Maria José Oliveira [6,7], Manuel Bicho [1, 2], Ricardo Ribeiro [1,2,3]

[1] Genetics Laboratory and Environmental Health Institute, Faculty of Medicine, Universidade de Lisboa, Lisbon, Portugal

[2] Rocha Cabral Institute, Lisbon, Portugal

[3] Molecular Oncology Group, Portuguese Institute of Oncology Porto Centre, Porto, Portugal

[4] Abel Salazar Biomedical Sciences Institute, University of Porto, Porto, Portugal

[5] Oncohematology Department, Portuguese Institute of Oncology Porto Centre, Porto, Portugal

[6] i3S-Instituto de Investigação e Inovação em Saúde/INEB-Institute of Biomedical Engineering, University of Porto, Porto, Portugal.

[7] Department of Pathology and Oncology, Faculty of Medicine, University of Porto, Porto, Portugal.

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Corresponding authors:

Dr. Andreia Matos
Genetics Laboratory
Faculty of Medicine, University of Lisbon
Avenida Prof. Egas Moniz
1649–028 Lisbon
Portugal
Phone: + 351 217 999 449
Fax: + 351 217 999 451
Email: andreiamatos@medicina.ulisboa.pt

Professor Ricardo Ribeiro
Genetics Laboratory
Faculty of Medicine, University of Lisbon
Avenida Prof. Egas Moniz
1649-028 Lisbon
Portugal
Phone: + 351 912 157 736
Fax: + 351 217 999 451
Email: oriebir.r@gmail.com

ABSTRACT

A solid body of knowledge indicates that overweight and obese subjects are more prone to have cancer, aggressive disease and death than their lean counterparts. Even though obesity has been causally associated with many cancers, only few studies looked for the link with Hodgkin's Lymphoma (HL). Recent findings from meta-analysis and prospective studies demonstrated an increased risk for subjects with higher body mass index to develop HL. Underlying epidemiological evidence, it is known that excess adiposity correlates with increased production of pro-tumoral and pro-angiogenic adipokines and is characterized by a chronic inflammatory state. Since inflammation is a cornerstone in HL development (pathophysiology) and aggressiveness, the role of adipose tissue, either through endocrine or paracrine mechanisms and both at its primary site and in bone marrow, must be of importance.

In this hypothesis generating review we explore the association between excess adiposity and HL, in light of recent basic and clinical data, to create a basis for understanding the mechanisms that underlie this epidemiological association, and to untangle hazy routes, fostering applied research. The establishment of excess adiposity as a significant risk factor for HL will determine public health preventive measures to fight obesity and eventually anti-obesity neoadjuvant therapeutic approaches in HL patients.

INTRODUCTION

Obesity has been described as a cause of morbidity and mortality and it is estimated that the number of deaths caused by cancer will double by 2030, as a result of the obesity epidemic [1]. The association between obesity and many cancer types has been consistently established [2]. In Hodgkin's Lymphoma (HL), despite an apparently variable association with overweight and obesity (according to the body mass index-based World Health Organization classification) [3-7], a contemporary meta-analysis on prospective studies established a significant association between body mass index (BMI) and risk for HL [8]. Concordantly, a new prospective study in over 1 million individuals demonstrated increased risk for HL development for each 10 $Kg.m^{-2}$ units of increase in BMI [9]. Although epidemiologically obesity seems to be associated with HL, the biological rationale and mechanisms behind this causal relation remain largely unexplained.

Hodgkin's Lymphoma is characterized by an inflammatory microenvironment at the lymph nodes primary tumor site [10, 11]. The distinctive HL's malignant Hodgkin & Reed-Sternberg (HRS) cells reciprocally interact with the inflammatory milieu resulting in tumors with survival and evasion advantages [10]. Besides age, inflammation and Epstein-Barr virus (EBV), little is known concerning predictive factors of HL aggressiveness [12, 13]. Noteworthy, despite the high cure rate, in advanced stage disease approximately 25% to 30% of patients are not cured with standard therapeutic regimens alone, and about 20% of the patients still die after relapse or disease progression [12, 14]. In advanced stages, where bone marrow infiltration is common, the international prognostic score and other risk stratification instruments

remain only modestly effective to predict disease progression and therapeutic response [15].

