

RODOLFO GONZALEZ CAMARGO

**Insulin resistance in Cancer cachexia and
Metabolic Syndrome: Role of insulin activated
macrophages and miRNA-21-5p**

Thesis presented to the Post-Graduate Program in Cell and Tissue Biology, Instituto de Ciências Biomédicas, Universidade de São Paulo and to the Post-Graduate Program in Nutritional Biochemistry, Institut für Ernährungswissenschaft, Universität Potsdam, to obtain the Ph.D. title.

São Paulo
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Area: Cell and Tissue Biology and Nutritional Biochemistry

Advisors: Dr. Marília Cerqueira Leite Seelaender and Dr. Gerhard Paul Püschel

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São Paulo
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**Resistência à Insulina na Caquexia Associada ao
Câncer e na Síndrome Metabólica: Papel dos
Macrófagos ativados pela Insulina e do miRNA-
21-5p**

Tese apresentada ao Departamento de Biologia Celular e do Desenvolvimento do Instituto de Ciências Biomédicas da Universidade de São Paulo e ao programa de Pós-Graduação em Bioquímica dos Alimentos do Instituto de Ciências dos Alimentos da Universidade de Potsdam para obtenção do título de doutor em Ciências.

Área: Biologia Celular e do Desenvolvimento e Bioquímica dos Alimentos

Orientadores: Dra. Marília Cerqueira Leite Seelaender e Dr. Gerhard Paul Püschel

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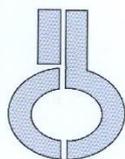
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São Paulo, 17 de maio de 2013.

PARECER 1109/CEP

A Comissão de *Ética em Pesquisas em Seres Humanos* do ICB, na data de 14.05.2013, **APROVOU** o projeto intitulado: "*Papel dos microRNAs do tecido adiposo subcutâneo no desenvolvimento e manutenção da caquexia associada ao câncer*" da pesquisadora *MARILIA CERQUEIRA LEITE SEELEANDER* e aluno *RODOLFO GONZALEZ CAMARGO*.

Cabe aos Pesquisadores executantes elaborar e apresentar a este Comitê, relatórios anuais (parciais ou final), de acordo com a resolução 196/06 do Conselho Nacional da Saúde, item IX. 2 letra c. conforme modelo constante no site.icb.usp.br.

Ao pesquisador cabe também finalizar o processo junto à Plataforma Brasil quando do encerramento deste.

O primeiro relatório deverá ser encaminhado à Secretaria deste CEP em **14.05.2014**.

Atenciosamente,

Prof. Dra. PAOLO M.A. ZANOTTO
Coordenador da Comissão de Ética em
Pesquisas com Seres Humanos - ICB/USP



São Paulo, 28 de julho de 2014.

Il^{mo}(a). Sr^a(a).

Profa. Dra. Marília Cerqueira Leite Seelaender
Departamento de Biologia Celular e do Desenvolvimento
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UNIVERSIDADE DE SÃO PAULO

REFERENTE: **Projeto de Pesquisa** "Papel dos microRNAs do tecido adiposo subcutâneo no desenvolvimento e manutenção da caquexia associada ao câncer"

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Pesquisador executante: Rodolfo Gonzalez Camargo

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Prezado(a) Senhor(a)

O Comitê de Ética em Pesquisa do Hospital Universitário da Universidade de São Paulo, em reunião ordinária realizada no dia 25 de julho de 2014, analisou o Projeto de Pesquisa acima citado, considerando-o como **APROVADO**, bem como o seu **Termo de Consentimento Livre e Esclarecido**.

Lembramos que cabe ao pesquisador elaborar e apresentar a este Comitê, relatórios parciais e final, de acordo com a Resolução nº 466/2012 do Conselho Nacional de Saúde, inciso XI.2, letra "d".

O primeiro relatório está previsto para 26 de janeiro de 2015.

Atenciosamente,

Dr. Mauricio Seckler
Coordenador do Comitê de Ética em Pesquisa
Hospital Universitário da USP

To my family, the reason for my existence and to the
hope that we can change the reality
of our country through education.

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“The most powerful force in the universe is faith”.

ABSTRACT

Camargo, RG. Insulin resistance in Cancer Cachexia and Metabolic Syndrome: Role of insulin activated macrophages and miRNA-21-5p. [Ph.D. Thesis (Cellular and Tissue biology)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2016. Potsdam: Institut für Ernährungswissenschaft, Universität Potsdam; 2016.

The ever-increasing fat content in Western diet, combined with decreased levels of physical activity, greatly enhance the incidence of metabolic-related diseases. Cancer cachexia (CC) and Metabolic syndrome (MetS) are both multifactorial highly complex metabolism related syndromes, whose etiology is not fully understood, as the mechanisms underlying their development are not completely unveiled. Nevertheless, despite being considered “opposite sides”, MetS and CC share several common issues such as insulin resistance and low-grade inflammation. In these scenarios, tissue macrophages act as key players, due to their capacity to produce and release inflammatory mediators. One of the main features of MetS is hyperinsulinemia, which is generally associated with an attempt of the β -cell to compensate for diminished insulin sensitivity (insulin resistance). There is growing evidence that hyperinsulinemia *per se* may contribute to the development of insulin resistance, through the establishment of low grade inflammation in insulin responsive tissues, especially in the liver (as insulin is secreted by the pancreas into the portal circulation). The hypothesis of the present study was that insulin may itself provoke an inflammatory response culminating in diminished hepatic insulin sensitivity. To address this premise, firstly, human cell line U937 differentiated macrophages were exposed to insulin, LPS and PGE₂. In these cells, insulin significantly augmented the gene expression of the pro-inflammatory mediators IL-1 β , IL-8, CCL2, Oncostatin M (OSM) and microsomal prostaglandin E₂ synthase (mPGES1), and of the anti-inflammatory mediator IL-10. Moreover, the synergism between insulin and LPS enhanced the induction provoked by LPS in IL-1 β , IL-8, IL-6, CCL2 and TNF- α gene. When combined with PGE₂, insulin enhanced the induction provoked by PGE₂ in IL-1 β , mPGES1 and COX2, and attenuated the inhibition induced by PGE₂ in CCL2 and TNF- α gene expression contributing to an enhanced inflammatory response by both mechanisms. Supernatants of insulin-treated U937 macrophages reduced the insulin-dependent induction of glucokinase in hepatocytes by 50%. Cytokines contained in the supernatant of insulin-treated U937 macrophages also activated hepatocytes ERK1/2, resulting in inhibitory serine phosphorylation of the insulin receptor substrate. Additionally, the transcription factor STAT3 was activated by phosphorylation resulting in the induction of SOCS3, which is capable of interrupting the insulin receptor signal chain. MicroRNAs, non-coding RNAs linked to protein expression regulation, nowadays recognized as active players in the generation of several inflammatory disorders such as cancer and type II diabetes are also of interest. Considering that in cancer cachexia, patients are highly affected by insulin resistance and inflammation, control, non-cachectic and cachectic cancer patients were selected and the respective circulating levels of pro-inflammatory mediators and microRNA-21-5p, a posttranscriptional regulator of STAT3 expression, assessed and correlated. Cachectic patients circulating cytokines IL-6 and IL-8 levels were significantly higher than those of non-cachectic and controls, and the expression of microRNA-21-5p was significantly lower. Additionally, microRNA-21-5p reduced expression correlated negatively with IL-6 plasma levels. These results indicate that hyperinsulinemia *per se* might contribute to the low grade inflammation prevailing in MetS patients and thereby promote the development

of insulin resistance particularly in the liver. Diminished MicroRNA-21-5p expression may enhance inflammation and STAT3 expression in cachectic patients, contributing to the development of insulin resistance.

Keywords: Cachexia. Metabolic Syndrome. Inflammation. Insulin Resistance. MicroRNAs. Insulin. Liver. Macrophages.

RESUMO

Camargo, RG. Resistência à Insulina na Caquexia Associada ao Câncer e na Síndrome Metabólica: Papel dos Macrófagos ativados pela Insulina e do miRNA-21-5p. [Tese (Doutorado em biologia celular e tecidual)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2016. Potsdam: Institut für Ernährungswissenschaft, Universität Potsdam; 2016.

O teor de gordura cada vez maior na dieta ocidental, combinada com a diminuição dos níveis de atividade física têm marcadamente aumentado a incidência de doenças relacionadas ao metabolismo. A caquexia associada ao câncer (CC) e a síndrome metabólica (SM) são síndromes de etiologia complexa e multifatorial, não totalmente compreendida, e com mecanismos subjacentes ao seu desenvolvimento não completamente revelados. No entanto, apesar de serem consideradas "lados opostos", a CC e a MetS apresentam várias características em comum, tais como resistência à insulina e inflamação de baixo grau, com macrófagos teciduais como importantes coadjuvantes, devido à sua capacidade de produzir e liberar mediadores inflamatórios, e microRNAs, descritos como RNAs não-codificantes ligados à regulação da expressão de proteínas e reconhecidos como participantes ativos na geração de várias doenças inflamatórias, tais como o câncer e diabetes tipo II. Uma das principais características da MetS é a hiperinsulinemia, que está geralmente associada com uma tentativa da célula β do pâncreas de compensar a diminuição da sensibilidade à insulina (resistência à insulina). Um número crescente de evidências sugere que a hiperinsulinemia "por si só", pode contribuir com o desenvolvimento de resistência à insulina através do estabelecimento de um quadro inflamatório de baixo grau, em tecidos sensíveis a insulina, e em particular no fígado, devido ao fato da insulina ser secretada pelo pâncreas na circulação portal. A hipótese do presente estudo foi que a insulina pode induzir uma resposta inflamatória em macrófagos e culminar em diminuição da sensibilidade hepática à insulina. Para confirmar esta hipótese, primeiramente, macrófagos diferenciados da linhagem de células humanas U937 foram expostos à insulina, LPS e PGE_2 . Nestas células, a insulina aumentou significativamente a expressão gênica dos mediadores pró-inflamatórios IL-1 β , IL-8, CCL2, oncostatina M (OSM) e prostaglandina E2 sintase microssomal (mPGES1), e do mediador anti-inflamatório IL-10. Além disso, o sinergismo entre insulina e LPS aumentou a indução provocada por LPS nos genes da IL-1 β , IL-8, IL-6, CCL2 e TNF- α . Quando combinado com PGE_2 , a insulina aumentou a indução provocada pela PGE_2 nos genes da IL-1 β , mPGES1 e COX2, e restaurou a inibição induzida pela PGE_2 no gene CCL2 e TNF- α . Subsequentemente, sobrenadantes dos macrófagos U937 tratados com insulina modulou negativamente a sinalização da insulina em culturas primárias de hepatócitos de rato, como observado pela atenuação de 50% da indução dependente de insulina da enzima glicocinase. Citocinas contidas no sobrenadante de macrófagos U937 tratados com insulina também ativaram em hepatócitos ERK1/2, resultando na fosforilação do resíduo de serina inibitório do substrato do receptor de insulina. Adicionalmente, o fator de transcrição STAT3 foi ativado por um elevado grau de fosforilação e a proteína SOCS3, capaz de interromper a via de sinalização do receptor de insulina, foi induzida. Considerando que na caquexia associada ao câncer, pacientes são altamente afetados pela resistência à insulina e inflamação, pacientes controle, não caquéticos e caquéticos foram selecionados e os respectivos níveis circulantes de mediadores pró-inflamatórios e microRNA-21-5p, um regulador pós-transcricional da expressão de STAT3, avaliados e correlacionados. Pacientes

caquéticos exibiram citocinas circulantes IL-6 e IL-8 significativamente maiores do que pacientes não caquéticos e controles, assim como a expressão de microRNA-21-5p significativamente diminuída. Além disso, a reduzida expressão de microRNA-21-5p correlaciona-se negativamente com níveis de IL-6 no plasma. Estes resultados indicam que a hiperinsulinemia pode, por si só contribuir para o desenvolvimento da inflamação de baixo grau prevalente em pacientes com excesso de peso e obesos e, assim, promover o desenvolvimento de resistência à insulina especialmente no fígado e o nível reduzido de miRNA-21-5p pode modular a inflamação e expressão de STAT3 em pacientes caquéticos, contribuindo para o desenvolvimento da resistência à insulina.

Palavras-chave: Caquexia. Síndrome Metabólica. Inflamação. Resistência à Insulina. MicroRNAs. Insulina. Fígado. Macrófagos.

ZUSAMMENFASSUNG

Camargo, RG. Insulinresistenz in Tumorkachexie und Metabolischem Syndrom: Die Rolle von insulin-aktivierten Makrophagen und miRNA-21-5p. [Thesis (Doctoral degree in Cellular and Tissue biology)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2016. Potsdam: Institut für Ernährungswissenschaft, Universität Potsdam; 2016.

Der stetig steigende Fettgehalt in westlicher Ernährung in Kombination mit reduzierter körperlicher Aktivität hat zu einem dramatischen Anstieg der Inzidenz metabolischer Erkrankungen geführt. Tumorkachexie (Cancer cachexia, CC) und Metabolisches Syndrom (MetS) sind sehr komplexe, multifaktorielle metabolische Erkrankungen, deren Ätiologie nicht vollständig verstanden ist. Die molekularen Ursachen, die zu diesen Symptomkomplexen führen, sind noch unzureichend aufgeklärt. Obwohl ihr äußeres Erscheinungsbild stark gegensätzlich ist, haben MetS und CC etliche Gemeinsamkeiten wie zum Beispiel Insulinresistenz und eine chronische unterschwellige Entzündung. Sowohl bei der Entstehung der Insulinresistenz als auch bei der chronischen Entzündung spielen Makrophagen eine Schlüsselrolle, weil sie in der Lage sind pro-inflammatorische Mediatoren zu produzieren und freizusetzen.

Eine der hervorstechendsten Auffälligkeiten des MetS ist die Hyperinsulinämie, die durch den Versuch der β -Zelle, die verminderte Insulinsensitivität (Insulinresistenz) zu kompensieren, zustande kommt. Es gibt zunehmend Hinweise darauf, dass die Hyperinsulinämie selber an der Entzündungsentstehung in Insulin-abhängigen Geweben beteiligt ist und dadurch zur Entwicklung und Verstärkung der Insulinresistenz beitragen kann. Dies trifft besonders auf die Leber zu, weil hier die Insulinspiegel besonders hoch sind, da Insulin vom Pankreas direkt in den Pfortaderkreislauf gelangt. Daher wurde in dieser Arbeit die Hypothese geprüft, ob Insulin selber eine Entzündungsantwort auslösen und dadurch die hepatische Insulinsensitivität senken kann. Zu diesem Zweck wurde die humane Zelllinie U937 durch PMA-Behandlung zu Makrophagen differenziert und diese Makrophagen mit Insulin, LPS und PGE_2 inkubiert. In diesen Zellen steigerte Insulin die Expression der pro-inflammatorischen Mediatoren IL-1 β , IL-8, CCL2, Oncostatin M (OSM) signifikant und induzierte die mikrosomale PGE-Synthase 1 (mPGES1) ebenso wie das anti-inflammatorische Cytokin IL-10. Ferner verstärkte Insulin die LPS-abhängige Induktion des IL-1 β -, IL-8-, IL-6-, CCL2- und TNF α -Gens. Ebenso verstärkte Insulin die PGE_2 -abhängige Induktion von IL-1 β , mPGES1 und COX2. Im Gegensatz dazu schwächte es die Hemmende Wirkung von PGE_2 auf Expression von TNF α und CCL2 ab und trug so auf beide Weisen zu einer Verstärkung der Entzündungsantwort bei. Überstände von Insulin-behandelten U937 Makrophagen reduzierten die Insulin-abhängige Induktion der Glukokinase in Hepatocyten um 50%. Die Cytokine, die im Überstand Insulin-behandelter Makrophagen enthalten waren, aktivierten in Hepatocyten ERK1/2, was zu einer inhibitorischen Serin-Phosphorylierung der Insulin Rezeptor Substrats (IRS) führte. Zusätzlich führten die Cytokine zu einer Phosphorylierung und Aktivierung von STAT3 und einer dadurch bedingten Induktion von SOCS3, das seinerseits die Insulinrezeptor-Signalkette unterbrechen kann.

MicroRNAs, nicht-codierende RNAs, die an der Regulation der Proteinexpression beteiligt sind und deren Beteiligung an der Regulation der Entzündungsantwort bei zahlreichen Erkrankungen, unter anderem Tumorerkrankungen und Typ II Diabetes gezeigt wurde, sind auch von Interesse. Unter dem Blickwinkel, dass Tumor-Kachexie Patienten sich durch eine

Insulinresistenz und eine systemische Entzündung auszeichnen, wurden in nicht-kachektische und tumorkachektische Patienten Plasmaspiegel von pro-inflammatorischen Mediatoren und der microRNA-21-5p bestimmt, von der bekannt ist, dass sie ein posttranskriptioneller Regulator der STAT3 Expression ist. Die Spiegel der pro-inflammatorischen Mediatoren und der miRNA-21-5p wurden korreliert. In kachektischen Patienten waren die Spiegel der Cytokine IL-6 und IL-8 signifikant höher, die der miRNA-21-5p signifikant niedriger als in nicht-kachektischen Patienten. Die Plasma IL-6-Spiegel korrelierten negativ mit den miRNA21-5p Spiegeln.

Insgesamt zeigen die Ergebnisse, dass eine Hyperinsulinämie selber zu der Entwicklung einer unterschwelliger Entzündung, wie sie in Patienten mit einem MetS vorherrscht, beitragen, und dadurch besonders in der Leber eine Insulinresistenz auslösen oder verstärken kann. Eine verringerte Expression der MicroRNA-21-5p kann in kachektischen Patienten die Entzündungsantwort, im Speziellen die STAT3 Expression, verstärken und dadurch zur Entwicklung einer Insulinresistenz beitragen

Schlüsselwörter: Kachexie. Metabolisches Syndrom. Entzündung. Insulinresistenz. MicroRNAs . Insulin. Leber. Makrophagen.