Excess adiposity is regarded as a chronic inflammatory state, hence excess adiposity-mediated inflammation may play a role in promoting HL growth, survival and tumor immune evasion [10]. We hypothesize that this accumulation of immune cells in HL, including T and B cells, neutrophils, eosinophils, and mast cells might be disturbed by excess adiposity, which is known to modulate the immune system function [16, 17]. In addition to immunoinflammatory derangements, there is a systemic metabolic dysfunction in obesity that prompts altered circulating levels of pro-tumoral adipokines. Indeed, some of these adipokines have been associated with HL and advanced stages of disease [18-20]. Albeit the mechanisms of the causally invoked association between obesity and HL remain undetermined, a reasonable biological rationale supports plausibility for the relation of adipose tissue-inflammation-HRS axis with HL aggressiveness. Taken together these obesity-linked endocrine and paracrine mechanisms may create a favourable microenvironment for growth and spread of HRS malignant cells. Moreover, albeit still experimentally unproven, it is expected that the known paracrine action of adipocytes in the bone marrow microenvironment [21, 22] might facilitate HRS cells to metastasize and grow within the bone marrow microenvironment.

Future research should establish definite biological mechanisms involved in the association between excess adiposity and HL, which will foster the development of new molecular markers based on adipokine's pathways as indicators of clinical outcome and incorporated into prediction models. If confirmed, the effects of bone marrow adipocytes in metastatic HL cells might represent new potential therapeutic targets in this advanced stage of disease.

MECHANISMS PROMOTING HODGKIN LYMPHOMA'S: LESSONS TO ESTABLISH NEW DRIVERS

HRS cells, despite their small representativeness in HLs (about 1%), are modulated by a mixed inflammatory microenvironment that influences tumor development [10, 23]. These unique features of HL are far outweighed by reactive and stromal cells [10]. Inflammation, among others, has been recently considered as a cancer hallmark [24]. In fact, survival, proliferation and immunoinflammatory mechanisms seem to have an important role in HL pathophysiology [10].

The HL tumor microenvironment, in particular, the cytokine/chemokine pattern secreted by HRS cells and non-neoplastic circulating cells may influence the proliferation and survival of malignant cells [25, 26]. Among others, HRS cells secrete several interleukins (ILs) (e.g., IL-1α, IL-4, IL-6, IL-7, IL-8, IL-9, IL-10, IL-13), chemokines (CCL17, TARC, CCL22, MDC), macrophage colony stimulating factor (M-CSF), tumor necrosis factor-α (TNF-α) and β (TNF-β), transforming growth factor beta (TGF-β), soluble CD30 and chitinase-3-like protein 1 (YKL-40), with autocrine tumor growth effect [25]. From these molecules, thymus and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC), produced by HRS cells, are partially responsible for whittling HL microenvironment by inducing Th2 and Treg cell responses [25], which by exerting anti-inflammatory and immunosuppressive activities, have been associated with reduced tumor immunosurveillance [27]. Moreover, paracrine actions were uncovered through upregulated pathways mediated by cytokine receptors (IL-2R, IL-6R, IL-9R, IL3R), macrophage colony-stimulating factor receptor (M-CSFR), tumor necrosis factor receptors (TNFR1, TNFR2), CD30 and

CD40 [28]. As an example, downstream signalling through the IL-3/IL3R pathway induces growth and extends survival in HL cells [29]. Paracrine signals may arise from non-malignant tumor-infiltrating cells in HL microenvironment (e.g., eosinophils, mast cells, neutrophils and macrophages) [10]. The nuclear factor-kappa B (NF-kB) and Janus Kinase-signal transducer and activator of transcription (JAK-STAT) pathways have been identified as important modulators in HL [26]. Activation of the NF-kB pathway, which is involved in the expression of multiple anti-apoptotic factors and pro-inflammatory cytokines, reduces the expression of CD99, a marker associated with HRS cells phenotype [10]. This cell surface receptor is involved in Tcell adhesion, leukocyte migration, and Tcell caspase-independent cell death, playing also a role in the induction on human thymocytes of Tcell receptor (TCR) and Major histocompatibility complex Class I and II molecules expression. It has been reported that CD99 deficiency leads to the arrest of MHC Class I molecules at the Golgi complex, impairing their transport to the cell surface, which constitutes one of the most frequent immune escape mechanisms adopted by cancer cells [132]..Some authors identified candidate-circulating biomarkers for HL, with prognostic and/or predictive response to therapy values. These molecules act at HL site and modulate disease outcome [30-32]. Furthermore, it was found that before treatment, HL patients have increased levels of serum interleukin 6 (IL-6) and YKL-40, both facts correlated with disease progression stage [18].

OBESITY AND HODGKIN LYMPHOMA

Obesity is currently considered epidemic worldwide [33]. Overweight and obesity are defined as excessive accumulation of adipose tissue and are associated with

increased risk of morbidity and premature mortality. Besides other related diseases, epidemiological studies reported obesity as a risk factor for cancer [2, 34, 35]. In the United States of America the estimated risk of death from cancer in morbidly obese (BMI $\geq$ 40 kg/m^2) was 1.5 in men and 1.6 in women [34], whereas in Europe, obese have 1.5 to 3.5 higher risk of having cancer [1]. Alongside, it has been estimated that approximately 30% of cancer deaths might be related with dietary and behavioural factors, namely, high BMI, low intake of fruits and vegetables, lack of exercise, smoking and alcohol abuse. A recent meta-analysis based on prospective studies reported that obesity (BMI $\geq$ 30 kg/m^2) are positively correlated with risk of HL [8]. Indeed, a number of studies reported a positive association of obesity with hematologic malignancies, including Hodgkin's lymphoma [3-7, 9, 35], even though others have not found such an association [36-42]. Noteworthy, 2 studies found a protective role for obesity in the development of HL. Despite the batch of positive association studies between obesity and HL, it is important to highlight the time when the anthropometric assessments were made and the outcomes considered. Indeed, some authors consider that this potential protective effect of lower BMI in cancer may be due to the effects of cancer-related cachexia, a more deleterious than the potential adverse events related to a higher BMI [131]. Obesity may increase the risk for developing HL, even though its effects in survivors might be paradoxal, thus influencing the natural history of disease [130].

Specifically in HL, the intake of saturated fats, which is also related with excess adiposity, can modulate the immune function through an anti-apoptotic action on T cells and increased expression of pro-inflammatory molecules [133]. Nevertheless, most studies rely on body mass index measures and World Health Organization BMI-based classification of obesity, which are imperfect estimates of adiposity and disease risk

[44-46], and do not account for local fat depots (e.g., visceral adipose tissue), which were shown to have adverse specific adipokine expression profiles, contributing towards more aggressive tumors [47-49]. Future epidemiological studies should address these issues by using more precise methods (e.g. visceral and subcutaneous fat determinations by computed tomography scan, whole body fatness by tetrapolar bioimpedance, or local and whole body fat measurements through magnetic resonance imaging, among others) to evaluate whole and local body fatness in association with HL.

Presently, the adipocyte is no longer considered a passive component of the human metabolism. It is known as an endocrine/paracrine c that exerts many biological effects, through production of growth factors, cytokines, chemokines and hormones [50]. These biologically active molecules secreted primarily, partially or exclusively by adipocytes, known as adipokines, have a significant role in regulating tissue angiogenesis and tumour growth [51]. In fact, the importance of the interaction between cancer cells and the surrounding stroma cells has been increasingly accepted. These interactions are particularly prominent in environments rich in adipocytes [52]. The excess body fatness is characterized by a chronic low-grade inflammatory state with altered circulating levels of adipokines, including IL-6, IL-8, leptin, adiponectin, TNF-α, vascular endothelial growth factor (VEGF), osteopontin (OPN), haptoglobin (Hp), YKL-40, among others [53-55] (Figure 1). These molecules impact cancer cell-related mechanisms such as proliferation, apoptosis and migration [56, 57]. The altered secretion of adipokines in obesity deranges metabolic homeostasis, together with influences in immunological status [58] (Figure 1). In this context, the evaluation of markers related to obesity and immune response in HL, might reveal new opportunities for understanding the mechanisms responsible by the association between obesity and

HL. Various adipokines have already been shown to be linked with HL risk and with advanced stage of disease, namely IL-6 and interleukin 7 (IL-7) [18, 19, 59], while others endure as promising targets for future studies (e.g., leptin, adiponectin, resistin, HGF, visfatin, …). Thus, the chronic inflammation sustained by expanding adipose tissue may modulate the host immunosurveillance [16] and exert a direct effect on both the local tumor microenvironment and on distant tumor cells, through the systemic effects of paracrine signals (Figure 1).

Angiogenesis is a well-established hallmark of tumor development both in solid tumours and hematological malignancies (including HL). Reasonable data, mostly supported by retrospective immunohistochemistry evaluations, stands for a relevant role of angiogenesis in HL. In this pathology, a shift towards an angiogenic phenotype is observed as result of unbalanced angiogenic versus anti-angiogenic stimulus [60]. Interestingly, many of the adipokines overexpressed by adipose tissue in obesity individuals are well-known for their potent pro-angiogenic effects [53]- Therefore, it seems plausible that these adipokines might mediate the causally invoked association between excess adiposity and HL, through a modulatory effect in angiogenesis (Figure 1).