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ABBREVIATURE LIST

AA	Amino acid;
AIDS	Acquired Immune Deficiency Syndrome;
AKT	Protein Kinase B;
ALB	Albumin;
AMP	Adenosine monophosphate;
ANOVA	Analysis of variance;
AP1	Activator protein 1;
APS	Ammonium Persulfate;
ATGL	Adipose Triglyceride Lipase;
ATP III	Adult Treatment Panel III;
BMI	Body mass index;
CC	Cancer Cachexia;
CCL2	'CC'-chemokine ligand 2 (monocyte chemoattractant protein 1);
CD68	Cluster of Differentiation 68;
cDNA	Complementary DNA;
CHF	Chronic Heart Failure;
COPD	Chronic Obstructive Pulmonary Disease;
COX-2	Cyclooxygenase-2;
CRP	C-Reactive protein;
Ct	Cycle Threshold;
CVD	Cardiovascular Disease;
DNA	Deoxyribonucleic acid;
ERK1/2	Extracellular signal-regulated kinases 1 and 2;
FCS	Fetal Calf serum;
FFA	Free-fatty Acid;
FOXO1	Forkhead box protein O1;
FPG	Fasting plasma glucose;
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase;
GK	Glucokinase;
GLUT	Glucose transporter;

GPS	Glasgow prognostic Score;
HDL-C	High density lipoprotein cholesterol;
HC	Hepatocyte;
HRP	Horseradish peroxidase;
HSL	Hormone Sensitive Lipase;
IDF	International Diabetes federation;
IκB	Inhibitor of kappa B;
IKK	Inhibitor of kappa B kinase;
IL-6-R	Interleukin-6 receptor;
IL	Interleukin;
INSR	Insulin receptor;
IR	Insulin resistance;
IRS	Insulin receptor substrate;
JAK	Janus kinase;
JNK	c-Jun N-terminal kinase;
LPS	Lipopolysaccharide;
LDL	Low-density lipoprotein;
LDL-R	Low-density lipoprotein receptor;
MAP	Mitogen Activated Protein;
MetS	Metabolic Syndrome;
miRNA	MicroRNA;
miRISC	MicroRNA-induced silencing complex;
mPGES1	Microsomal prostaglandin E₂ synthase 1;
mRNA	Messenger RNA;
NCS	Neonatal Calf serum;
NFκB	Nuclear factor kappa B;
nt	Nucleotide;
OSM	oncostatin M;
PCR	Polymerase chain reaction;
PDK	Phosphoinositide-dependent kinase;
PGE₂	Prostaglandin E₂;
PKC	Protein Kinase C;
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase;

PIF	Proteolysis inducing factor;
PIP₂	Phosphatidylinositol (4,5)-bisphosphate;
PIP₃	Phosphatidylinositol (3,4,5)-trisphosphate;
PLIN	Perilipin;
PMA	Phorbol-myristate acetate;
pre-miRNA	Precursor microRNA;
pri-miRNA	Primary microRNA;
PTEN	Phosphatase and tensin homolog;
PTP1B	Protein-tyrosine phosphatase 1B;
PVDF	Polyvinylidene difluoride;
TG	Triglyceride;
TNF-α	Tumor necrosis factor alpha;
TLR	Toll-like receptor;
STAT3	Signal transducer and activator of transcription 3;
SOCS3	Suppressor of cytokine signaling 3;
RNA	Ribonucleic acid;
SDS-PAGE	sodium dodecylsulfate polyacrylamid electrophoresis;
SREBP	Sterol regulatory element-binding protein;
VLDL	Very-low-density lipoprotein;
TPCA-1	2-[(Aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide;
TEMED	Tetramethylethylenediamine;
UTR	Untranslated region;

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1 INTRODUCTION

1.1 Cancer cachexia and the Metabolic Syndrome

Cancer cachexia (CC) and Metabolic Syndrome (MetS) are both recognized as multifactorial conditions with marked impairment of intermediate metabolism. Cachexia was described as a wasting condition two thousand years ago and is frequently reported as a common negative consequence of diseases such as cancer, Chronic Heart Failure (CHF), Chronic Obstructive Pulmonary Disease (COPD), Acquired Immune Deficiency Syndrome (AIDS) and sepsis, among other. MetS is a consequence of excessive calorie intake combined with reduced energy expenditure due to a sedentary life style. It is considered a risk factor for heart disease and other health problems, such as stroke and/or type II diabetes. Both syndromes share several common issues, in spite of a major differences regarding the final outcome: the excessive uptake and storage of energy substrates in MetS leading to overweight or obesity, while in CC extensive catabolism of fat and lean mass is the most prominent feature (1).

Cachexia is present in approximately half of all cancer patients, and in 15% of patients with Chronic Heart Failure (CHF) (2). This scenario rises up to 80% in cancer patients with advanced disease (3). In addition to markedly increasing cancer-associated morbidity, leading to a gradual loss of peripheral organ function (1), cachexia is directly responsible for the death of a large proportion of cancer patients, reaching up to 20% (3). Furthermore, the quality of life of cachectic patients is compromised, implying in reduced effect of cancer treatment (4). In spite of its clinical relevance, to the present date there is no nutritional (5) or pharmacological treatment able to prevent, attenuate or to stop the progression of the syndrome (6), frustrating physicians and patients.

The Metabolic syndrome (MetS), on the other hand, is one of the most intriguing and complex conditions of modern life (7), and is associated with high morbidity and mortality among adults. The prevalence of MetS is around 20-30% in developed countries (8), this figure rises up to over 40% in adults over the age of 50 (9) and even more alarmingly, the high fat content in Western diets, combined with low physical activity cause a significant number of children to develop some of MetS symptoms (10). The definition for MetS, which is also known as the “insulin resistance syndrome”, may be found below on the item 1.2.2,

and among its main features, lipid metabolism abnormalities and insulin resistance are the most prominent. This disease is linked to a 2-fold increase in cardio-vascular disease risk and to 1.5-fold augmented mortality, as well as to a 5-fold increase in the risk of developing *type II diabetes* (11). The global epidemics of obesity and related outcomes, such as *type II diabetes* and cardiovascular disease have raised the attention addressed to MetS, which has thus become a major focus in clinical research. Dietary modification and lifestyle intervention may prevent the development of MetS (12).

1.2 Definitions and main symptoms

1.2.1 Cachexia

Cachexia is still a sub-diagnosed syndrome, and the mechanisms underlying its development are not fully understood. In order to better understand the syndrome and its mechanisms, in December 2007 a group of researchers and clinicians combined efforts to discuss what would be the best way to define cachexia. The definition that emerged was: “Cachexia is a complex metabolic syndrome associated with underlying illness and characterized by loss of muscle with or without loss of fat mass”. In this international meeting, the group additionally discussed the possible diagnostic criteria. According to Evans and colleagues (13), the patient should present involuntary weight loss > 5% in the past 12 months or Body Mass Index (BMI) < 20 kg/m², and additionally, three of five other symptoms: Decreased muscle strength, Fatigue, Anorexia, Low fat-free mass index and Abnormal biochemistry as increased inflammatory markers such as C-Reactive protein (CRP) and Interleukin-6 (IL-6), anemia or low serum albumin. This was the first proposition in which clear criteria for diagnosis were established. In the year 2011, cancer cachexia experts gathered in a formal consensus process to update the definition and diagnosis criteria (14). The updated definition proposed was: Cachexia is a “multifactorial syndrome defined by ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment. Its pathophysiology is characterized by a negative protein and energy balance, driven by a variable combination of reduced food intake and abnormal metabolism”. Additionally, a new and most relevant aspect was added to cachexia clinical assessment; the concept of three different stages: pre-cachexia, in which

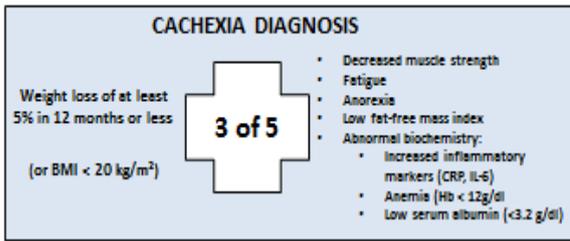
anorexia and metabolic changes may be observed before weight loss; cachexia itself, including weight loss > 5% in the past six months or weight loss between 2% and 5% and BMI < 22 in the past six months or, still the presence of sarcopenia with often reduced food intake and systemic inflammation; and refractory cachexia, in which a low performance score and poor responsiveness to cancer treatment is observed; usually, life expectancy does not exceed three months (14).

1.2.2 Metabolic Syndrome

The definition of MetS has been proposed by the experts of the Adult Treatment Panel III (ATP III, 2001-2005), and, by the International Diabetes Federation (IDF - 2005) (15). The best way to define MetS is still frequently the focus of debate in the literature, and despite the criteria may vary to some degree in all of them; in general, MetS definitions target a population with increased risk for the development of *type II diabetes mellitus* and increased risk for cardiovascular disease (CVD). A cluster of five factors was selected for MetS diagnosis, in which three or more of these components confirm the presence of the condition: Increased waist circumference or central obesity, dyslipidemia (high triglycerides (TGs) and/or low high-density lipoprotein cholesterol (HDL-C), high fasting plasma glucose (FPG) and high systolic blood pressure (9, 15). Besides that, additional features such as liver steatosis or non-alcoholic steatohepatitis (NASH) and sarcopenia are frequently pointed out as part of the syndrome. MetS is also known to be associated with a state of chronic low-grade inflammation (10) and oxidative stress.

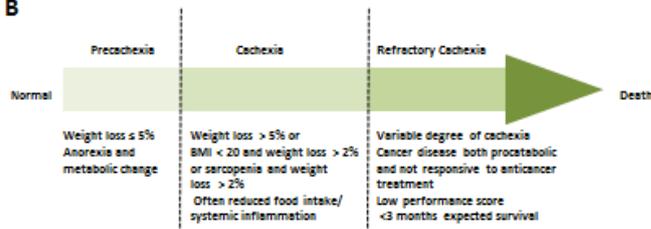
Figure 1 – Cancer cachexia and Metabolic Syndrome main diagnosis criteria

A



Adapted from Evans et. al., 2008 (13)

B



Adapted from Fearon et. al., 2011 (14)

C

ATP III Clinical Identification of the Metabolic Syndrome	
Risk factor	Defining level
Abdominal obesity, given as waist circumference	
Men	>102 cm (>40 in)
Women	>88 cm (>35 in)
Triglycerides	≥ 150mg/dl
HDL cholesterol	
Men	< 40 mg/dl
Women	< 50 mg/dl
Blood pressure	≥ 130 / ≥ 85 mm Hg
Fasting glucose	≥ 110 mg / dl

Adapted from Grundy et. al., 2004 (16)

Cancer Cachexia (A and B) and Metabolic Syndrome (C) diagnosis criteria.

Table 1 – Main characteristics of Cancer cachexia and Metabolic Syndrome

Cancer Cachexia	Metabolic Syndrome
Anorexia	Hyperphagia
Weight loss	Central Obesity
Sarcopenia	Sarcopenia
Dyslipidemia	Dyslipidemia
Insulin resistance	Insulin resistance
Chronic low grade inflammation	Chronic low grade inflammation
Oxidative Stress	Oxidative stress
Liver Steatosis	Liver Steatosis
Hypotension	Hypertension

1.3 Inflammation

In the recent years, it has become widely accepted that inflammation is a pivotal player underlying the development and progression of chronic diseases, such as cancer, obesity and *type II diabetes*, being this, the most relevant feature pointing out to poor clinical and metabolic outcome in CC and MetS (10, 17). The nature of chronic systemic inflammation in MetS and CC is very similar, including the exacerbated production of cytokines, such as Tumor Necrosis factor alpha (TNF- α), IL-1 β , IL-6, and also of adipokines, as Leptin and Resistin (17-19). Augmented serum levels of the cytokines IL-6, IL-1 and TNF- α and of acute-phase proteins, such as (C-reactive protein) are the most relevant markers (8, 13, 20). The role of cytokines in the development of CC was confirmed in rodent models, in which exogenous administration of TNF- α and IL-6 was shown to induce cachexia-like effects, with or without the presence of tumors (21, 22). In MetS, patients show increased cytokine levels in plasma and saliva (23), and as of other pro-inflammatory mediators, such as C-Reactive protein and CCL2 (24). Despite the unequivocal importance of the low grade inflammatory process in both syndromes, the source(s) are not clear. In cachexia, Susuki and colleagues showed that it is not yet certain whether the primary source of cytokine production is the tumor (Cancer Cachexia) or the host's inflammatory cells (25). The adipose tissue is also a

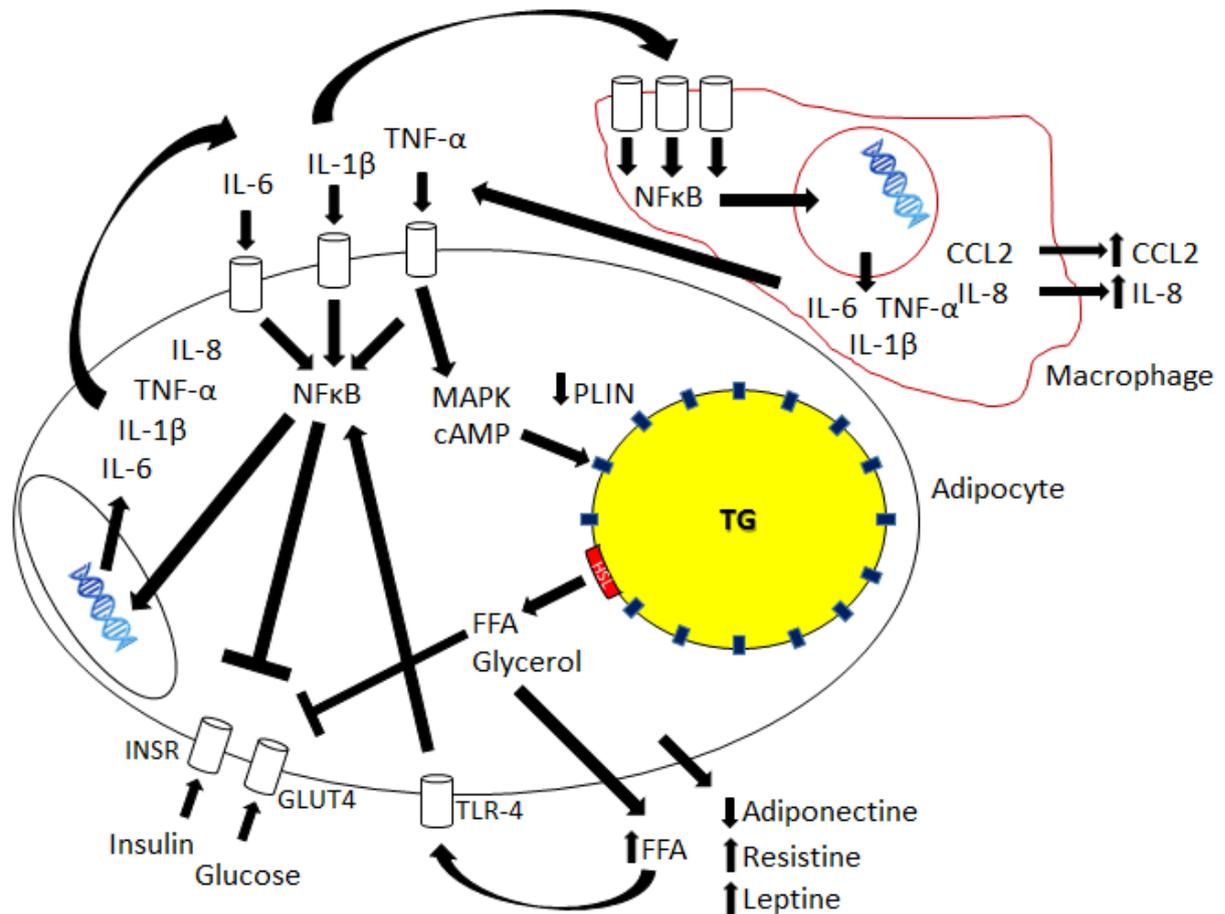
candidate tissue due to its altered pro-inflammatory secretion and conspicuous mononuclear cell infiltration during cachexia (26). In MetS, the most accepted hypothesis is linked to the broad expansion of the adipose tissue. This would trigger adipocyte apoptosis and culminate in the development of local low-grade inflammation that would later affect other tissues. Despite some evidence supporting this hypothesis, it is believed that both in MetS and CC, an orchestrated response of the organism involving several different organs and tissues is contributing for a sustained inflammatory state (27). The adipose tissue, muscle and liver, in this scenario, are often infiltrated with inflammatory cells, with great capacity to produce and release pro-inflammatory mediators (28).

1.3.1 Inflammation in the adipose tissue

Alterations in lipid metabolism are a very frequent metabolic abnormality described in both CC and MetS (19, 29, 30 2013). This tissue is nowadays recognized as more than just as an energy storage compartment. It is indeed an endocrine organ, capable of expressing and secreting several peptides with paracrine, autocrine and endocrine functions (31). In MetS, the expansion of the adipose tissue beyond healthy limits due to a constant positive caloric balance, leads to lipid accumulation and consequently, to adipocyte hypertrophy, culminating in metabolic stress and consequently, immune system cell recruitment and infiltration, which triggers cytokine expression and release, along with chemokines and adipokines. In CC, the adipose tissue is an active player in the development and maintenance of the inflammatory state. The metabolism of this tissue is altered under the influence of cytokines, while this compartment responds to the inflammation by modifying the secretion of adipokines, cytokines and chemokines (26). In both syndromes, chemokines play a most relevant role, as these molecules signal to and recruit monocytes from the bloodstream to the adipose depots, increasing monocyte infiltration (28, 29, 32). The molecular mechanisms and consequences of adipose tissue inflammation are described for MetS and CC (8, 26, 33), and share several common characteristics. Initially, the inflammatory scenario provokes augmented lipolysis, which is partly explained by the stimulus provided by cytokines such as TNF- α through the activation of MAP kinases (34). This leads subsequently, to the activation of other signaling cascades, what culminates in the phosphorylation and decreased production of perilipin (PLIN), an essential protein in the process of lipid storage (35). The

lower the synthesis of PLINs, the more profound the lipolysis induced by enhanced accessibility of lipolytic enzymes (ATGL) to the surface of the triglyceride (36) droplet. This, results in augmented triglyceride hydrolysis and release of free fatty acids (FFA) and glycerol (37). Other cytokines, such as IL-6, IL-1 β and TNF- α also enhance the activation of pro-inflammatory transcription factors in the adipose tissue, among which NF κ B (38), causing another relevant effect in the adipose pads: Down-regulation and decreased activation of insulin signaling proteins (GLUT4 and IRS), impairing insulin action and establishing a state of insulin resistance (39). This, in turn also dysregulates the secretion of adipokines such as leptin, adiponectin and resistin, all of which, in combination with cytokines, act in a paracrine or autocrine way, exacerbating adipose tissue inflammation (40), and the state of insulin resistance, in a vicious cycle.

Figure 2 - Molecular mechanisms underlying the setting of inflammation and insulin resistance in CC and MetS in the white adipose tissue



Pro-inflammatory mediators, including IL-6, IL-1 β and TNF- α bind to specific membrane receptors and trigger several signaling cascades, such as those of as the NF κ B and MAP kinases. Perilipins (PLIN) expression is decreased, elevating the levels of free-fatty acids (FFA) and glycerol. These FFA bind to Toll-like receptor 4 (TLR-4) and maintain the state of local inflammation. Macrophages are part of the process, as cells that are sensitive to pro-inflammatory mediators, responding with further release of several pro-inflammatory mediators such as CCL2, adding to the recruitment of monocytes from the bloodstream and maintaining local inflammation in the adipose tissue. Release of adipokines involved in the regulation of inflammation amplifies the process.

1.4 Insulin resistance

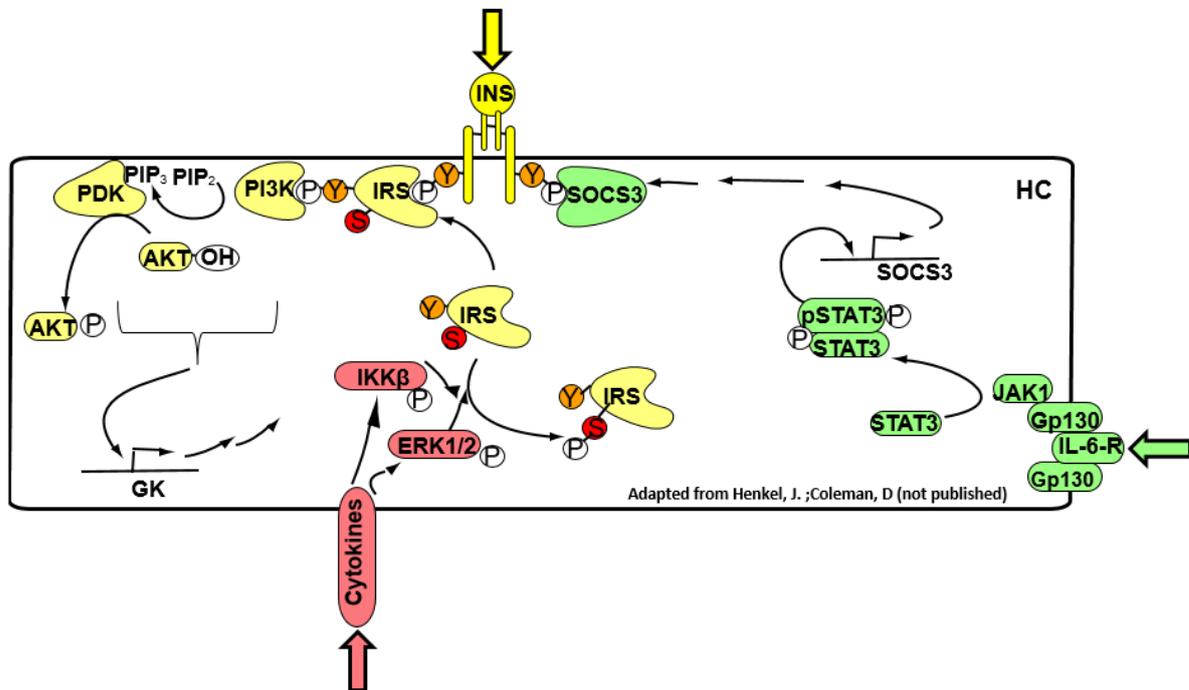
Insulin is a peptide hormone composed of fifty one amino acids, which signals through binding to membrane receptors, modulating diverse cellular functions, including energy storage and nutrient uptake (41). Insulin signals via a receptor that belongs to the class of

receptor tyrosine kinases (Figure 3). Upon ligand binding the receptor is auto phosphorylated on tyrosine residues. This results in the recruitment of SH2-domain containing downstream signaling proteins, in particular the insulin receptor substrates (IRS), which in turn also get phosphorylated at tyrosine residues. The tyrosine phosphorylated IRS then activates two downstream signal cascades, one of which results in the activation of the protein kinase B (Akt), part of the insulin signaling pathway and the protein responsible for the signal that triggers cell glucose uptake via the stimulation of glucose transporter IV vesicles translocation to the plasma membrane and subsequently glucose transport (42) in skeletal muscle and adipose tissue. Insulin is regarded as the primary anabolic hormone (43). In order to re-synthesize ATP to maintain cell homeostasis, glucose is one of the main substrates, although, if the demand for energy in the cell is low and the availability of glucose is high over an extended period, signals that impair the function of the insulin related cascade may be triggered within the cell or, still by extracellular signals. When insulin signaling pathway is inhibited, binding of insulin to its receptor no longer transduces into downstream signaling and thus the cell is considered to be in a state of insulin resistance (IR). One such state is defined as a significant decrease in insulin sensitivity (30). It occurs when tissues that normally are sensitive to the hormone lose the ability to respond properly to stimulation (32). This will provoke impaired tissue glucose uptake and impaired inhibition of hepatic glucose production (44). IR is considered a metabolic component of cachexia (27, 44), and one of the most prominent features of MetS pathogenesis (18, 45, 46). Interestingly, a similar degree of IR in CC patients and in obese and *type II diabetes* patients may be found (44).

Diverse molecular mechanisms have been proposed to explain the development of insulin resistance. One of them is related to the augmented synthesis of triglycerides, once this process is dependent on an intermediate product, diacylglycerol, capable of activating specific protein kinases, such as those of the PKC family (47). Once activated, protein kinase C interrupts intracellular insulin receptor signal chain by direct phosphorylation of the insulin receptor substrate in inhibitory serine residues (IRS). In contrast to the insulin receptor-dependent phosphorylation of tyrosine residues, the serine phosphorylation of the IRS leads to uncoupling from the downstream signal chain and IRS degradation (48). Recent studies (49-51) also link the development of insulin resistance to low-grade and chronic state of local inflammation in insulin responsive tissues such as the adipose tissue, liver and muscle.

The precise mechanisms, as well as the mediators involved in this interaction are not completely unveiled yet (19, 32, 44, 52), although inflammatory cytokines as IL-1 β or TNF α bind to receptors on insulin target cell membrane and culminate in kinase activation, (e.g. NF κ B inhibitor kinase, IKK β). This protein is also capable of provoking the inhibitory serine phosphorylation of the IRS (53), impairing the insulin signaling chain. Other cytokines as those of the IL-6 cytokines family through canonic signaling chains trigger the synthesis of suppressors of cytokine signaling (SOCS) that act as feed-back inhibitors of their proper signaling chains. However, SOCS can also bind unspecifically to the tyrosine phosphorylated insulin receptor or IRS reduce insulin signaling by uncoupling the tyrosine-phosphorylated insulin receptor from its downstream signaling chain and enhancing the degradation of the IRS (54). Other mediators are also described as capable of impairing insulin signaling, including prostaglandin E₂ (55). Its mechanism of action in insulin target-tissues involves the direct phosphorylation of IRS-inactivating serine kinases such as the Extracellular signal-regulated kinases 1 and 2 (ERK1/2) (56). In addition, PGE₂ may also induce cytokine production by resident or recruited immune cells such as macrophages (56), which in turn, will release inflammatory mediators, decreasing insulin sensitivity.

Figure 3 – Main hepatocyte Insulin and cytokine signaling pathways



Insulin binds to its receptor in hepatocytes (HC) inducing recruitment of insulin receptor substrates (IRS), which when phosphorylated in tyrosine residues, will activate protein PI3K and subsequently, the conversion of PIP₂ into PIP₃, activating PDK and subsequently, Akt, by phosphorylation. These stimuli culminate in elevated glucokinase (GK) expression. Cytokines bind to specific membrane receptors and trigger the phosphorylation of MAP kinases such as ERK 1/2 and the NFκB inhibitor kinase (IKK). These kinases are capable of phosphorylating the IRS inhibitory residue, impairing the insulin signaling pathway. Interleukin-6 family cytokines bind to membrane receptors (IL-6-R) and activate the transcription factor STAT3, which induces the suppressor of cytokine signaling (SOCS3). This suppressor binds directly to the insulin receptor, impairing the signaling cascade.

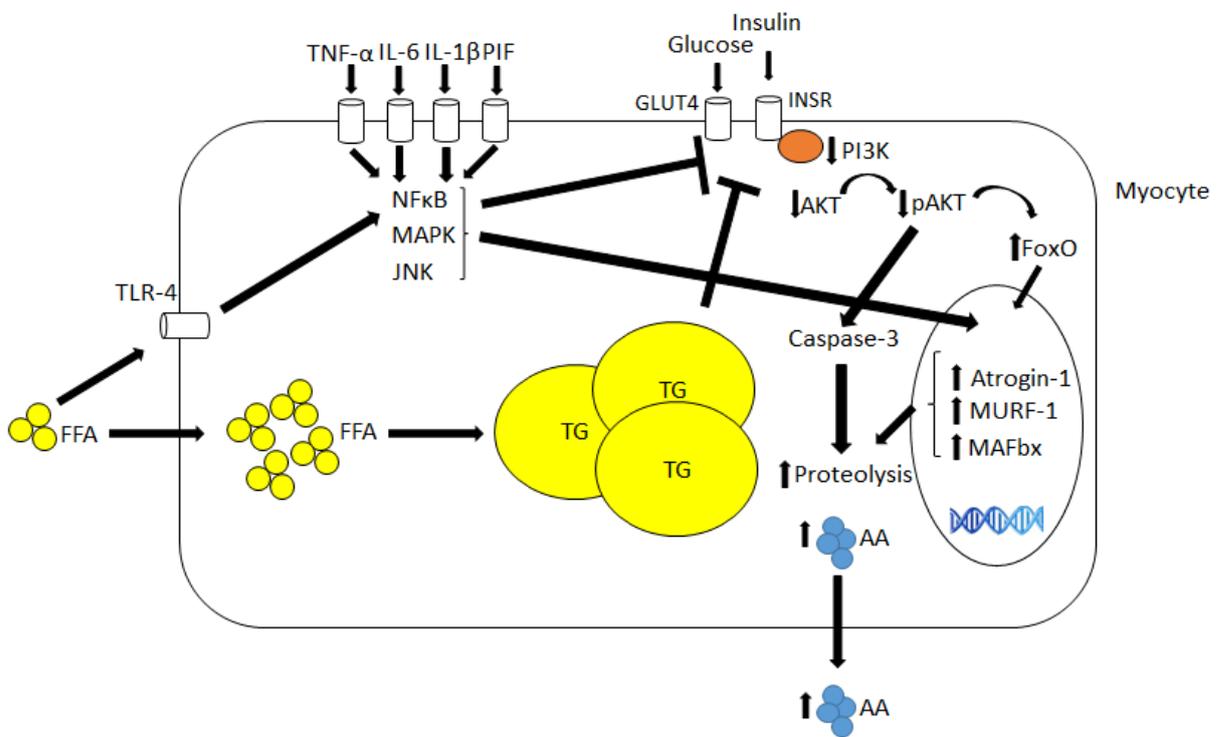
1.4.1 Effects of inflammation and insulin resistance on the Muscle

Skeletal muscle is the primary tissue in which insulin-mediated glucose uptake occurs. The most prominent clinical characteristic of CC is accentuated loss of lean mass, and in special, of skeletal muscle mass, inducing fatigue, impairment of muscle function, and marked consequences upon quality of life and survival. In MetS, the expression “obesity–related sarcopenia” is frequently employed to describe patients that, despite augmented body mass, show reduced lean mass, due to proteolysis and diminished protein synthesis (57). As in cachexia, these symptoms are also associated with poor prognosis and decreased quality of life. The mechanisms involved in the loss of skeletal muscle mass comprise several pathways at the molecular level, giving rise to imbalance between the processes of protein synthesis

and degradation (58). The utilization of muscle amino acids as carbon skeletons for gluconeogenesis and as a source of energy (Krebs cycle intermediates) is frequently observed in cachectic patients (59). Argiles (17), pointed out that cytokines *per se* are capable of modulating muscle catabolism, causing wasting and consequently, weight loss. Tumor-derived factors, such as the proteolysis-inducing factor (PIF) may as well provoke muscle loss (60). Nuclear factor κ B (NF κ B) and the Mitogen-activated protein kinases (MAPKs) are the main modulators of skeletal muscle metabolism in response to inflammatory stimulus. The chronic activation of these signaling pathways is directly involved in the development and maintenance of wasting conditions such as Cachexia and Diabetes (61). Protein degradation occurs in the skeletal muscle through different mechanisms: a) The lysosomal system, b) The Calcium-activated calpains I and II, and c) The ubiquitin–proteasome pathway and the augmented expression of the ubiquitin–proteasome pathway proteins (60). Insulin resistance contributes to sarcopenia because insulin increases amino acid uptake into skeletal muscle cells, increases translation of mRNA by activating eukaryotic translation initiation factors, among others and inhibits both autophagy and proteasomal protein degradation (62, 63).

In MetS, excessive circulating free-fatty acids are a common finding (64). These FFA reach and are taken up by the muscle, which, in order to compensate for this increased levels of FFA, enhances fatty acid uptake and re-esterification (65). During triglyceride synthesis, the intermediate metabolite diacylglycerol is formed and activates proteins, which directly impair insulin signaling, compromising sensitivity to the hormone and exacerbating the state of insulin resistance. This will lead to increased glucose plasma levels and characterize a state of hyperglycemia (65).

Figure 4 - Muscle-related molecular mechanisms underlying inflammation and insulin resistance in CC and MetS



Pro-inflammatory mediators as TNF- α , IL-6, IL-1 β and proteolysis inducing factor (PIF) bind to membrane receptors in myocytes and activate several signaling pathways as those of MAP kinases, JNK and NF κ B, which are capable of impairing insulin signaling cascade and inducing the expression of several proteins involved in proteolysis such as Atrogin-1, MURF-1 and FoxO. Diminished insulin signaling enhances proteolysis, while augmented influx of FFA and its binding to receptors such as TLR-4 contributes to insulin signaling impairment.

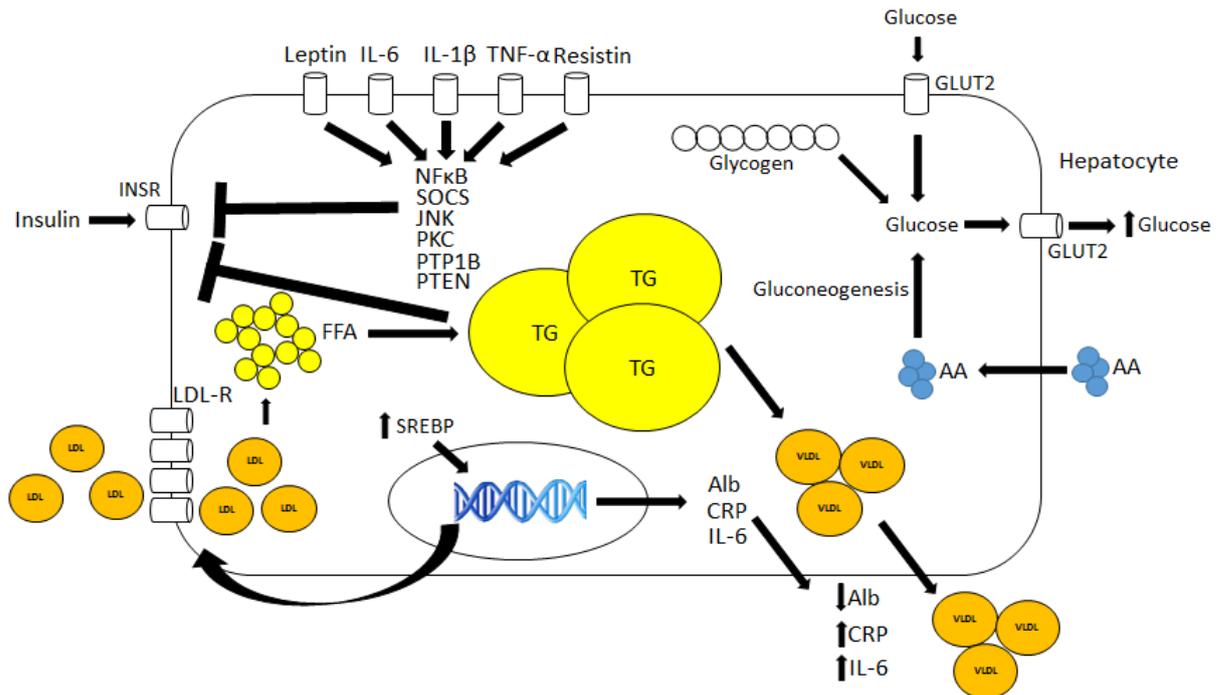
1.4.2 Effects of hepatic inflammation and insulin resistance

The liver is a central organ responsible for the coordination of intermediate metabolism and is highly affected by MetS and CC, both in terms of its microstructure and function (66, 67). Carbohydrate metabolism is precisely regulated by the liver, as excess of glucose will be stored in the form of glycogen. Conversely, when glycaemia falls, the liver releases glucose by breakdown of glycogen. Under conditions in which excessive adipose tissue lipolysis is present as in CC and MetS, the liver increases the uptake of adipose tissue-derived free fatty acid (68). Increased TG accumulation impairs liver metabolism and may evolve to the condition known as fatty liver or steatosis (1). In fact, there is often relationship between increased accumulated intracellular fat is associated with different degrees of inflammation (66), which reflects into a propitious scenario for the establishment of insulin resistance in

hepatocytes and results in impaired glycogen synthesis, failed suppression of gluconeogenesis, enhanced lipid accumulation and increased synthesis of acute phase proteins, such as C-Reactive protein (CRP) (18). In both MetS and CC, insulin resistance is highly associated with hepatic steatosis (69), confirming the role of increased flux of free fatty acid and triglyceride-rich lipoprotein remnants in its development. The organ per se may contribute for higher intracellular content by presenting abnormal glucose uptake in combination with reduced gluconeogenesis-related protein gene expression (70) and despite insulin resistance, conversion of glucose to fatty acids and triglycerides in the liver may be high because (1) ChREBP-dependent induction of enzymes of fatty acid synthesis and triglyceride synthesis and (2) the excessive "substrate load" in hyperglycemic patients. Adipose tissue dysfunction may also impair insulin signaling in the liver, through the diminished secretion of adiponectin, which contributes for glucose uptake in the liver (71). As triglyceride stores in adipocytes increase, the production of adiponectin falls (72). The lower the circulating content of this hormone, the lower sensitivity to insulin stimulated glucose uptake and utilization and, consequently, more glucose is mobilized and released from the liver, despite the stimulation by insulin (71). This scenario contributes to the development of insulin resistance not only locally, but also systemically. As sustained inflammation is considered a pivotal feature in the development of hepatic insulin resistance, cytokines play a major role in impaired hepatic metabolism. Interleukin-6 is the main mediator of hepatic acute phase response in cancer cachexia (22). It inhibits albumin production and induces the synthesis of acute phase protein such as CRP. TNF- α and other cytokines are able to impair insulin signaling pathway (73, 74). This will result in decreased insulin sensitivity, and insulin will no longer stimulate feeding-associated glucose storage in the liver, neither effectively diminishes excess glucose production between meals (75). Thus, constant hyperglycemia is observed. Acute-phase proteins are induced by pro-inflammatory stimuli. The concentration of CRP and Albumin has been suggested (76) as a marker of inflammation in conditions such as cancer and other inflammatory diseases. Interleukin-6 correlates positively with serum levels of CRP in CC (17). This scenario of chronic inflammation also associates positively with high levels of circulating FFA and insulin resistance in the liver. In summary, the molecular mechanisms underlying insulin resistance are composed of several pro-inflammatory factors, which will inhibit insulin signaling in hepatocytes by the activation of gene and protein expression of a plethora of proteins as the

suppressor of cytokine signaling (SOCS) and also of different kinases as JNK, IKK- β and of PKC, as of protein tyrosine phosphatases such as the protein-tyrosine phosphatase 1B (PTP1B) and the phosphatase and tensin homolog (PTEN), which in turn, may impair insulin signaling directly at the receptor or receptor substrate (IRS) level (18).

Figure 5 - Molecular mechanisms associated with CC and MetS in the Liver



Interleukin-6, TNF- α , IL-1 β , Resistin and leptin induce several pro-inflammatory signaling pathways that impair insulin signaling. Free-fatty acid enhanced uptake similarly induces insulin impairment and augmented acute-phase protein synthesis. Gluconeogenesis is also induced due to insulin signaling impairment.

1.4.3 Hyperinsulinemia

Either as a consequence of the insulin resistance or, in the case of patients in early stages of the metabolic syndrome, as a consequence of the repeated intake of processed carbohydrates from which glucose is rapidly available, these patients experience repeated episodes not only of hyperglycemia but in particular of hyperinsulinemia. Insulin levels may rise above the normal range (Table 2), and may remain elevated for prolonged periods extending beyond the normal transient peaks. Because insulin resistance does not affect all intracellular signaling chains to the same extent (77), this may result in the activation of

some processes downstream of the insulin receptor, despite the impaired insulin-dependent stimulation of glucose disposal. Among others, these include tumor promoting and antiapoptotic signals (78), as well as cell differentiation leading to inflammation (79).