Besides adipokine secretion, the expanding adipose tissue is also infiltrated by macrophages ongoing M2-to-M1 differentiation, further contributing towards the obesity-associated systemic chronic inflammation and insulin resistance [58, 61] (Figure 1). Leptin and adiponectin, hormones exclusively produced by adipocytes, and with opposing effects in obesity and cancer, contribute to the polarization of macrophages with a pro-inflammatory phenotype [62] and towards anti-inflammatory M2 [63], respectively. Interestingly, in HLs the presence of $CD68^{+}$ tumor-associated macrophages indicates poor prognosis [23, 64] and higher adiponectin levels were

found in paediatric HL patients [65]. Recently, the soluble circulating CD163 and TARC were identified as possible biomarkers of HL [30]. Also interestingly, CD163 is a known marker for M2 macrophages polarization and a receptor for Hp, which is a major acute phase protein overexpressed in conditions such as obesity and HL [66, 67]. Nonetheless, Hp is induced not only by HL-associated IL-6 downstream transcription factor STAT3, but also by hypoxia-inducible factor-1α (HIF-1α) that is overexpressed in HL [68]. Therefore, excess adiposity seems to interfere with tumor cell's signalling pathways and to modulate macrophages differentiation, both of which may impact the tumor. This obesity-driven inflammatory environment exerts tumor-promoting effects, due to alter inflammation pathways implicated in cell proliferation, survival, angiogenesis, and metastasis associated with cancer (Figure 1). Taken together, these evidences demonstrate potential unrevealed links that should foster experimental validation in order to uncover the impact of these obesity-associated molecules in HL.

BONE MARROW ADIPOSITY AND HODGKIN LYMPHOMA - BONE MARROW ADIPOCYTES HAVE A ROLE IN METASTATIC NICHE?

Adipocytes in the bone marrow have been implicated as regulators of marrow microenvironment [21, 69], and present a distinctive phenotype, which resembles both, white and brown adipose tissue [70]. Besides representativeness, bone marrow adipocytes present an unilocular lipid morphology similar to adipose tissue, and are a unique adipose depot that overexpresses genes associated with cell differentiation and with inflammation [71, 72]. Amplified bone marrow adiposity, due to diet-induced obesity in mice, was recently implicated in altered bone metabolism, and inflammation within the bone microenvironment [73]. Adiposity in bone marrow is modulated by

high fat diet, diabetes, aging, dyslipidemia and obesity, through diverse pathways that comes together to regulate the expression and activity of a key pro-adipogenic transcription factor, the peroxisome proliferator-activated receptor γ2 (PPAR-γ2) [74].

Bone marrow adipocytes behave as energy suppliers to bone physiological functions, including bone remodelling [69, 75]. In addition to energy storage, these adipocytes secrete adipokines and fatty acids that impact significantly on metabolism and function of other neighboring cells, present within the bone microenvironment [72, 76]. From this interaction in bone marrow milieu, an inverse relationship has been described between osteoblastogenesis and adipogenesis, with a negative correlation of marrow adiposity with osteoblast number and bone mineral density [74]. In fact, several factors produced in the bone marrow may exert a regulatory role in local adipocytes, as well as adipokines secreted in marrow adipocytes might influence other cellular players through a paracrine effect [76] (Figure 2).

Hodgkin's lymphoma involving the bone marrow ranges between 2 to 32%, with an average incidence of 10% [77]. Although the chronic low-grade inflammation and the upregulated secretory profile, associated with obesity, may exert endocrine effects, we should not overlook paracrine actions of adipocytes in bone marrow microenvironment bearing metastasis from primary HL tumors [22]. The crosstalk between adipocytes and cancer cells has been demonstrated to support progression and aggressiveness of tumors in other oncologic models [52, 78-80], as well as metastatic cell growth in the bone marrow [81]. Fat cells seem to be able of translocating stored lipids to metastatic tumor cells, ultimately driving cancer growth and motility [78, 82]. The complex interaction between components of bone marrow, including adipocytes and eventually tumor cells, is depicted in Figure 2.

In other oncologic models, the fatty acid binding protein 4 (FABP4) was

implicated in adipocyte-tumor cell interactions [78, 81]. Notwithstanding FABP4 is transcriptionally regulated by PPARγ, this lipid chaperone also controls PPARγ [83, 84], while both seem to be involved in adipocyte-induced metabolic switching with the in cancer microenvironment. Interestingly, in B lymphoma cells the decreased PPARγ expression was related with increased proliferation and survival, and initiation of inflammatory pathways, specifically the activation of nuclear factor kappa B (NFκB) [85], further underline the link between cellular metabolism and neoplastic progression.