Table 2 – Reference Range of Insulin Levels

	Insulin Level	Insulin Level (SI Units*)
Fasting	< 25 mIU/L	< 174 pmol/L
30 minutes after glucose administration	30-230 mIU/L	208-1597 pmol/L
1 hour after glucose administration	18-276 mIU/L	125-1917 pmol/L
2 hour after glucose administration	16-166 mIU/L	111-1153 pmol/L
≥ 3 hour after glucose administration	< 25 mIU/L	< 174 pmol/L
*SI unit: conventional units x 6.945		

Adapted from Melmed, 2011 (80)

1.5 MicroRNAs in inflammatory states and metabolic conditions

MicroRNAs are small non-coding RNAs of approximately 19–25 nucleotides (nt), regulating a wide panel of cellular functions, such as chromatin structure, chromosome segregation, transcription, RNA processing, RNA stability, and translation (81). miRNAs are described in several organisms including plants, nematodes, fruit flies and mammals, and one of their main characteristics is to be highly conserved during evolution (82). MicroRNA biogenesis is a very well described process in the literature; the first step is the transcription of genomic DNA by the enzyme RNA polymerase II. This yields a primary miRNA transcript, the so called “pri-miRNA”. Then, a complex of enzymes (Drosha-DGCR8 RNase complex) continues an intermediate step in miRNA production, through the cleavage of a stem loop into the pri-miRNA (83). After cleavage, a 60 to 70 nucleotide-long miRNA precursor (pre-miRNA) is formed with an overhang of 2-3 nucleotides (84). The pre-miRNA is then subsequently transported out of the nucleus, into the cytoplasm, by exportin 5 and is processed to a double-stranded RNA molecule of about 19 to 25 nucleotides in length, by the Dicer enzyme (85). In order to exert its functions, the newly synthesized miRNA requires incorporation into an effector complex, the miRISC (miRNA-induced silencing complex). The double stranded miRNA then is cleaved to a single stranded miRNA (86, 87), and one of the strands is either degraded, incorporated into another miRISC or, still, exported to the periphery by exosomes. When the latter occurs, miRNA will act in an endocrine way (88, 89). In order to have an

effect and regulate protein expression, the complex miRISC binds directly to the mRNA as to repress the translation of target genes by cleavage (perfect or near-perfect binding) or by forming a “hairpin” in the 3’UTR, through imperfect base pairing (90). Base-pairing of approximately 7 nucleotides must be complementary to provoke effects in the target mRNA (91). These base-pairing complementary nucleotides sequences are known as the “seed region” and consist on the most important pre requisite to the miRISC action. The binding site of the miRISC may also not correspond to the 3’UTR, but the ORF or 5’UTR region of the target, causing target gene repression or when binding to the 5’UTR, even stimulating the translation of target genes (92).

More than half of all described human genes are susceptible to modulation by miRNAs (93). MicroRNAs expression depends on to the local of synthesis, metabolic status and presence of disrupt states as diseases (94). Several studies point out a role for miRNAs as important regulators of diverse biological processes as cellular differentiation, proliferation, tissue development and cell-type specific function and homeostasis. Moreover, several inflammatory conditions have been linked in the past years to altered miRNAs expression (95, 96). MicroRNAs apparently modulate the magnitude of inflammatory responses (97) and act as pivotal players in the maintenance of inflammatory conditions such as cachexia and MetS.

1.5.1 *MicroRNA-21-5p*

Among over the six thousand described microRNAs on the international database for microRNAs (www.mirbase.org), some are known to interfere with the regulation of inflammatory processes. microRNA-21-5p is one of the most highly expressed members in several mammalian cell types (98) and was one of the first microRNAs to be identified in mammals. MicroRNA-21-5p mature sequence is highly conserved throughout evolution and its coding gene is located at the plus strand of chromosome 17q23.2 (55273409–55273480). This is an intronic region, and despite that, miRNA-21-5p shows particular promoter sites and when transcribed, originates an approximately 3433 nucleotides long primary transcript (pri-miR-21), and stem–loop precursor (pre-miR-21) located between nucleotides 2445 and 2516 of the pri-miR-21 (99). MicroRNA-21-5p dysregulated expression is frequently linked to the development of inflammation and consequently, of diseases, as

cancer (100-102) and obesity (103, 104). Specifically in regard to cachexia, one study showed that dysregulated miRNA-21-5p expression was linked to the wasting condition prevalent in patients and rodents (105). Among the specific types of cancer, including colorectal cancer, up-regulation of microRNA-21-5p is the most frequent observation, despite some of the studies not identifying differences in the expression in cancer patients, compared to non-cancer patients (11). In obesity, the opposite is observed, diminished microRNA-21-5p expression is linked to inflammation and obesity (103, 104). According to *mirbase*, microRNA-21-5p targets several essential proteins in the development of inflammation, such as PTEN and STAT3. A link between STAT3 activation and microRNA-21-5p is described (101, 102), as well as a negative correlation between miRNA-21-5p and pro-inflammatory mediators (102). Due to a high relevance in inflammatory processes, immune system cells were also focus of various studies in which the modulation by miRNA-21-5p was evaluated. Curiously, miRNA-21-5p is induced by STAT3 activation (106), but inhibits STAT3 through two sites of interaction in the 3'UTR region of STAT3 mRNA (101). Whether this represents a feedback mechanism is not fully clear. Since MetS and CC are inflammatory conditions, miRNA-21-5p appears as good candidate for the modulation of imbalanced inflammatory response.

1.6 Integrated metabolism and main hypothesis

Insulin resistance develops with inflammation as background (32, 107). Due to diminished insulin sensitivity in responsive tissues, the pancreas β cells produce and release more insulin, in order to maintain normal blood glucose levels even for long periods (108). However, as a consequence of the increased insulin release a hyperinsulinaemic condition develops. Hyperinsulinemia in turn, might further impact on insulin sensitivity even before hyperglycemia is established. The liver is an organ in which this is most likely to happen, because pancreatic hormones are secreted into the portal vein (109) and liver is exposed to much higher insulin concentrations than any other organ of the body, with the exception of the endocrine pancreas itself. The mechanisms behind such an insulin-induced insulin resistance are still poorly understood. Apart from desensitization on the receptor level, inflammation might play a role (110). In equines, a disease related with the sterile inflammation of the hoof corium, laminitis, develops after carbohydrate overload or after a

prolonged hyperinsulinaemic, euglycaemic clamp (111). This suggests that hyperinsulinemia itself might trigger local inflammation and subsequently, provoke insulin resistance. Once immune system cells are known to be active players in tissue local inflammation due to their capacity of secreting pro-inflammatory mediators, the main hypothesis of this study was to answer the question: Does hyperinsulinemia “per se” in some way contribute to the development or progression of insulin resistance, through the induction of inflammation?

Therefore we chose to examine whether insulin might elicit an inflammatory response in macrophages that could on its turn contribute to the development of insulin resistance. Moreover, although cachectic patients have lower insulin levels than patients with MetS, they present inflammation and insulin resistance. Among other mechanisms, miRNA-21-5p appears to be important to keep the inflammatory response in check. Therefore, the hypothesis was addressed that this miRNA might be diminished in cachectic patients resulting in an unrestrained inflammatory response.

2 AIMS

2.1 General

- To test the hypothesis whether insulin might elicit an inflammatory response in macrophages that could in turn contribute to the development of hepatic insulin resistance.
- To verify whether cachectic patients present difference in circulating microRNA expression, as compared to non-cachectic and control patients.

2.2 Specific

- To assess specific gene and protein expression of pro-inflammatory mediators and insulin signaling pathway proteins in cultured differentiated macrophages and primary rat hepatocytes;
- To assess specific circulating microRNA-21-5p and cytokine expression in cachectic patients, as compared to non-cachectic and control patients; and to correlate these results.

3 MATERIAL E METHODS

3.1 Part I – Insulin-induced insulin resistance: Cell culture and rat primary hepatocyte study

3.1.1 *Materials*

The antibiotics, fetal calf serum (FCS), calf serum and M199 culture medium were obtained from Biochrom AG (Berlin, Germany). The RPMI1640 medium was purchased from Gibco (Eggenstein, Germany). Dexamethasone, phorbol 12-myristate 13-acetate (PMA), Insulin and lipopolysaccharide (LPS) were obtained from Sigma-Aldrich (Deisenhofen, Germany). Oligo(89)₁₂₋₁₈ and acetone were obtained from Roth (Karlsruhe, Germany). The Maxima™ SYBR Green qPCR Master Mix and the RevertAid™ M-MuLV reverse transcriptase were obtained from ThermoFisher Scientific (Darmstadt, Germany). The antibodies against phospho-Ser306-IRS1, phospho-Ser636/639-IRS1, IRS1, p44/42 MAPK, phospho-p44/42 MAPK (Thr202/Tyr204), STAT3, phospho-STAT3 (Tyr705), phospho-Akt (Ser473), Akt and IL-1 β were purchased from Cell Signaling Technology (Frankfurt, Germany). The antibody against GAPDH was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). All oligonucleotides were customized by Biolegio (Nijmegen, The Netherlands) or Eurofins Genomics (Ebersberg, Germany).

3.1.2 *Animals*

Male Wistar rats (200-300 g, Charles River, Sulzfeld, Germany) were maintained on a 12-hour light/dark cycle with free access to standard rat diet #1320 (Altromin, Lage, Germany) and water. The principles of laboratory animal care were followed. Treatment of the animals followed the German animal protection laws and was performed with permission of the State Animal Welfare Committee (LUGV Brandenburg, V3-2347).

3.1.3 U937 cell culture

The buffers and solutions detailed preparation protocols are described in the supplementary material.

3.1.3.1 U937 cell culture differentiation experiment

The human monocytic cell line U937 was cultivated in medium RPMI1640 with 10% heat-inactivated FCS and 1% antibiotics (cell culture medium I). Cells were seeded in 35-mm diameter culture plates (1×10^6 cells per plate). Monocytes were tested for differentiation capacity into macrophages by the addition of 100 ng/ml Phorbol-myristate acetate (PMA) or 100 nM insulin for 24 h. The control cells were cultivated in cell culture medium I under no stimulation. After removal of the medium, macrophages were washed with RPMI1640, at room temperature and the number of differentiated cells estimated by counting under a microscope at 10 times magnification.

3.1.3.2 U937 macrophages stimulation with Insulin and LPS

The human monocytic cell line U937 was cultivated as described previously (3.1.3.1). Monocytes were differentiated into macrophages by addition of 100 ng/ml PMA for 24 h. After removal of the medium, macrophages were washed with RPMI1640 medium at room temperature and incubated in RPMI1640 medium with 0.5% heat-inactivated FCS and 1% antibiotics (cell culture medium II), in the absence of PMA for 24 h. Macrophages were then stimulated for another 24 h, with 100 nM insulin and/or 100 ng/ml LPS from *Escherichia coli* Serotype 0127:B8 to evaluate the effect of insulin, LPS and the possible synergism. Cells were shock-frozen in liquid nitrogen and stored at -70 °C for further analysis and the supernatant collected and centrifuged (10,000 x g, 15 min, and 4 °C).

3.1.4 Rat primary hepatocytes culture

Buffers and solutions detailed preparation protocols are described in the supplementary material. The experiments involving rat hepatocytes followed three initial steps: Isolation,

purification and culture. The hepatocytes isolation and purification was performed following the protocol of Meredith, 1988 (112). Hepatocytes were released from the cell structure of the liver by perfusion with an EDTA-containing buffer without calcium. This leads to cell separation once calcium is needed for cell-cell contacts. After perfusion, cells were purified by centrifugation, separated from non-parenchymal cells and debris with continuous Percoll density gradient and cultured in the appropriate culture medium.

3.1.4.1 *Non-recirculating in situ perfusion of rat liver for hepatocyte isolation*

One male Wistar rat (200-350 g body weight) was anesthetized by intraperitoneal injection of 100 μ l Narcoren/100 g body weight. After removing the skin of the abdomen, a longitudinal incision along the *linea alba* to the *processus xyphoideus* was made. The hepatic portal vein was exposed by shifting the intestinal miscellany. The portal vein was then cannulated and the liver was perfused with Perfusion buffer (140 mM NaCl, 5 mM KCl, 0.8 mM MgCl₂ x 6 H₂O, 1.6 mM Na₂HPO₄ x 2 H₂O, 0.4 mM KH₂PO₄, 25 mM NaHCO₃, 2 mM EDTA, 1.5 mM Glucose, 0.2 mM Lactate, 0.02 mM Pyruvate – pH 7.4) at a flow rate of 40 ml / min at 37 °C (2l per 45 - 60 min). After opening the inferior *vena cava*, the cannula in the portal vein was attached at the desired position, while the liver was flushed bloodless and the thorax was opened by extending the clavicle paramedian through a longitudinal incision (112).

3.1.4.2 *Preparation of rat hepatocytes*

After perfusion, the liver was dissected from the abdomen and placed on a glass plate filled with wash buffer (140 mM NaCl, 5 mM KCl, 0.8 mM MgCl₂ x 6 H₂O, 1.6 mM Na₂HPO₄ x 2 H₂O, 0.4 mM KH₂PO₄, 0.1 mM CaCl₂ x H₂O). The liver was torn and completely separated from the hepatic portal vein, and the cells detached. In order to remove any remaining connective tissue and larger cell aggregates, the cell suspension was filtered with washing buffer through a moistened cloth nylon sieve (pore size 80-100 microns). The cell suspension was centrifuged for 2 min at 20 x g (300 rpm, Heraeus Biofuge Primo), at room temperature. The supernatant was then aspirated and the cell sediment resuspended twice in 50 ml of wash buffer and centrifuged for 2 min at 20 x g.

3.1.4.3 *Rat hepatocytes isolation by density gradient centrifugation*

After the third centrifugation, the cell sediment was resuspended in wash buffer to a volume of 16ml and mixed with 32ml of Percoll gradient medium (27.8 ml Percoll and 4.2 ml Perfusion solution 10 X (1400 mM NaCl, 50 mM KCl, 8 mM MgCl₂ x 6 H₂O, 16 mM Na₂HPO₄ x 2 H₂O, 4 mM KH₂PO₄). Percoll final concentration was 58% (v/v). The suspension was centrifuged for 5 min at 800 x g (2100 rpm, Hettich centrifuge Rotina 35), at room temperature. Non-parenchymal cells, dead hepatocytes and cell debris were aspirated, along with the excess of buffer.

3.1.4.4 *Cell counting*

The counting of hepatocytes was performed in a Neubauer chamber (0.0025 mm² / 0.1 mm) by pipetting 96 µl of M199 cell culture medium mixed with 4 µl of Hepatocyte suspension (1:25 dilution), under a microscope at 10 times magnification.

3.1.4.5 *Rat hepatocyte culture*

Hepatocytes were plated and immediately treated with M199 cell culture medium with 4% (v/v) neonatal calf serum for optimal attachment to the bottom of the polystyrene trays, with 1% (v/v) penicillin/streptomycin, 0.1 mM Dexamethasone and 0.5 nM insulin (= M199 cell culture medium I). The hepatocytes were cultured in 35 mm cell culture plates at a density of 1.0×10^6 cells (1.5 ml). After an initial period of 4 hours, the medium was changed to 1.5 ml M199 culture medium II, in the absence of neonatal calf serum (NCS). Another Medium change with M199 culture medium II was carried out after 24 h. The cell culture experiments occurred after a period of 40 hours from seeding. The incubation of hepatocytes was performed in a steam saturated atmosphere with 5% (v/v) CO₂, at 37 °C. Experimental treatments were performed after 44 h of culture, in M199 cell culture medium containing 1% antibiotics and 100 nM Dexamethasone for 4 h. Subsequently, hepatocytes were incubated with medium containing 50% of the supernatants obtained from U937 macrophages incubation with or without 100 nM insulin for 15 or 120 minutes. Cells were shock-frozen in liquid nitrogen and stored at -70 °C for further analysis.

3.1.4.6 U937 macrophages and rat primary hepatocytes protein expression analysis by Western blotting

The Western Blot experiment was performed following in three steps: protein separation by molecular size, protein transfer from a gel to the membrane, and target protein labeling, with a proper primary and secondary antibodies as to allow detection (113).

3.1.4.6.1 Preparation of cell lysates

Rat primary hepatocytes and U937 macrophages were ruptured in 100ul of lysis buffer with protease inhibitors and Sodium orthovanadate [20 mM Tris / HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100 (v/v), 2.5 mM Sodium pyrophosphate, 1 mM β -Glycerol phosphate, 50 mM NaF, 200 μ M Pefablock, 10 μ g/ml Leupeptin, 10 μ g/ml Trypsin inhibitor, 1 mM Na_3VO_4] to each cell culture plate. Subsequently, the cells were scraped off and transferred to the lysates in 1.5 ml reaction tubes. For optimal cell disruption, the cell suspension was homogenized for 10 seconds under ultrasound (Ultrasound Wand Bandelin UW 2070 Power 55%, Cycle 5). Then, cell homogenates were centrifuged for 15 min at 10,000 x g (Sigma 3K30 centrifuge rotor 1215/4-H), and the supernatant transferred to 1.5 ml reaction tubes. The cell lysates were stored at -20 °C. Determination of the protein content was performed by the method described by Bradford (114).

3.1.4.6.2 Measurement of sample protein content

The samples were diluted 1:100 by adding into 3 μ l of the lysate, 27 μ l H_2O , and then 10 μ l of the prediluted lysate to 90 μ l H_2O (duplicates). The Standard series consisted of decreasing concentration dilutions of a BSA (1 mg/ml) stock solution ranging from 0.0 mg/ml to 0.01 mg/ml, 0.02 mg/ml, 0.04 mg/ml, 0.06 mg/ml, 0.08 mg/ml and 0.1 mg/ml. The measurement was performed in a 96-well plate by adding (duplicates) 20 μ l per sample / standard series to 180 μ l of the Bradford reagent. The absorbance (570 nm) was determined after 10 minutes with the MRX II Microplate Reader (Dynex Technologies, Denkendorf, Germany).

3.1.4.6.3 Preparation of the samples for SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The protein content of cell lysates was determined as described in item 3.1.4.10.4 and all the samples were diluted with lysis buffer to 40 µg of protein, in a maximum volume of 45 µl. The samples were then mixed with 1/3 volume of sample buffer [80 mM Tris / HCl (pH 7.5), 2% SDS (w/v), 5% Glycerol (w/v), 0.0025% Bromophenol blue (w/v), 20% β-mercaptoethanol], briefly centrifuged (spin) and then denaturated on heating block for 5 min at 95 °C, followed by another centrifugation for 5 min at 13,000 rpm (16,050 x g, Biofuge Pico). For the detection of IL-1β in the supernatants of the U937 macrophage culture, the medium was mixed with 1:5 acetone, stored over night at -20 °C, and centrifuged (4696 x g, 5 min, 4 °C). The pellet was resuspended in 55 µl of the sample buffer.

3.1.4.6.4 Protein separation by SDS-PAGE

For the analytical separation of proteins by molecular weight, discontinuous polyacrylamide gels were used according to Laemmli (115). Gels were set and run in the electrophoresis system Mini Protean II (BIO-RAD). The resolving gels were cast with 10% polyacrylamide [30% Acrylamide (30% w/v), 22.5% Resolving gel buffer (1.5 M TRIS, 0.4% SDS (w/v), pH 8.8), 0.15% TEMED and 0.75% APS (10% w/v)] for the detection ERK 1/2, STAT3 and 6% acrylamide [18% Acrylamide (30% w/v), 22.5% Resolving gel buffer (1.5 M TRIS, 0.4% SDS (w/v), pH 8.8), 0.15% TEMED and 0.75% APS 10% (w/v)]for IRS1. The stacking gel was cast with 4.45% polyacrylamide [25% Acrylamide (30% w/v), 30% Stacking gel buffer (0.5 M TRIS, 0.4% SDS (w/v), pH 6.8), 2% TEMED and 10% Ammonium persulfate (APS, 10% w/v)]. Samples (45 µl) and the protein standard were pipetted to the gels. Electrophoresis was set at 15 mA / gel for the stacking gel and 20 mA / gel for the resolving gel.

3.1.4.6.5 Protein transfer to PVDF membranes

After gel electrophoresis, the stacking gel was carefully separated, removed from the upper left corner of the separating gel and equilibrated for 15 minutes in Transferbuffer C [25 mM TRIS, 0.1% SDS (w/v), 20% Methanol (v/v), pH 9.0]. Then, the PVDF membrane was briefly dropped in pure methanol for activation and equilibrated in Transferbuffer B [2 mM TRIS,

0.1% SDS (w/v), 20% Methanol (v/v), pH 10.5] for about 15 minutes. The transfer took place at a constant current of 1.2 mA / cm² gel area (= 56 mA / gel), over a period of 45 minutes (Akt, ERK 1/2, STAT3, IL-1 β) or 120 minutes (IRS1, IRS2).

3.1.4.6.6 Detection of transferred proteins by Ponceau S staining

The PVDF membrane rested briefly in a bowl with Ponceau S staining solution [0.25% Ponceau S, 40% Methanol (v/v), 15% Acetic acid (v/v)] and the protein bands were reversibly stained. The membrane was then washed with TBS / Tween buffer [TRIS 0.02 M, NaCl 0.136 M, 20% TWEEN (0.1% v/v)], three times for 5 minutes.

3.1.4.6.7 Immunological detection of proteins by the peroxidase-mediated chemiluminescent reaction

For the saturation of nonspecific binding sites, the membrane was incubated in blocking solution (5% non-fat milk powder (w/v) in TBS / Tween Buffer) for 60 minutes at room temperature. Thereafter, it was washed three times for 10 min with TBS / Tween buffer and incubated overnight at 4 °C with the primary antibody incubation (Table 3). On the next day, the primary antibody was removed, and the membrane washed again three times for 10 minutes each with TBS / Tween buffer. The incubation with the peroxidase-coupled secondary antibody (1:4000) was performed for 2 h at room temperature in blocking solution. Depending on the primary antibody, the secondary antibody was conjugated with a goat anti-mouse or goat anti-rabbit-HRP (see Table 7). After incubation, the membrane was again washed three times for 10 min with TBS / Tween buffer. The experiment was completed through a chemiluminescent reaction with the SuperSignal® West Pico Chemiluminescent Substrate (ThermoFisher Scientific Inc., Waltham, MA, USA), by mixing solutions 1 and 2 of the kit in equal parts to a final volume of 2 ml / cm² PVDF membrane. After an incubation time of 5 minutes, the membrane was inserted in cling film and the excess of chemiluminescent substrate removed by carefully rolling with a glass tube. The resulting chemiluminescence was directly detected by the ChemiDoc™ system (BIO-RAD) and the stained bands were quantified with the software program Imagelab (BIO-RAD).

Table 3 - Primary and secondary antibodies adopted in the Western blot analysis

Primary antibody	Dilution in 5 % (w/v) BSA in TBS/Tween	Secondary antibody
pAkt(Ser473)	1:1500	Goat-anti-Mouse (1:4000)
Akt	1:1500	Goat-anti-Rabbit (1:4000)
pERK1/2	1:1500	Goat-anti-Mouse (1:4000)
ERK1/2	1:1500	Goat-anti-Rabbit (1:4000)
pSTAT3	1:1500	Goat-anti-Rabbit (1:4000)
STAT3	1:1500	Goat-anti-Mouse (1:4000)
pSerin636-IRS1	1:1000	Goat-anti-Rabbit (1:4000)
pTyrosin632-IRS1	1:500	Goat-anti-Rabbit (1:4000)
IRS1	1:1000	Goat-anti-Rabbit (1:4000)
IRS2	1:1000	Goat-anti-Rabbit (1:4000)

3.1.4.7 U937 Macrophage and rat primary hepatocyte gene expression analysis by Real time PCR

The PCR experiments were preceded by the synthesis of complementary DNA through reverse transcription reaction from isolated RNA, followed by polymerase chain reaction. This technic has been proven to be an adequate and powerful method to quantify gene expression (36).

3.1.4.7.1 Total RNA extraction

Total RNA was isolated from frozen rat hepatocytes and U937 macrophages adopting the peqGold Total RNA Kit (Peqlab, Erlangen, Germany), according to the manufactures protocol. Briefly, after culture medium aspiration, 400 µl RNA lysis buffer was applied directly to the cells. The lysate was transferred to a DNA removing column, placed in a 2.0 ml collection tube. A centrifugation step was performed (12,000 x g, 1 minute at room temperature). Then, 400 µl of a 70% ethanol solution was added and mixed by vortexing and the mixture transferred to a RNA binding column, followed by another centrifugation step (10,000 x g, 1 min at room temperature). The column was then washed two times with the supplied wash buffer and the elution step performed with RNase-free water. The quantification was performed by measuring the absorption rate at 260/280 nm. One A260-unit is about 40 µg RNA/ml. The RNA concentration was calculated as follows:

RNA concentration ($\mu\text{g/ml}$) = Absorption 260 \times 40 \times Dilution Factor.

The samples were stored at -70°C .

3.1.4.7.2 Complementary DNA synthesis

Complementary DNA was synthesized from 600 – 1500 ng purified RNA and 500 ng Oligo(89)₁₂₋₁₈ in DEPC treated water (13.0 μl), 5X reaction buffer (250 mM Tris-HCl pH 8.3, 250 mM KCl, 20 mM MgCl_2 , 50 mM DTT) (4 μl), 200 U of RevertAid™ M-MuLV reverse transcriptase (1 μl) and 10 mM dNTP Mix (2 μl), in a total volume of 20 μl . The reaction was incubated for 60 min at 42°C in a T3000 Thermocycler (Biometra GmbH, Göttingen, Germany).

3.1.4.7.3 Real-time PCR amplification

Real-time PCR for the quantification of each transcript was performed in triplicates in a reaction mixture of 2x SybrGreen qPCR Master Mix®, 250 nM of forward and reverse oligonucleotides (table 4), and 0.3 μl cDNA, in a total volume of 10 μl . qPCR was performed with an initial enzyme activation step at 95°C for 10 min, followed by fifty cycles of denaturation at 95°C for 20 sec, annealing at $57\text{--}60^\circ\text{C}$ for 30 sec and extension at 72°C for 20 sec, and subsequent melting curve analysis in a CFX96™ Thermal Cycler (Bio-Rad, Munich, Germany). The expression level was calculated as the n-fold induction of the gene of interest in treated versus control cells with β -actin as a reference gene. The calculation was based on the differences in the threshold cycles between control (c) and treated (treat) groups according to the formula: $2^{-(c\text{-treat})_{\text{interest gene}}} / 2^{-(c\text{-treat})_{\text{reference gene}}}$ (36).

Table 4 - U937 Cell culture and rat primary hepatocytes study primer list

Gene	Species	Forward (5'-3')	Reverse (5'-3')	Acc. No.
β -Actin	human	CCCCAAGGCCAACCGCGAGAAGATG	AGGTCCCGGCCAGCCAGGTCCAG	NM_001101.3
β -Actin	rat	CCCTAAGGCCAACCGTGAAAAGATG	AGGTCCCGGCCAGCCAGGTCCAG	NM_031144.2
CCL2	human	TCAGCCAGATGCAATCAATG	AGCTTCTTTGGGACACTTGC	NM_002982.3
COX2	human	TGTGCCTGATGATTGCCCGACTCC	TGTTGTGTTCCCGCAGCCAGATTG	NM_000963.2
Glucokinase	rat	GCCGTGCCTGTGAAAGCGTGTC	CCAGGGGTAGCAGCAGAATAGGTC	NM_012565.1
IL-1 β	human	CCAGCTACGAATCTCCGACC	AGAACACCACTTGTGCTCCA	NM_000576.2
IL-6	human	CATCCTCGACGGCATCTCAG	TCACCAGGCAAGTCTCCTCA	NM_000600.3
IL-8	human	CAGTTTTGCCAAGGAGTGCTAA	AACTTCTCCACAACCTCTHC	NM_000584.2
mPGES1	human	GAAGAAGGCCTTTGCCAACCC	GTGCATCCAGGCGACAAAAG	NM_004878.4
OSM	human	CACCCAGCATGGGGGTACT	GCACGCGGTACTCTTTCGAG	NM_020530.3
SOCS3	rat	GACCTTCAGCTCCAAGAGCGAGTA	CGCCCCAGAATAGATGTAGTAAGC	NM_053565.1
TNF- α	human	GGCTCCAGGCGGTGCTTGTT	GTAGGAGACGGCGATGCGGC	NM_000594.2
IL-10	human	CGAGATGCCTTCAGCAGAGT	AATCGATGACAGCGCCGTAG	NM_000572.2
TLR2	human	GAGTTCTCCAGTGTTTGGTG	ATTGTCCAGTGCTTCAACCCA	NM_003264.3
TLR4	human	GGTGCCTCCATTTAGCTCT	ACTGCCAGGTCTGAGCAATC	NM_138554.4
				NM_003266.3
				NM_138557.2

3.1.4.8 Statistics

All data are expressed as means \pm SE. Differences between the groups were analyzed by Student's unpaired T-test and considered statistically significant when $p < 0.05$.

3.2 Part II – Study with cachectic cancer patients

3.2.1 Patient recruitment

3.2.1.1 Ethics Committee Approval

The Project was approved by the University of São Paulo Biomedical Sciences Institute Ethics Committee (1109/CEP) and the University Hospital Ethics Committee (HU/USP: 1387/14) and registered on the Brazilian Platform for Human Studies (CAAE:13803213.0.0000.5467).

3.2.1.2 Inclusion criteria

Patients (n = 48) were recruited between August 2014 and July 2015 at the University Hospital of the University of Sao Paulo. The recruitment was carried out by the Hospital physicians and consisted in selecting patients engaged in the treatment of hernia (Control group, n = 16) and cancer (weight stable group, n = 16; cachectic group, n = 16). The inclusion criteria were not having received prior anticancer or anti-inflammatory treatment (the hospital operates only with patients who will undergo surgery, but no adjuvant treatment, as determined by the in-charge physicians, according to individual clinical recommendations); and willingness to participate. The criterion for the diagnosis of gastric cancer was the based on the TNM Classification of Malignant Tumors (116) (Esophagus, Stomach, Small intestine, Colon and rectum, Anal canal, Liver, Gallbladder, Extra bile liver pathways, Vater Ampoule and Pancreas). The exclusion criteria were liver failure, renal failure, AIDS, inflammatory diseases of the bowel; autoimmune disorders and continuous anti-inflammatory medication.

3.2.1.3 Consent term signature obtainment and clinical evaluation

After the selection, anthropometric measurements were obtained (height, weight, weight loss delta) and the patients were interviewed with a cancer patient oriented Quality of life questionnaire, EORTC QLC-C30 (117), validated for Portuguese (118), which evaluates three parameters of quality of life: Global Health Status, Functional Scales and Symptoms scales. A

high score for Global Health Status and Functional Scales means better quality of life; a high score for Symptoms scale means worsened quality of life. The patients under cancer treatment were then divided into two groups, based on the answers and on the anthropometric measurements. The cachectic group (TC) consisted of patients with a self-declared weight loss > 5% in the past six months or weight loss between 2% and 5% in the past six months, with concomitant presence of sarcopenia; reduced food intake and/or systemic inflammation (14). The cancer weight stable group (WSC) consisted of patients in treatment for cancer without declared weight loss > 5% in the past 12 months. Full written consent form was obtained from each patient (Supplementary material). All procedures were carried out in the hospital premises.

3.2.2 *Assessment of Clinical and biochemical parameters*

Height and weight were assessed and, approximately 20 mL of blood, collected prior to the surgical procedure (by a certificated trained health professional). Blood was placed in tubes with or without anticoagulant (EDTA) and then, centrifuged at 3000 rpm for 15 minutes, at 4°C to obtain plasma and serum, respectively. Then, plasma and serum were transferred to plastic microtubes and stored at -80 °C for analysis. Serum concentration of hemoglobin was determined by the University Hospital, while C-reactive protein and albumin were measured with commercial kits (PCR Plus Ultra Turbiquest ref 335 – Labtest diagnostica SA, Lagoa Santa, MG, Brazil; range 0.5 – 10 mg/L) and Albumin - Diagnostic Labtest ref 19 - Labtest diagnostica SA, Lagoa Santa, MG, Brazil; range 0-6.0 g/dL), according to the manufacturer's protocols.

3.2.3 *Real time PCR analysis*

3.2.3.1 *Total RNA extraction*

Total RNA was isolated from patients' plasma employing the *mirVana*[™] miRNA Isolation Kit (Thermofisher Scientific Inc., Waltham, MA, USA), according to manufacturer's instructions. Briefly, the samples were first lysed in a denaturing lysis solution, which stabilize RNA and inactivated RNAses. The lysate was then extracted with 50% Acid-Phenol:Chloroform (119),

which removed most of the other cellular components, leaving a semi-pure RNA sample. This RNA was further purified over a glass-fiber filter to yield total RNA and 5 µl of total extracted RNA was aliquot for further cDNA synthesis.

3.2.3.2 *Complementary DNA synthesis*

Complementary DNA (cDNA) was synthesized from 5 µl purified RNA with the TaqMan® MicroRNA Reverse Transcription Kit (Thermofisher Scientific Inc., Waltham, MA, USA). Specific cDNA was synthesized for the miRNA-21-5p and RNU6B by adding to the cDNA Mastermix the miRNA-21-5p (Thermofisher Scientific Inc., Waltham, MA, USA, CAT# 4427975) or RNU6B specific primers (Thermofisher Scientific Inc., Waltham, MA, USA, CAT# 4427795). The reaction was setup in a mixture of 0.15 µl of 100nM dNTPs (with dTTP), 1 µl MultiScribe™ Reverse transcriptase (50 U/µl), 1.50 µl 10X Reverse Transcription Buffer, 0.19 µl RNase Inhibitor (20 U/µl), 4.16 µl Nuclease-free Water in a volume of 7 µl. Then, 3 µl of 5X RT specific primers and 5 µl of miRNA sample were added to the mixture, in a total of 15 µl reaction volume. The reaction was performed in the 96 well VERITI thermocycler (Thermofisher Scientific Inc., Waltham, MA, USA), at the following parameters: Step I, 30 minutes at 16 °C; step II, 30 minutes at 42 °C; step III, 5 minutes at 85 °C. Complementary DNA synthesis for large molecular weight RNAs was carried out using the high capacity cDNA reverse transcription kit (Thermofisher Scientific Inc., Waltham, MA, USA), which consisted of an assay mix containing 5 µl purified RNA, 2 µl 10× RT Buffer, 0.8 µl 25× dNTP mix (100 mM), 2 µl 10× Random primers, 1 µl MultiScribe™ Reverse Transcriptase and 9.2 µl of nuclease-free water in a final volume of 20 µl. The thermal cycler conditions were: 25 °C for 10 min, then 37 °C for 120 min followed by 85 °C for 5 min.

3.2.3.3 *Primer validation*

The choice of the endogenous control for the quantification of miRNA-21-5p was based on a microRNA array (Human miRNome miScript® miRNA PCR Array, Qiagen, Valencia, CA, USA), in which six endogenous controls were tested in one pool per group of human plasma samples: SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A and RNU6B (supplementary figure S1). The RNU6B was shown to be the least prone to variation gene in the samples.

However, when tested in each sample, RNU6B amplification presented cycle threshold (Ct) for the vast majority of samples was over the value of 35 Cts. This way, large molecular weights RNAs were also tested as endogenous controls to avoid possible misleading results. Primers for 18S, HPRT and RPL-27 were tested in one pool per group of human plasma samples (supplementary figure S2). The primer for 18S displayed good linearity ($r^2 = 0.999$) and was shown to be the least prone to variation. Then, gene expression results for miRNA-21-5p were calculated with RNU6B or 18S as reference genes, and the results correlated by the Spearman's test ($r = 0.5835$; $p = 0.0028$), confirming a positive correlation, and validating the results for both primers. The primer for 18S was chosen as reference gene, as it was the most stable in samples.

3.2.3.4 Real-time PCR Amplification

Realtime PCR for the quantification of each miRNA-21-5p and RNU6B transcripts was performed in samples duplicates by mixing 1.00 μ l of TaqMan® Small RNA Assay (20 \times), 1.33 μ l of the specific synthesized cDNA for miRNA-21-5p or RNU6B, 10 μ l of TaqMan® Universal PCR Master Mix II (2 \times), no UNG and 7.67 μ l of nuclease-free water, in a total volume of 20 μ l. The reaction was performed with an initial enzyme activation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 seconds, annealing and extension at 60 °C for 60 seconds, with a subsequent melt curve analysis in the Quantstudio 12K™ Thermal Cycler (Thermofisher Scientific Inc., Waltham, MA, USA). Real-time PCR for the quantification of each of the large molecular weight RNAs 18S, RPL-27 and HPRT transcripts was carried out in duplicates in a reaction mixture of 2x SybrGreen® qPCR Master Mix, 400 nM forward and reverse oligonucleotides (table 5), and 4.2 μ l cDNA in a total volume of 10 μ l qPCR was performed with an initial enzyme activation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95 °C for 20 sec, annealing at 60 °C for 30 sec and extension at 72 °C for 20 sec with a subsequent melt curve analysis in a Quantstudio 12K™ Thermal Cycler (Thermofisher Scientific Inc., Waltham, MA, USA). The expression level was calculated as the n-fold induction of the gene of interest in treated versus control cells with 18S as reference gene. The calculation is based on the differences in the threshold cycles between control (c) and treated (treat) groups according to the formula: fold induction = $2^{-(c_{\text{treated}})_{\text{interest}}} / 2^{-(c_{\text{treated}})_{\text{reference}}}$ (36).

Table 5 – Large molecular weight RNA Primer List

Gene	Species	Forward (5'-3')	Reverse (5'-3')	Acc. No.
18S	Human	CCTGCGGCTTAATTTGACTC	ATGCCAGAGTCTCGTTCGTT	NR_003286.2
RPL-27	Human	CCGAAATGGGCAAGTTCAT	CCATCATCAATGTTCTTCACGA	NM_000988.3
HPRT	Human	TGGCGTCGTGATTAGTGATG	CTTGAGCACACAGAGGGCTA	NM_000194.2

3.2.4 Measurement of plasma cytokine and chemokine content (IL-1 β , IL-6, IFN- γ , TNF- α , IL-10, IL-8, CCL2)

The protein measurement protocol consisted of incubating the samples (50 μ l) with a mixture of MagPlex® beads coated with different antibodies for 2 hours, followed by the detection of target antigens bound to the microspheres with a mixture of biotinylated capture antibody incubated for 1 hour; then followed by incubation with phycoerythrin labeled streptavidin for 30 minutes. The identification of microspheres was performed with the Luminex instrument MAGPIX (ThermoFisher Scientific Inc., Waltham, MA, USA). After reading, the value for each cytokine was analyzed with the Analyst 5.1 software, and expressed as pg/ml.

3.2.5 Statistics

Data were expressed as mean \pm standard error or median [1st. quartile; 3rd. quartile]. The groups were compared using One-way ANOVA followed, as needed, for multiple comparisons by Tukey's post-test. Data with non-parametric distribution were compared employing Kruskal-Wallis method, followed by Dunn's post-test. The Spearman correlation coefficient was obtained to evaluate the linear relationship between the variables of interest. The significance level was set at $p < 0.05$. Human experiments data were also tested and corrected for outliers. All Human data statistical procedures were performed with the assistance of the Statistics Sector of the Institute of Biomedical Sciences, USP, under the supervision of Mrs. Rosana Duarte Prisco. The statistical software Statgraphics® Centurion XVI version 2.16.04 (Statpoint Technologies, Inc. Warrenton, Virginia, USA), and the Graphpad Prism 5.0 (San Diego, California, USA) were employed in the calculation.