Primary cancer-derived metastases that home to the bone are by themselves incapable of inducing bone resorption. However, these aggressive malignant cells interact with bone constituents and influence the function of bone-degrading cells (osteoclasts), inducing osteolytic lesions [86]. Bone metastases from HLs have been described as osteolytic [87, 88]. The complex interaction of tumor cells with bone marrow microenvironment, including adipocytes, exerts profound influence in proteolytic degradation and bone resorption, enabling metastasis allocation. Obesity and aging are known effectors of bone remodelling by forming adipocytes instead of osteoblasts, which will lead to increased osteoclast activity and osteoporosis [76, 89]. A key enzyme for bone matrix osteoclastic bone resorption is cathepsin K (CTK) that degrades the bone matrix protein collagen I and other proteins of the [90]. CTK expression within the bone marrow milieu is high in osteoclasts and adipocytes, and results in accelerated bone turnover [91] and in a potential contribution to the metastatic process. CTK production was also described in cancer cells that metastasize to bone [92]. CTK acts by upregulating the processing of its substracts extracellularly, including the secreted protein acidic and rich in cysteine (SPARC or osteonectin) that interacts with collagen I and other matrix proteins to attract and anchor malignant cells in the bone [91]. In addition, VEGF, a growth factor known to be involved in tumor cell

migration and in osteoclast differentiation and migration has been proposed to be modulated in the bone microenvironment by CTK cleavage [90, 93]. Besides the key role of CTK in degrading collagen I, it also seems to be relevant for adiponectin cleavage, which may be a mechanism to promote osteoclastogenesis via increased expression of receptor activator of NFκB ligand (RANKL), to modulate marrow fatness, or to influence adiponectin-mediated suppression of tumorigenesis [94, 95]. On their tutone marrow adipocytes also stimulate osteoclast differentiation and activity by directly secreting RANKL [96].

Bone marrow adipocytes are a significant secondary source of leptin and IL-6, whereas only trace amounts of IL-1β and TNF-α were found [97]. These molecules exert interactive regulatory mechanisms between them, in order to modulate the marrow environment, controlling the proliferation and differentiation of hematopoietic precursors as well as the maturation of stromal cells [98].

Given the importance of JAK/STAT signalling in HL malignant cells and that leptin and IL-6 downstream signals are mediated by this pathway [99, 100], we hypothesize that these adipokines might influence HRS cell survival and proliferation, both through an endocrine mechanism in lymph nodes and by a paracrine effect in the bone marrow. Since leptin and IL-6 are upregulated in the serum of obese subjects, they can partially explain the association between excess adiposity and HL. Only few unpowered studies have measured serum leptin and adiponectin levels in HL patients, mostly children, with inconclusive mixed results [65, 101, 102]. With respect to IL-6, several reports demonstrated that it was a relevant cytokine for HRS proliferation and survival and a useful biomarker of aggressiveness [103, 104]. It is largely unknown whether bone marrow adipocytes behave differently in presence of HL malignant cells. Thus, further investigation on the interactions of bone marrow adipocytes with HRS

cells is required to clarify many unanswered questions and advance knowledge with potential clinical translation.

Here, we hypothesize that when HRS cells are metastasized to bone marrow, resident adipocytes may have a role in bone remodelling, yielding tumor cells with adipokines and fatty acids to boost growth and survival, concurring towards worst prognosis. Further clinical studies should follow disease behaviour in HL obese patients and evaluate the impact of intervening in obesity on HL prognosis. Therefore, obesity and its effect on bone marrow adipocytes may represent a potential therapeutic target in the future.

FUTURE PERSPECTIVES

Obese subjects are at increased risk of progression of certain cancers, as seems to be the case of HL. Knowledge of adipocyte biology in the context of malignancy is therefore crucial for understanding the pathophysiological basis of obesity associated HL. The excess adiposity setting provides a unique microenvironment with concomitant systemic endocrine alterations that may, by affecting cancer cell activities or by impairing tumour immunosurveillance, favor tumor progression [134]. The reprogrammed tumor-educated behavior of adipocytes likely provides a tumor-permissive metabolic, inflammatory, fibrotic and angiogenic microenvironment that promote both primary and metastatic HRS cells survival. Therefore, understanding the heterotypic interactions between adipocytes and HRS cells could lead to the identification of further novel targets for HL therapy.

Although intuitively relevant for future studies, the effects of lifestyle intervention in HL prevention and treatment are not yet clear. The impact of these

interventions on weight loss also offers a means for testing the reversibility of some mechanisms triggered by obesity. Thus, research endeavour to ascertain the influence of weight loss and other lifestyle changes (e.g. physical exercise or diet) on HL risk or outcomes is both required and feasible. Randomized controlled trials including physical exercise, nutrition or both interventions are urgently needed to guide recommendations for susceptible groups and for HL survivors, and to support implementation of chemoprevention strategies and weight management programs. Indeed, the Clinician-patient relationship after cancer diagnosis is an opportunity to encourage lifestyle modifications, which might impact cancer recurrence, risk of other diseases and overall quality and length of life [135].