4 RESULTS

4.1 Part I – Insulin-induced insulin resistance: Cell culture and rat primary hepatocyte study

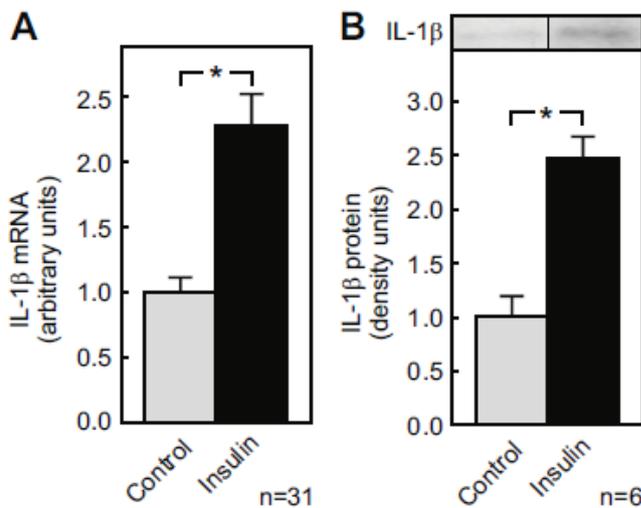
The experiments were performed in the laboratory of Nutritional Biochemistry at the University of Potsdam, in collaboration with Ms. Julia Manowsky, who increased the number of samples in several experiments and performed assays yielding complementary data. Part of the results has been already published in the American Journal of Physiology, Endocrinology and Metabolism (120), and are also included in this Thesis.

4.1.1 *Insulin-dependent induction of IL-1 β production in U937 macrophages*

U937 cells were differentiated into macrophages by PMA treatment. Macrophage-differentiated U937 cells were stimulated with insulin (100 nM) for 24 h. This stimulation induced a significant increase in IL-1 β expression (2 to 2.5-fold), both in terms of mRNA and of protein expression (Figures 6a and 6b). Based on the experiments above a number of experiments were performed in the laboratory (Julia Manowsky) to corroborate the hypothesis that the increase in IL-1 β formation was really a direct consequence of the stimulation with insulin. Firstly, to exclude that a minor contamination of the insulin preparation with LPS elicited the IL-1 β induction, experiments were performed in which polymyxine B (LPS inhibitor) was included in the cell culture medium. While an LPS-elicited induction of IL-1 β was completely abolished by polymyxine B, the insulin-dependent induction was not affected. By contrast, the insulin-dependent induction was abrogated by the insulin receptor antagonist (GSK1838705), which, however, did not attenuate the LPS-dependent induction. Thus, the insulin-dependent induction of IL-1 β most likely reflected a specific effect of insulin that was mediated via its receptor. Once confirmed that insulin stimulates IL-1 β gene and protein expression in U937 cells, after differentiation with PMA, U937 differentiated cells exposed to insulin (100 nM) for 24 h were tested for gene expression of several other factors with pro-inflammatory and chemoattractant action, such as IL-8, CCL2, IL-6, OSM, and TNF- α (Table 5). In addition of IL-1 β , Insulin induced in a significant manner the gene expression of IL-8, CCL2, OSM COX2 and mPGES1. This might

indicate that insulin in addition to increasing the production of cytokines and chemokines increased the production of prostaglandin E₂ in U937 macrophages. Thus, prostaglandin E₂ might further modulate the function of the macrophages in an autocrine loop (see below).

Figure 6 - Insulin-dependent induction of IL-1 β in U937 macrophages



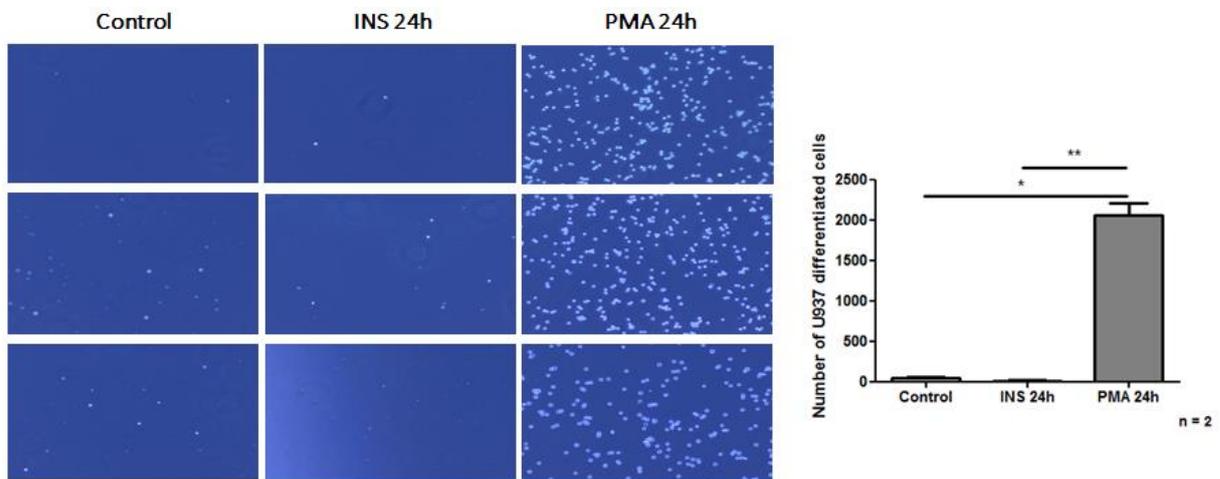
Adapted from Manowsky and Camargo (120)

U937 macrophages were stimulated with 100 nM insulin for 24 h. (A) IL-1 β mRNA was quantified by Real time PCR with β -actin as reference gene. (B) IL-1 β protein was measured in cell culture supernatants by Western Blotting. Values are means \pm SE of the number of independent experiments indicated. Student's T-test for unpaired samples; *: p < 0.05. (A) Data generated in collaboration with Ms. Julia Manowsky, (B) Data generated solely by Ms. Julia Manowsky.

4.1.2 *Exclusion of an insulin-dependent induction of U937 monocyte differentiation into macrophages*

Treatment of U937 monocytes with PMA for 24 h resulted in a transformation into macrophages. While monocytes grew in suspension culture, the PMA-treated cells firmly attached to the cell culture plates. Stimulation with insulin (100 nM) for 24 hours did not provoke any significant increase in the number of U937 macrophage-differentiated attached cells, and did not modulate the PMA-dependent attachment, rendering it very unlikely that insulin could stimulate or modulate monocyte differentiation into macrophages (figure 7).

Figure 7 - U937 monocyte differentiation test



Control cells were kept in RPMI1640 medium with 10% heat-inactivated FCS and 1% antibiotics (cell culture medium I) for 24 h (Control) and stimulated cells were treated for 24 h with 100 nM insulin (INS 24 h) or PMA (PMA 24 h). The number of differentiated cells was estimated by counting under light microscope (10 x magnification). Values are means \pm SE of three measurements of each number of independent experiments indicated. One-way ANOVA with Tukey's post-test. *, **: $p < 0.0001$.

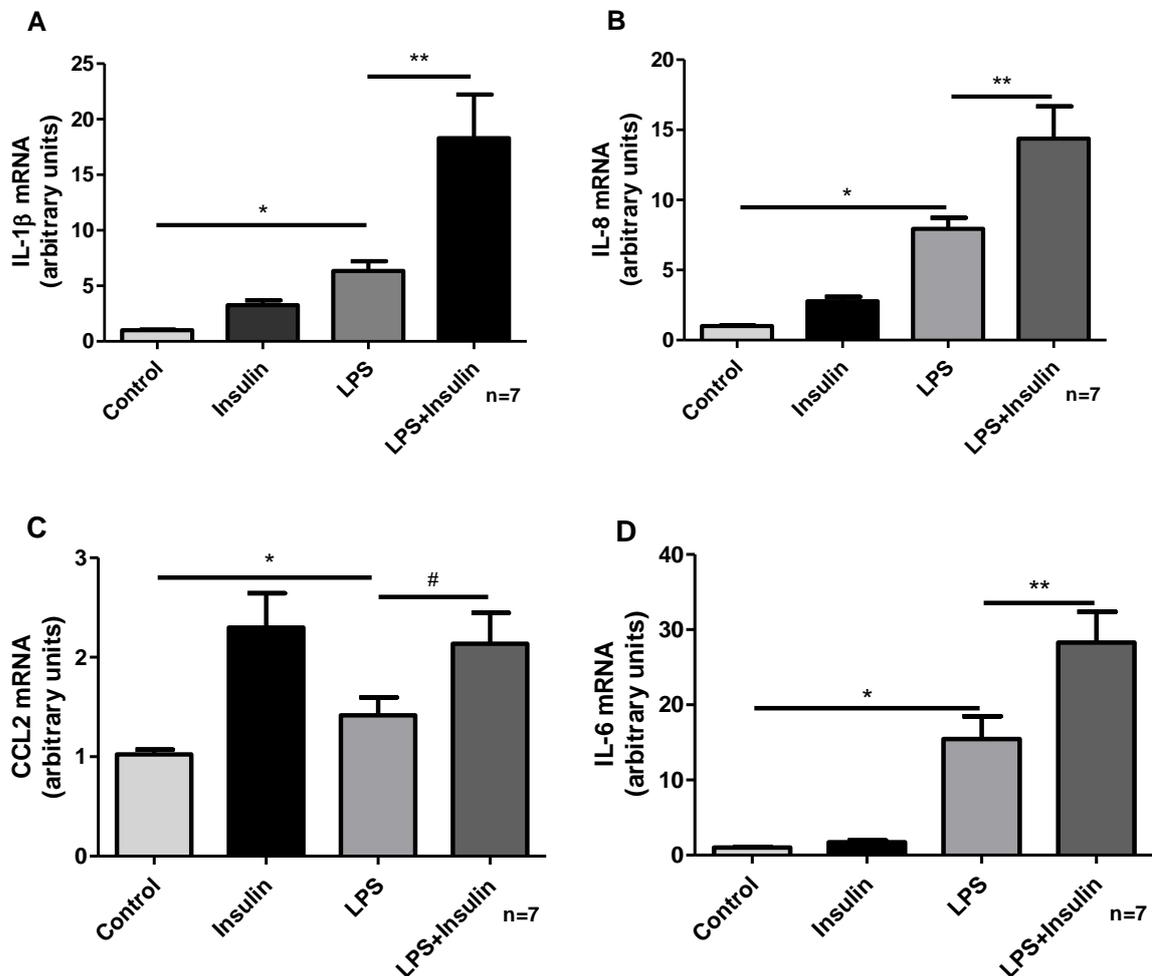
4.1.3 *Insulin-dependent induction of cytokine production in U937 macrophages and synergism with pro-inflammatory mediators*

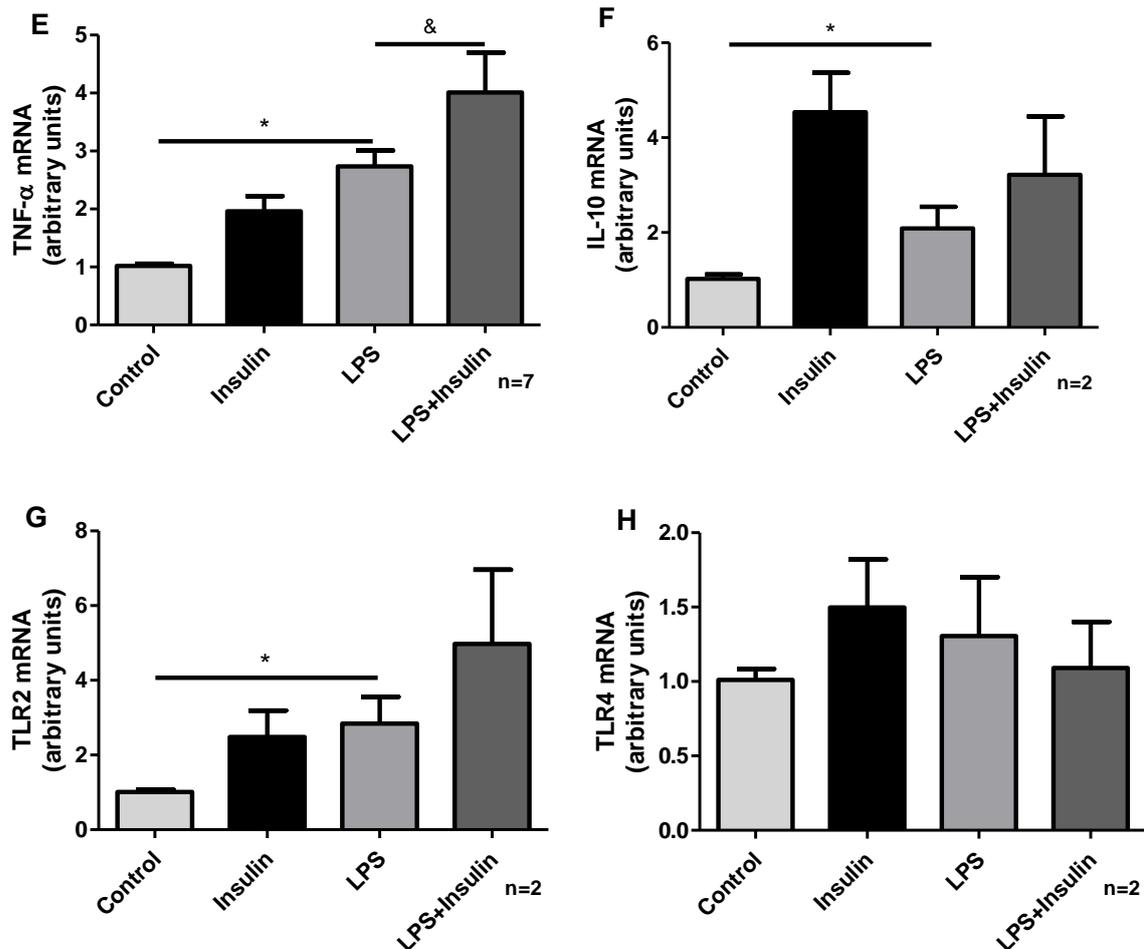
Patients with metabolic syndrome often display slightly elevated plasma endotoxin levels and Insulin induced key enzymes of PGE₂ synthesis in U937 macrophages. Therefore, the synergism among insulin, Lipopolysaccharide and PGE₂ was also tested (table 6 and table 7). Lipopolysaccharide stimulation provoked a significant increase in pro-inflammatory mediator gene expression, as verified by the induction of IL-1 β , IL-8, CCL2, IL-6, TNF- α and TLR2. Insulin potentiated this induction in IL-1 β , IL-8 and IL-6 genes. For CCL2 there is a similar trend. As for TNF- α and IL-10 insulin caused an induction that, although not significant, was larger than the LPS-dependent induction, highlighting that there is in some cases a synergism between Insulin and LPS, which potentiates pro-inflammatory stimuli (figure 8).

Like insulin, PGE₂ increased the expression of IL-1 β . The induction by a combination of insulin and PGE₂ was larger than for either stimulus alone. Similarly, PGE₂ induced IL-6 mRNA, however, this induction was not further enhanced by the presence of insulin. By contrast, PGE₂ reduced the basal expression of TNF- α and CCL2, while Insulin tended to

increased their expression (although not significantly). When applied together, Insulin attenuated or abolished the inhibitory effect of PGE₂ on the CCL2 and TNF α production, thus disrupting a putative autocrine feedback inhibition loop. Notably, PGE₂ induced the two key enzymes of its own synthesis, namely COX2 and mPGES1. This induction was further enhanced by the simultaneous presence of insulin (figure 9d and 9e). Finally, Insulin did not induce significantly either of the toll-like receptors, rendering unlikely the hypothesis, that this would be the cause for the synergism between insulin and LPS.

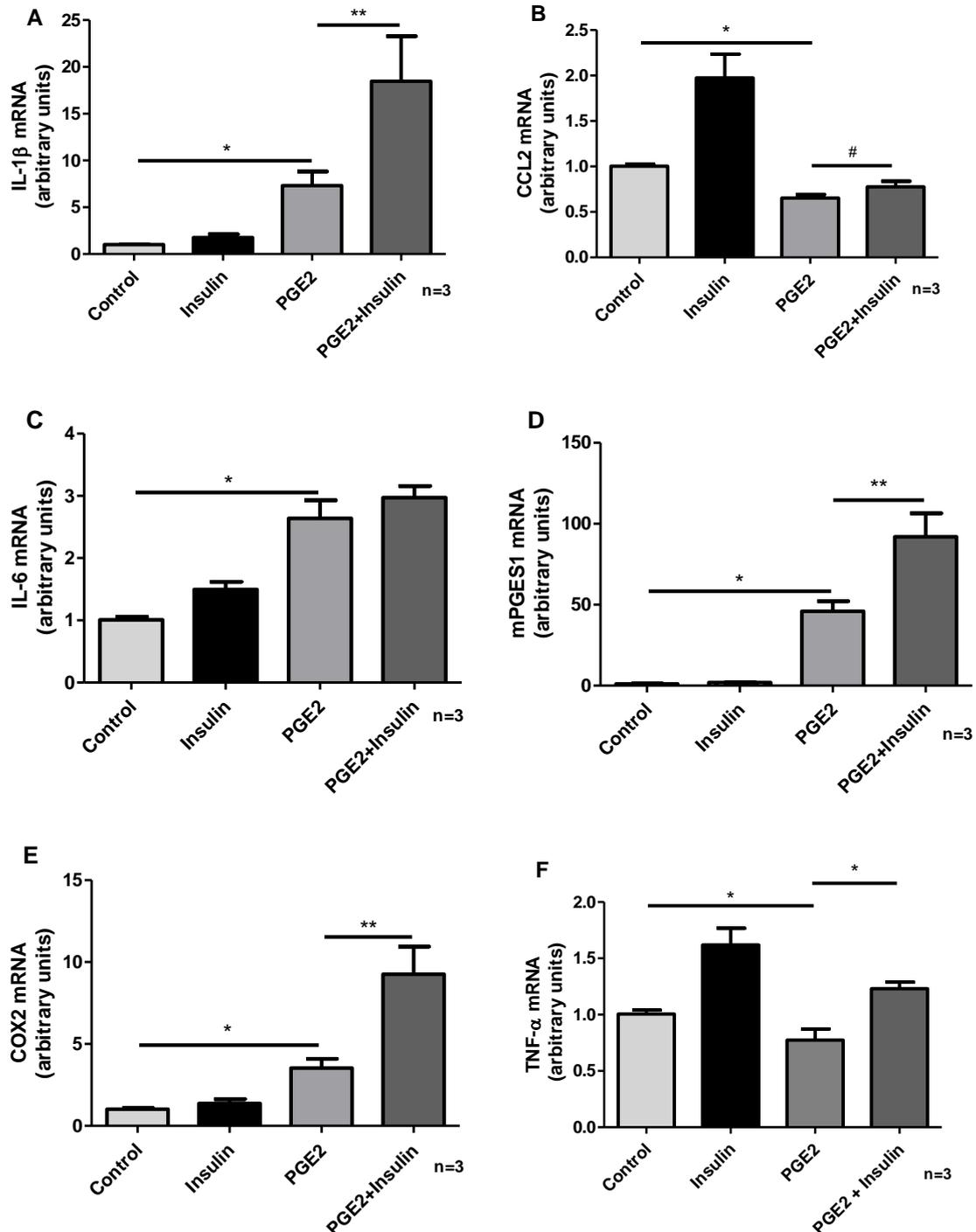
Figure 8 - IL-1 β , IL-8, CCL2, IL-6, TNF- α , IL-10, TLR2 and TLR4 gene expression modulation under Insulin, LPS and Insulin + LPS stimulation in U937 cells





The synergism between insulin and LPS was confirmed in U937 cells. (A) Lipopolysaccharide caused a 5-fold increase in IL-1 β gene expression and the combination with insulin a 20-fold increased expression. (B) LPS caused a 7-fold increase in IL-8 gene expression and the combination with insulin a 14-fold increased expression. (C) LPS caused a 1.4-fold increase in CCL2 gene expression and the combination with insulin a 2-fold increased expression. (D) LPS caused a 15-fold increase in IL-6 gene expression and the combination with insulin a 28-fold increased expression. (E) LPS caused a 2.7-fold increase in TNF- α gene expression and the combination with insulin a 4-fold increased expression. (F) LPS caused a 2-fold increase in IL-10 gene expression. (G) LPS caused a 2.8-fold increase in TLR2 gene expression. (H) LPS and the combination with insulin did not provoke a significant change in TLR4 gene expression. Values are means \pm SE of three measurements of each number of independent experiments indicated. Statistics: Student's T-test for unpaired samples; *, **: $p < 0.05$, #: $p = 0.052$, &: $p = 0.08$.

Figure 9 – IL-1 β , CCL2, IL-6, mPGES1, COX2 and TNF- α gene expression under Insulin, PGE₂ and insulin + PGE₂ stimulation in U937 cells



The synergism between Insulin and PGE₂ was confirmed in U937 cells. (A) PGE₂ caused a 7-fold increase in IL-1 β gene expression and the combination with insulin an 18-fold increased expression. (B) PGE₂ caused a 1.6-fold decrease in CCL2 gene expression. (C) PGE₂ caused a 2.6-fold increase in IL-6 gene expression. (D) PGE₂ caused a 45-fold increase in mPGES1 gene expression and the combination with insulin a 91-fold increased expression. (E) PGE₂ caused a 3.5-fold increase in COX2 gene expression and the combination with insulin a 9-fold increased expression. (F) PGE₂ elicited a significant decrease (1.4-fold) in TNF- α expression and the combination with insulin caused reversion of one such inhibition. Values are means \pm SE of three measurements of each of the number of independent experiments indicated. Statistics: Student's T-test for unpaired samples; *: $p < 0.05$; #: $p = 0.08$.

Table 6 - Pro-inflammatory mediator gene expression in insulin stimulated U937 cells

gene	mRNA expression level [arbitrary units]		significance	n
	control	+ 100 nM insulin		
IL-1 β	1.00 \pm 0.11	2.29 \pm 0.24	p < 0.001	31
IL-8	1.00 \pm 0.11	2.16 \pm 0.20	p < 0.001	12
CCL2	1.00 \pm 0.09	1.92 \pm 0.12	p < 0.001	13
IL-6	1.00 \pm 0.22	1.73 \pm 0.55	n.s.	10
OSM	1.00 \pm 0.14	1.61 \pm 0.13	p < 0.01	14
mPGES1	1.00 \pm 0.08	1.53 \pm 0.10	p < 0.001	9
COX2	1.00 \pm 0.15	1.41 \pm 0.20	n.s.	9
TNF- α	1.00 \pm 0.12	1.25 \pm 0.10	n.s.	16
IL-10	1.02 \pm 0.09	4.53 \pm 0.83	p = 0.0012	2
TLR2	1.00 \pm 0.06	2.48 \pm 0.71	p = 0.09	2
TLR4	1.01 \pm 0.07	1.49 \pm 0.32	n.s.	2

Adapted from Manowsky and Camargo (120)

IL-1 β : Interleukin 1 Beta, IL-8: Interleukin 8, CCL2: Chemokine (C-C motif) ligand 2, IL-6: Interleukin 6, OSM: Oncostatin M, mPGES1: Microsomal prostaglandin E synthase-1, COX2: Cyclooxygenase-2, TNF- α : Tumor necrosis factor alpha. Values are means \pm SE of the number of independent experiments indicated for IL-1 β , IL-8, CCL2, IL-6, OSM, mPGES1, COX2 and TNF- α . Values are means \pm SE of three measurements of each number of independent experiments indicated. Statistics: Student's T-test for unpaired samples. Data for IL-1 β , IL-8, CCL2, IL-6, OSM, mPGES1, COX2 and TNF- α generated in collaboration with Ms. Julia Manowsky.

Table 7 - Pro-inflammatory mediator gene expression in LPS and LPS + insulin stimulated U937 cells

gene	mRNA expression level [arbitrary units]				n	
	control	+ 100 ng/ml LPS	significance	+ 100ng/ml LPS + 100 nM Insulin		significance
IL-1 β	1.01 \pm 0.04	6.33 \pm 0.88	p < 0.0001	18.31 \pm 3.92	p < 0.0001	7
IL-8	1.01 \pm 0.04	7.94 \pm 0.79	p < 0.0001	14.38 \pm 2.29	p < 0.0001	7
CCL2	1.02 \pm 0.05	1.42 \pm 0.18	p = 0.04	2.14 \pm 0.31	p = 0.01	7
IL-6	1.03 \pm 0.05	15.46 \pm 3.04	p < 0.0001	28.29 \pm 4.08	p < 0.0001	7
TNF- α	1.02 \pm 0.04	2.74 \pm 0.27	p < 0.0001	4.01 \pm 0.69	p < 0.0001	7
IL-10	1.02 \pm 0.09	4.54 \pm 0.83	p = 0.0012	3.21 \pm 1.23	n.s.	2
TLR2	1.00 \pm 0.06	2.48 \pm 0.71	p = 0.0951	4.98 \pm 1.99	n.s.	2
TLR4	1.01 \pm 0.07	1.49 \pm 0.33	n.s.	1.09 \pm 0.31	n.s.	2

IL-1 β : Interleukin 1 Beta, IL-8: Interleukin 8, CCL2: Chemokine (C-C motif) ligand 2, IL-6: Interleukin 6, TNF- α : Tumor necrosis factor alpha, IL-10: Interleukin 10, TLR2: Toll-like receptor 2, TLR4: Toll-like receptor 4. Values are means \pm SE of three measurements of each number of independent experiments indicated. Statistics: Student's T-test for unpaired samples.

Table 8 - Pro-inflammatory mediator gene expression in PGE₂ and PGE₂ + insulin U937 stimulated cells

gene	mRNA expression level [arbitrary units]					n
	control	+ 10 μ M PGE ₂	significance	+ 10 μ M PGE ₂ + 100 nM Insulin	significance	
IL-1 β	1.01 \pm 0.04	7.33 \pm 1.50	p = 0.0007	18.48 \pm 4.79	p = 0.0022	3
CCL2	1.00 \pm 0.02	0.65 \pm 0.04	p < 0.0001	0.77 \pm 0.06	p = 0.04	3
IL-6	1.01 \pm 0.05	2.64 \pm 0.29	p < 0.0001	2.97 \pm 0.18	p < 0.0001	3
mPGES1	1.10 \pm 0.19	45.91 \pm 6.33	p < 0.0001	91.91 \pm 14.58	p < 0.0001	3
COX2	1.02 \pm 0.07	3.54 \pm 0.56	p = 0.0004	9.26 \pm 1.70	p = 0.0002	3
TNF- α	1.01 \pm 0.04	0.77 \pm 0.01	p = 0.043	1.23 \pm 0.06	p = 0.0049	3

IL-1 β : Interleukin 1 Beta, IL-8: Interleukin 8, CCL2: Chemokine (C-C motif) ligand 2, IL-6: Interleukin 6, mPGES1: Microsomal prostaglandin E₂ synthase-1, COX2: Cyclooxygenase 2, TNF- α : Tumor necrosis factor alpha. Values are means \pm SE of three measurements of each number of independent experiments indicated. Statistics: Student's T-test for unpaired samples.

4.1.4 Induction of insulin resistance in hepatocytes by supernatants of insulin-treated U937 macrophages

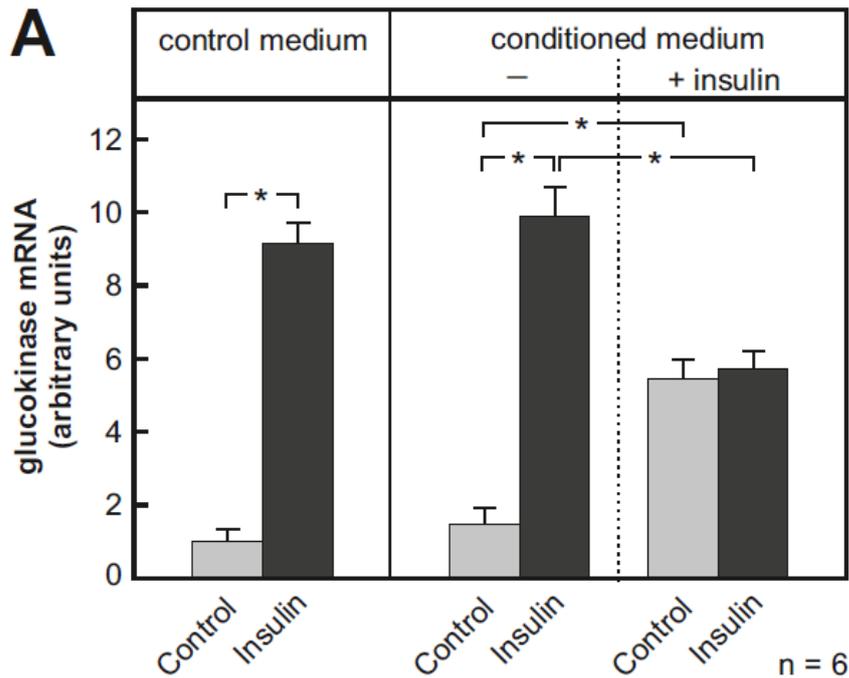
In order to test the hypothesis that insulin-induced cytokine production in U937 macrophages would have physiological relevance, insulin signaling pathway impairment in rat hepatocytes was evaluated under stimulation with U937 supernatant. One of the most sensitive enzymes in hepatocytes under insulin stimulation is glucokinase. This enzyme is responsible for the phosphorylation of glucose in the first step of glycolysis, in which glucose is converted to glucose-6-phosphate. Insulin stimulus in this study induced an increase of glucokinase mRNA content up to nine-fold in control hepatocytes (no treatment with supernatant of U937 macrophages, figure 10), and also in the hepatocytes treated with the supernatant of non-stimulated U937 macrophages. Hepatocytes exposed to the supernatant of insulin-treated U937 macrophages, exhibited as 5-fold increase in glucokinase induction as compared to control hepatocytes, probably due to residual insulin content in the supernatant of insulin-stimulated macrophages. However, when treated acutely with 100nM insulin, these cells did not enhance glucokinase mRNA induction, showing an inhibition of the insulin signaling pathway provoked by the supernatant of insulin-treated U937

macrophages, which even in high insulin concentrations provoked an induction in a maximum range of only 5-fold (figure 10).

Subsequently, the JAK-STAT and MAPK kinases were addressed, since all of them may impair insulin-mediated stimulation in hepatocytes and could be activated by the cytokines contained in the supernatant of insulin-stimulated U937 cells. The transcription factor STAT3 was phosphorylated to a higher extent (5-fold) in hepatocytes treated with the supernatant of insulin-stimulated U937 macrophages, compared to control hepatocytes (figure 11a). The supernatant of non-stimulated U937 cells induced a 4-fold increase of SOCS3 mRNA, which demonstrates that even non-stimulated U937 cells present basal IL-6 family cytokine production at sufficient amount for STAT3 signaling chain activation. The U937 insulin-stimulated macrophage supernatant elicited an 8-fold induction in SOCS3 mRNA (figure 11b). When acute insulin stimulation (100nM) was offered to hepatocytes, no further effect was observed. MAP kinase activation by insulin was not found in a significant manner in hepatocytes maintained in control medium. In hepatocytes treated with the supernatant of non-stimulated U937 cells, the phosphorylation of ERK 1/2 was significantly higher, in a similar way to that of hepatocytes treated with the supernatant of the insulin-treated U937 macrophages (2-fold). Moreover, phosphorylation was increased as additional insulin (100nM) was provided (figure 12a). Serine residues (Ser306 and Ser636) of the insulin receptor substrate (IRS) are susceptible to MAP kinases phosphorylation (48, 53). This causes an inhibition in the IRS and resulted in impaired insulin signaling (48). The phosphorylation of both residues was evaluated by Western Blotting (figure 12). In hepatocytes maintained in the control medium, the phosphorylation of the Ser306/636 residues was enhanced by the addition of insulin (2.5-fold, figure 12b), similarly to hepatocytes treated with the supernatant of non-stimulated U937 macrophages. The acute insulin stimulus did not further enhance phosphorylation. Nevertheless, phosphorylation of IRS induced in hepatocytes treated with the supernatant of insulin treated U937 macrophages (2-fold), compared to hepatocytes treated with the supernatant of non-treated U937 cells. The acute insulin stimulus failed to further enhance phosphorylation. Finally, the protein Akt phosphorylation was assessed. Insulin acutely induced a phosphorylation of Akt in control hepatocytes and hepatocytes that were treated with supernatants of control U937 cells. In hepatocytes incubated with supernatants of insulin-treated U937 cells Akt was phosphorylated to a similar extent that in acutely stimulated control hepatocytes. In

contrast to what was observed with glucokinase, this phosphorylation was further enhanced by the addition of fresh insulin to the hepatocytes exposed to the supernatants of insulin-treated macrophages (figure 13).

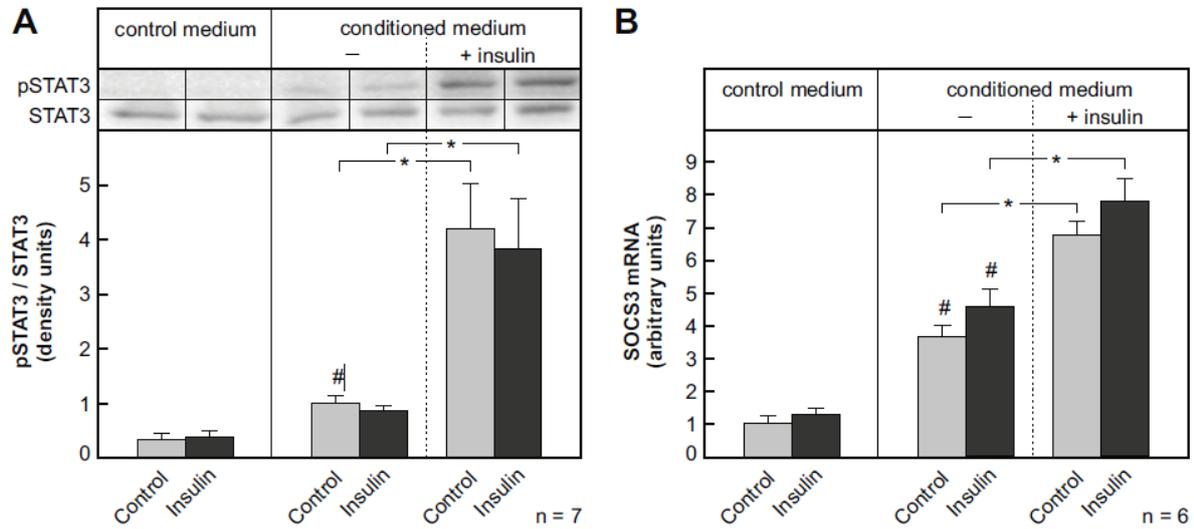
Figure 10 - Inhibition of insulin-induced Glucokinase induction in hepatocytes by supernatants of insulin-treated U937 macrophages



Manowsky and Camargo (120)

Primary rat hepatocytes were stimulated with 100 nM insulin for 2h in control medium containing 50% RPMI or with medium containing 50% of U937 macrophage conditioned medium of control or insulin-stimulated U937 macrophages. Glucokinase mRNA expression was quantified by Realtime PCR with β -actin as reference gene. Values are means \pm SE of the number of independent experiments indicated. Student's T-test for unpaired samples; *: $p < 0.05$. Data generated in collaboration with Ms. Julia Manowsky.

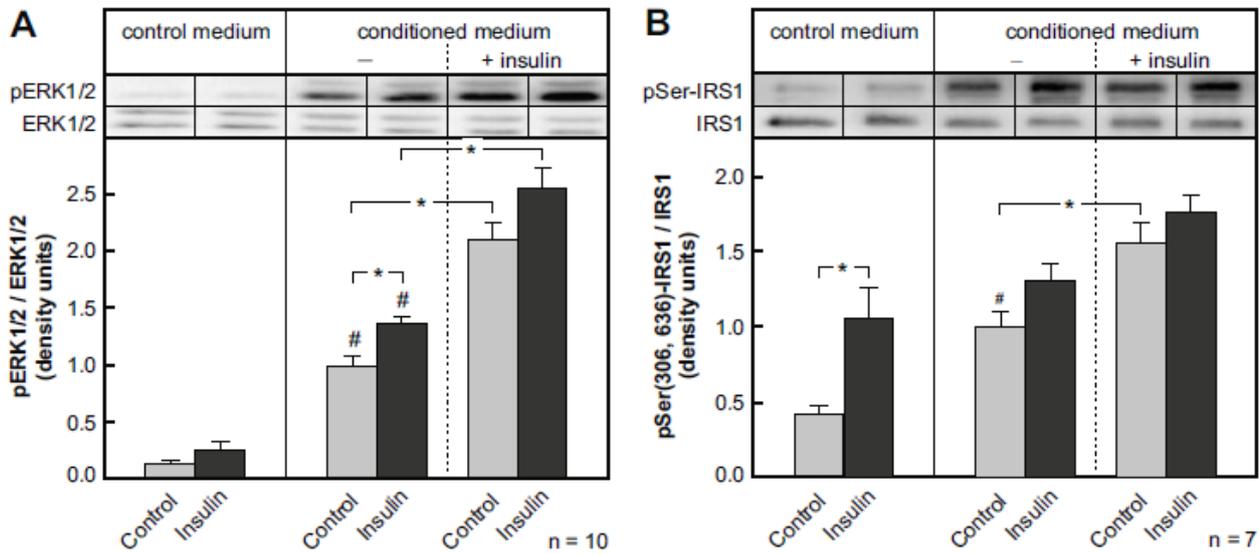
Figure 11 - Activation of STAT3 and induction of SOCS3 in hepatocytes by supernatants of insulin-treated U937 macrophages



Manowsky and Camargo (120)

Primary rat hepatocytes were stimulated as indicated for 15 min. (A) STAT3 phosphorylation non-phosphorylation ratio was quantified in hepatocyte lysates by Western Blot. (B) SOCS3 mRNA expression was quantified by Realtime PCR with β -actin as reference gene. Student's T-test for unpaired samples; *: $p < 0.05$; #: significant versus equal stimulus in control medium with $p < 0.05$. Data generated in collaboration with Ms. Julia Manowsky.