Targeting adipose tissue with anti-obesity and/or antidiabetic agents, such as metformin and thiazolidinediones, might be a promising option to treat patients with cancer who are overweight or have obesity. However, despite metformin is highly active against cancer growth, so far there are neither experimental studies nor clinical trials in HL. Accordingly, also for thiazolidinediones (a class of peroxisome proliferator-activated receptor γ agonists) that have growth-inhibitory effects on transformed cells, no studies have been conducted in HL. At a time of personalized cancer therapy, there is an unmet need for targeted therapeutic approaches for patients with obesity and cancer, namely HL. Thereby, future research should focus on the manipulation of adipocyte biology (e.g. enhance adiponectin synthesis, stimulate intra-adipocyte lipid oxidation and promote reactivation/conversion of brown adipose tissue) [136], in order to promote health and benefit patients. With a pace of discovery indicating that adipose tissue-cancer field is rapidly growing, with new insights unfolding for many cancer types, it remains to be seen how well we will translate these discoveries into HL, and further to HL patients.

CONCLUSIONS

Notwithstanding our present inability to explain potential mechanisms underlying the association between adiposity and improved prognosis following chemotherapy in HL, we believe that the role of adiposity should be explored in prospective cohorts. Although recent studies support a positive but weak causally related epidemiological association between obesity and HL, here we propose that stronger evidence would emanate from using more precise methods for evaluating both whole body and local fatness.

Though the molecular rationale has not yet been convincingly elucidated in the literature, inflammation is common to obesity and Hodgkin's lymphoma. In fact, the tumor microenvironment, particularly the repertoire of non-neoplastic cells and molecules produced by them, seem to exert a strong influence on tumor cell growth, evasion, survival and diversion of immunological mechanisms. The obesity-HL association could reflect the interaction of molecules produced by adipocytes in the tumor microenvironment and the HRS cells. Some molecules, such as IL-6, IL3, IL-7, TNF-α, YKL-40, and NF-kB and JAK/STAT pathways seem to support this association. In a topic where the literature has not provided the luxury of clinical trials and data, the development of new molecular biomarkers, based on adipokine pathways, may add information as clinical outcome predictors. Their incorporation into prognostic models may improve our understanding of the biologic correlates of obesity and HL. Moreover, if demonstrated, mediators of the effect of adipocytes in bone marrow metastatic Hodgkin’s lymphoma microenvironment represent an additional potential therapeutic target.

DECLARATION OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure 1. Endocrine effects of obesity impact Hodgkin Reed-Sternberg lymphoma cells. Excess adiposity modulates HRS aggressiveness in lymph nodes through a systemic effect mediated by adipokines and migrating adipose stem cells. In obesity states the adipose tissue acquires the following characteristics: hypertrophied adipocytes, neoangiogenesis with increased vessel density, infiltration with M1 type macrophages, increased amount of adipose stem cells and upregulated secretion of pro-tumoral adipokines whereas anti-tumoral adipokines are downexpressed (e.g. adiponectin, SHBG and LOX). The full black arrow represents adipokines and adipose stem cells entering peripheral blood. The circulation levels of these adipokines are significantly increased in obese (as opposed to adiponectin levels), reaching lymph nodes, where they may induce either direct effects to HRS cells through direct binding to cell receptors or indirect actions by interaction with cells in microenvironment modulating their crosstalk with HRS cells. Ultimately, adipokines may induce intracellular signalling pathways (represented by solid black arrows within HRS cell) and

mechanisms that will lead to angiogenesis, and to cell proliferation, cell migration, DNA damage or anti-apoptosis of HRS cells. AKT, Akt kinase; C/EBPβ, CCAAT/enhancer binding protein beta; CRP, C-reactive protein; ER, endoplasmic reticulum; ECM, extracellular matrix; FGF, fibroblast growth factor; FFA, free-fatty acids; HGF, hepatocyte growth factor; HRS, Hodgkin Reed-Sternberg cell; IGF-1, insulin-like growth factor-1; IL-1β, interleukin 1 beta; IL-6, interleukin 6; IL-8, interleukin 8; JAK, Janus kinase; LOX, lysyl oxidase; mTOR, mammalian target of rapamycin; MMP, matrix metalloproteinase; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein 1; NGF, nerve growth factor; NF-kB, nuclear factor kappa B; PAI-1, plasminogen activator inhibitor–1; PI3K, phosphatidylinositol 3'–kinase; PlGF, placental growth factor; PPARγ, peroxisome proliferator-activated receptor gamma; RANTES (CCL5), regulated on activation, normal T cell expressed and secreted; ROS, reactive oxygen species; SHBG, sex hormone binding globulin; STAT, signal transducer and activator of transcription; SDF-1, stromal derived factor 1; TGFβ, transforming growth factor beta; TNF-α, tumor necrosis factor alpha; VCAM-1, vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor

Figure 2. Hypothetical role of obesity and adipocytes in the bone marrow microenvironment invaded by HRS cells. This figure depicts the complex interaction between cellular components in the bone marrow and its mediators, particularly the contribution of bone marrow adipocytes to bone homeostasis and metastatic progression. Most adipokines produced in adipocytes (leptin, VEGF, osteopontin, TNF-α, MMP9, IL-1, IL-6, TGF-β, IGF-1, MCP-1) may contribute either directly (through an effect in HRS tumor cells mediated by their specific receptor downstream signalling

– impacting HRS cell motility, survival and proliferation) or indirectly (by influencing other cells in the microenvironment, including osteoblasts, osteoclasts, lymphocytes, macrophages, endothelial and mesenchymal stem cells, to acquire an pro-tumoral behaviour – immunological modulation, increased chemotaxis, cell differentiation, matrix reorganization, neoangiogenesis, increased osteoclast recruitment and activation, ultimately resulting in bone resorption and osteolytic metastatic lesions) to this stage aggressiveness and HL prognosis. Solid black lines with arrows represent secretion or the effect of a given adipokine, whereas dashed lines denote inhibitory action. Multiple light yellow arrows mean the reciprocal impact other cells might have in adipocytes. AdipoQ, adiponectin; CTSK, cathepsin K; DKK-1, dickkopf-related protein 1; FAs, fatty acids; IL-1, interleukin 1; IL-3, interleukin 3; IL-7, interleukin 7; IL-10, interleukin 10; MMP9, matrix metalloproteinase 9; MSCs, mesenchymal stem cells; OPG, osteoprotegerin; PGE2, prostaglandin E2; RANKL, receptor activator of nuclear factor kappa-B ligand; SPARC, osteonectin.

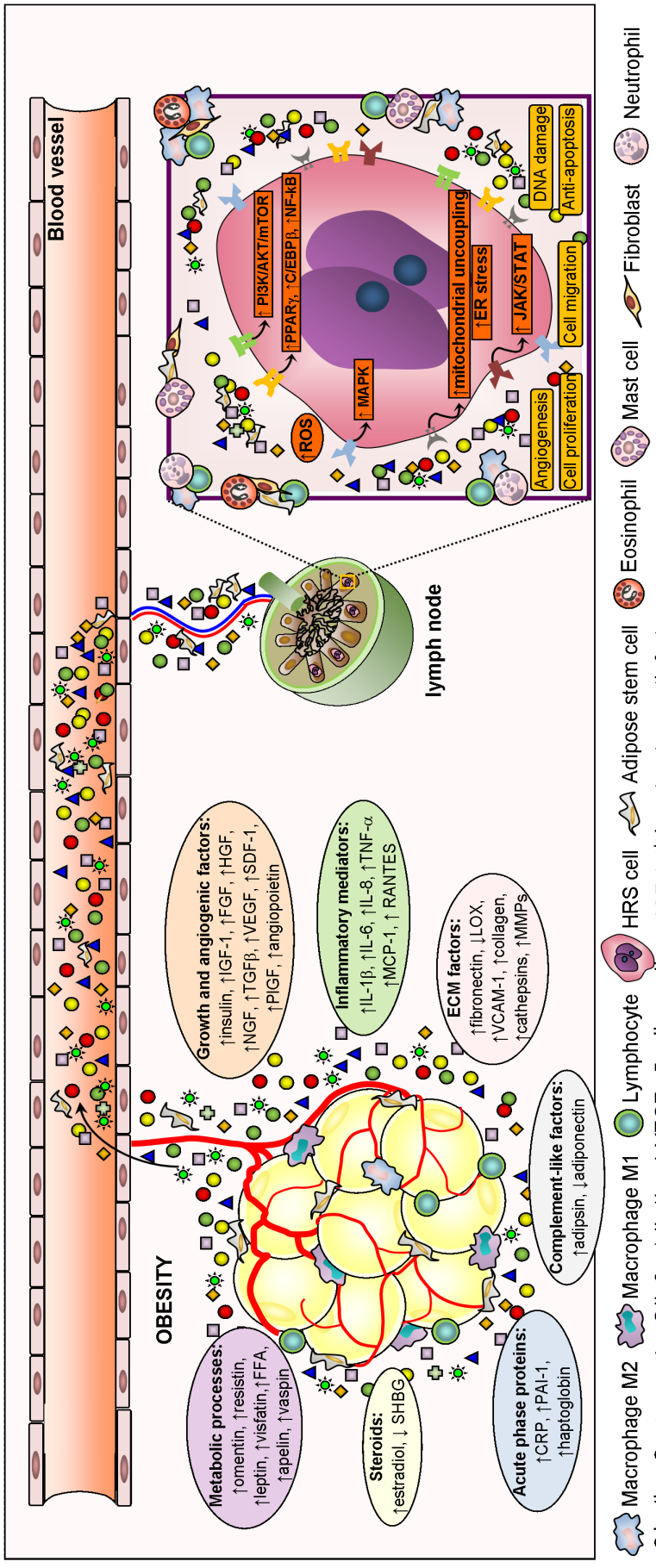
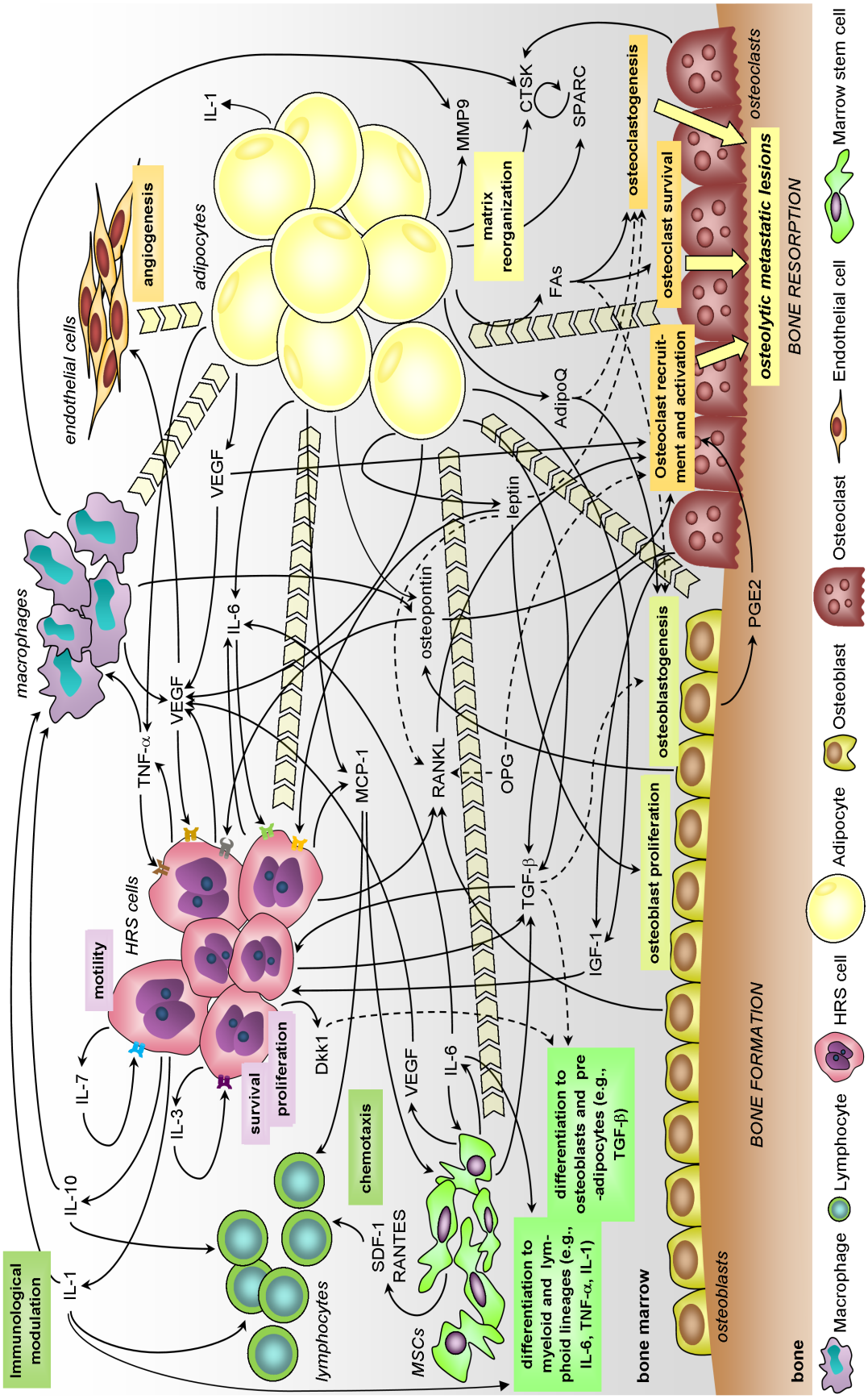


Figure 1: Endocrine effects of obesity impact Hodgkin Reed-Sternberg lymphoma cells.



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