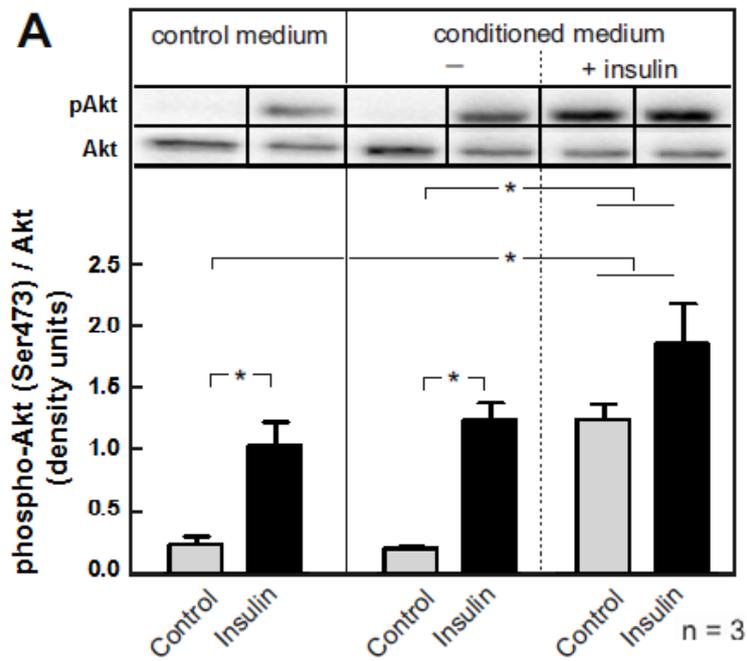
Figure 12 - Activation of ERK1/2 and inhibitory IRS Ser-phosphorylation in hepatocyte induced by supernatants of insulin-treated U937 macrophage cultures



Adapted from Manowsky and Camargo (120)

Primary rat hepatocytes were cultured as detailed in the methods section. They were then stimulated for 15 min. (A) ERK1/2 and (B) IRS as well as their phosphorylation were quantified in hepatocyte lysates by Western Blotting. Values are means \pm SE of the number of independent experiments indicated. Statistics: Student's T-test for unpaired samples; *: $p < 0.05$; #: significant versus equal stimulus in control medium with $p < 0.05$. Data generated in collaboration with Ms. Julia Manowsky.

Figure 13 - Activation of AKT in hepatocyte induced by supernatants of insulin-treated U937 macrophage cultures



Primary rat hepatocytes were cultured as detailed in the methods section. They were then stimulated for 15 min. (A) Akt (Ser473) as well as its phosphorylation counterpart was quantified in hepatocyte lysates by Western Blotting. Values are means \pm SE of the number of independent experiments indicated. Statistics: Student's T-test for unpaired samples; *: $p < 0.05$.

4.2 Part II – Study with Humans

4.2.1 Clinical findings

Patients' characteristics are shown in Table 8. The individuals of the three groups did not present significant differences in age, height and/or previously declared body mass. The Current body mass ($p < 0.0003$), Body mass loss (%) ($p < 0.0001$) and Body Mass Index (BMI) ($p = 0.0018$) were significantly different among the groups. Tumor staging was similar between non-cachectic and cachectic patients.

Table 8 - General characteristics of patients in each group.

	N	WSC	TC	<i>p</i>	<i>Significance</i>
<i>n</i>	16	16	16		
Male/Female (<i>n</i>)	9/7	10/6	5/11		
Age (years)	60.19 ± 9.71	66.25 ± 8.20	62.94 ± 14.37	0.3102	
Height (m)	1.65 ± 0.09	1.63 ± 0.09	1.59 ± 0.10	0.1984	
Previous body mass (Kg)	71.39 ± 7.97	73.18 ± 14.87	68.91 ± 12.59	0.6111	
Current body mass (Kg)	71.39 ± 7.97	72.78 ± 14.80	56.82 ± 10.09	< 0.0003*	N vs TC; WSC vs TC
Δ Body mass (%)	0.00 ± 0.00	0.50 ± 1.40	-17.10 ± 7.70	< 0.0001*	N vs TC; WSC vs TC
BMI (kg/m ²)	26.27 ± 2.36	26.03 ± 8.87	22.37 ± 2.99	0.0018*	N vs TC; WSC vs TC
Tumor stage					
I	-	12.5%	6.3%	-	
IIA/IIIB/IIC	-	31.3%	37.5%	-	
IIIA/IIIB/IIIC	-	25.0%	31.3%	-	
IVA/IVB	-	6.3%	5.0%	-	
Not determined	-	25.0%	-	-	
Primary tumor site					
Colon and rectum	-	100%	81.3%	-	
Other	-	0%	18.8%	-	

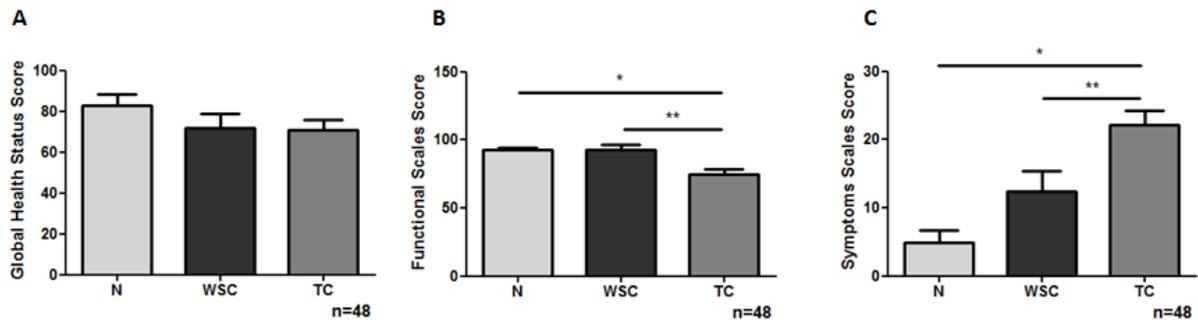
Data are expressed as mean ± standard deviation. Δ: Difference between declared previously body mass and current body mass. One-way ANOVA followed by Tukey's post-test for parametric data or Kruskal Wallis followed by Dunn's post-test for non-parametric data.

4.2.2 Quality of life assessment

Cachectic patients were evaluated through three different quality of life Scales: Functional Scales, which evaluate physical, emotional, cognitive and social functioning, Symptom Scales, which include fatigue, nausea, pain, dyspnea, sleep, appetite loss, constipation,

diarrhea and financial difficulties and Global Health Status. Cachectic patients exhibited differences in comparison to WSC and N in regard to the Functional and Symptom Scales (figure 14).

Figure 14 - Quality of life assessment

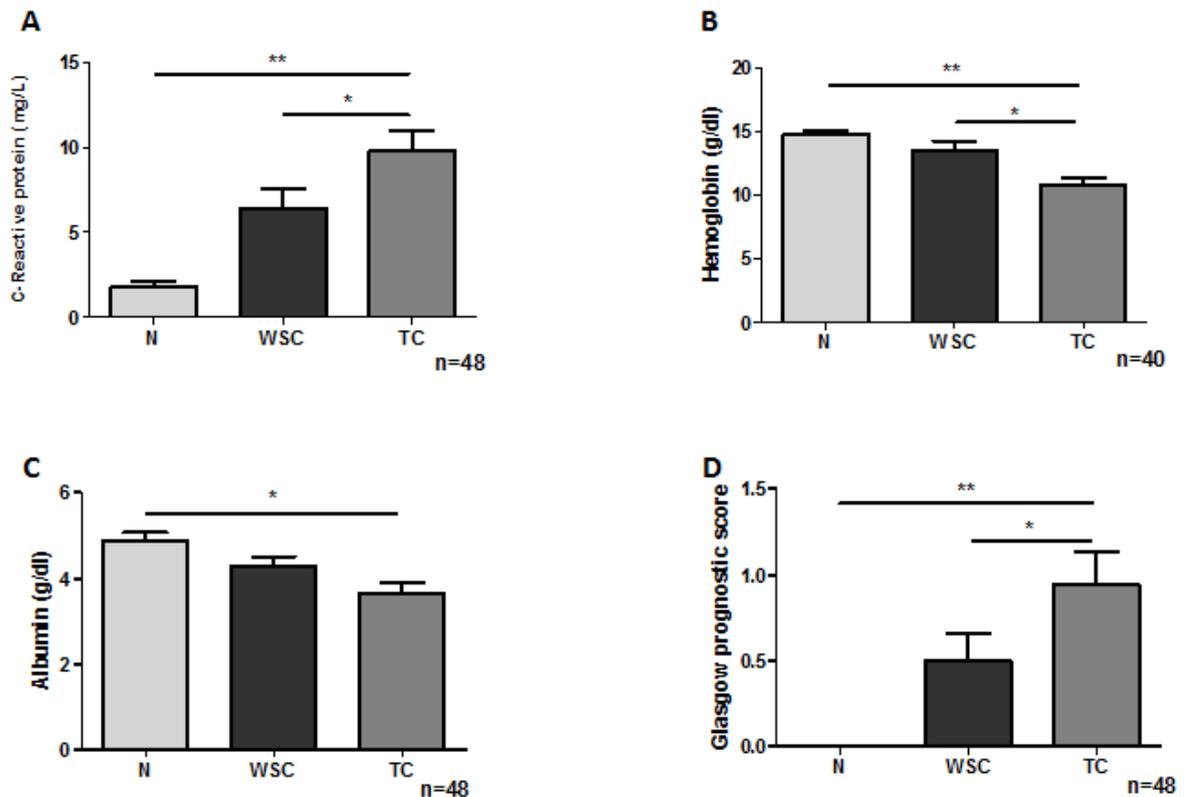


The quality of life of patients was evaluated in Control patients (N), non-cachectic cancer patients (WSC) and cachectic cancer patients (TC) by the QLC-C30 questionnaire, which assessed three different quality of life parameters: Global Health Status (A), Functional Scales (B) and Symptoms Scales (C). One-way ANOVA followed by Tukey's post-test for parametric data or Kruskal Wallis followed by Dunn's post-test for non-parametric data; * $p < 0.05$.

4.2.3 Biochemical parameters

The analysis of the biochemical parameters show that cachectic cancer patients present a higher degree of inflammation, as compared to non-cachectic cancer and control patients, reflected by plasma C-Reactive protein concentration (Figure 15a). These patients present other characteristics of cachexia such as anemia, with significantly diminished hemoglobin concentration (Figure 15b) and malnutrition, as indicated by the significantly lower albumin concentration in regard to the WSC and Control groups (Figure 15c). The Glasgow Prognostic Score (GPS) evaluates the ratio between C-Reactive protein and albumin, and the higher the Score, the higher the degree of inflammation (76). In this study, the GPS was confirmed as an efficient tool to evaluate the degrees of inflammation, demonstrating that differences could be assessed among the groups (figure 15d).

Figure 15 - Biochemical parameters and the Glasgow Prognostic Score

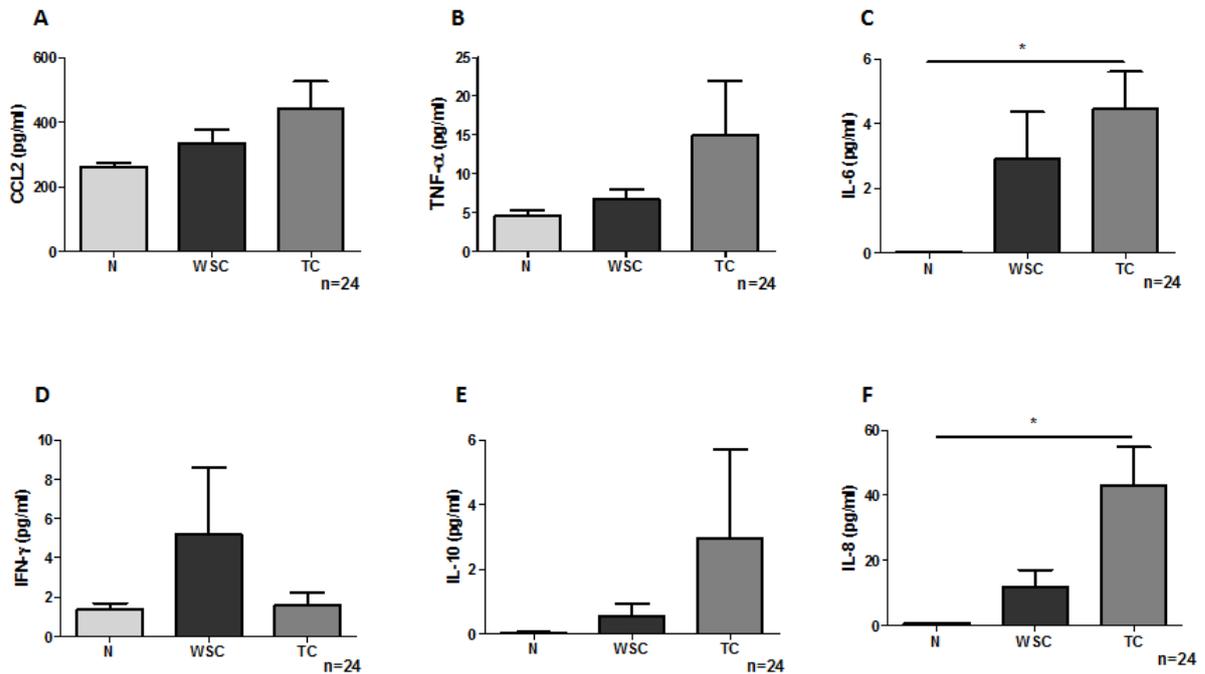


The concentration of serum C-Reactive protein (A), Hemoglobin (B), Albumin (C) and the Glasgow Prognostic Score (D) were evaluated in Control patients (N), non-cachectic cancer patients (WSC) and cachectic cancer patients (TC). Data expressed as mean \pm standard error. One-way ANOVA followed by Tukey's post-test for parametric data or Kruskal Wallis followed by Dunn's post-test for non-parametric data; * $p < 0.05$.

4.2.4 Cytokine expression assay

The plasma content of six cytokines was evaluated (figure 16). From these, two cytokines were significantly up-regulated in the cachectic patients compared to control patients (IL-6 and IL-8). Additionally, TNF- α presented a tendency ($p = 0.08$) for up-regulation in the cachectic patients.

Figure 16 – Plasma cytokine expression

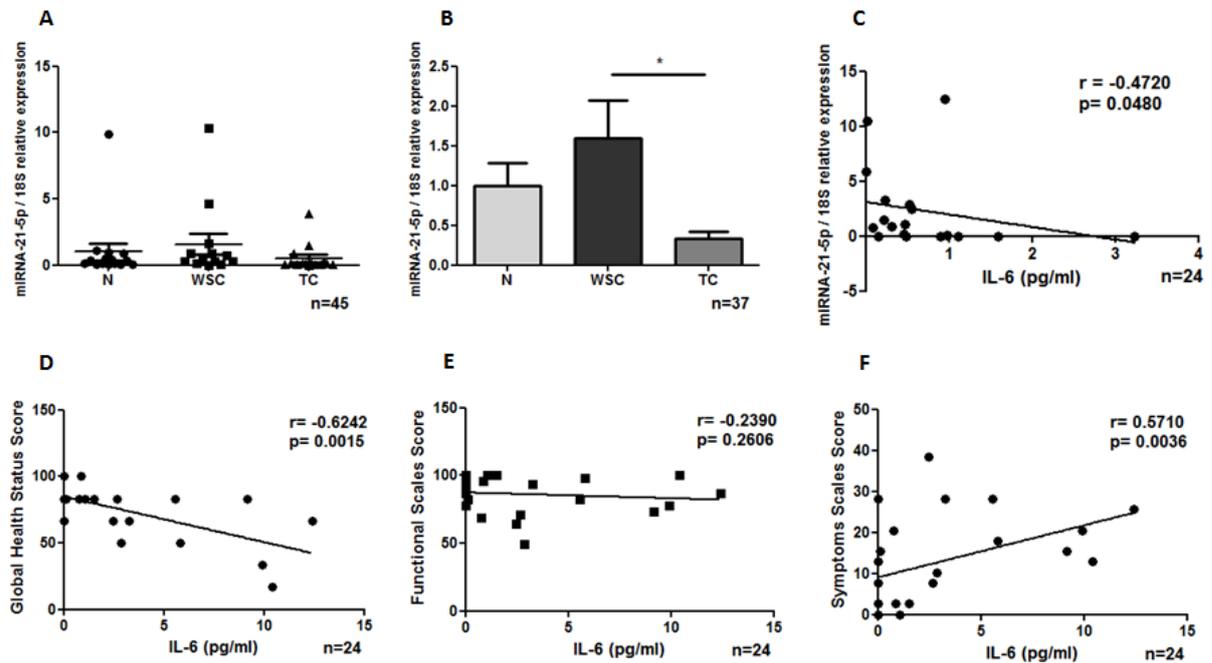


The concentration of CCL2 (A), TNF- α (B), IL-6 (C), INF- γ (D), IL-10 (E) and IL-8 (F) were determined by Multiplex analysis in Control patients (N), non-cachectic cancer patients (WSC) and cachectic cancer patients (TC). Data are expressed as mean \pm standard error. One-way ANOVA followed by Tukey's post-test for parametric data or Kruskal Wallis followed by Dunn's post-test for non-parametric data; * $p < 0.05$.

4.2.5 MicroRNA expression assay

The microRNA expression assay showed miRNA-21-5p to be significantly diminished in the cachectic patients, compared to the non-cachectic counterparts. Moreover, IL-6 correlated negatively with microRNA-21-5p expression in patient plasma (figure 17).

Figure 17 – MicroRNA-21-5p plasma expression and IL-6 Spearman's correlation with miRNA-21-5p and quality of life assessments



The expression of microRNA-21-5p was evaluated in the plasma of human samples in control patients (N), non-cachectic cancer patients (WSC) and cachectic cancer patients (TC). The results are shown for all samples (A) and after correction for outliers (B). The results were then correlated (Spearman correlation) with the pro-inflammatory cytokine IL-6 (C) and the Scores of the quality of life assessment: Global Health Status (D), Functional Scales (E) and Symptoms Scales (F). MicroRNA-21-5p data expressed as mean \pm standard error. One-way ANOVA followed by Tukey's post-test for parametric data or Kruskal Wallis followed by Dunn's post-test for non-parametric data; * $p < 0.05$.

5 DISCUSSION

Cachexia and Metabolic Syndrome are both clinically relevant multifactorial syndromes in which metabolism is disrupted. Obesity and related diseases are considered nowadays a worldwide epidemic (9), while cachexia reaches up to 80% of cancer patients in treatment of advanced stages of the disease (3). Despite being considered for a long time opposite sides of the coin, these two syndromes interestingly share several common features, as low-grade inflammation and insulin resistance appearing as key players underlying the etiology and progression of the diseases (10, 11, 14).

Insulin resistance is defined as diminished insulin sensitivity and is highly present in Cachexia and Metabolic Syndrome (44, 45). In MetS, IR is accompanied by hyperglycemia (16), while in cachexia, by hypoglycemia. Insulin resistance is considered the most important feature in MetS, being also-called “the insulin resistance syndrome” (121). In cachexia, the importance of insulin resistance was also reinforced by a recent publication, in which it was stated that “impaired insulin signaling is itself a direct cause of cancer cachexia development” (122), and anti-diabetic drugs were suggested as possible treatments for CC (123). Insulin resistance may develop through different mechanisms, such as augmented free fatty acids in the interstitium and in the circulation, impairing insulin signaling through the activation of proteins by triglyceride synthesis intermediate diacylglyceride, or by several inflammatory mediators, such as PGE₂ and cytokines, which will directly or indirectly impair insulin signaling and cause insulin resistance (56). Inflammation is a pivotal feature in insulin resistance development.

MicroRNAs are small non-coding RNAs, which modulate a wide range of cellular functions, including RNA transcription, processing, stability, and translation (81). More than half of human genes are modulate by miRNAs (93), which expression vary depending on metabolic status and presence of disrupt states as diseases (94). MicroRNAs have a role in diverse biological processes as cellular differentiation, proliferation, tissue development and cell-type specific function and homeostasis, and altered miRNAs expression have been linked to dysregulated magnitude of inflammatory responses (97) and to the development and maintenance of inflammatory conditions as cancer and *type II diabetes*.

One of the main characteristics of *type II diabetes* and MetS is hyperinsulinemia, which for many years, was thought to be a consequence of insulin resistance; however,

studies (108) show that hyperinsulinemia may actually precede hyperglycemia and insulin resistance, with a high impact on the liver, since insulin is secreted by the pancreas in the portal vein (109). This raised the first question of the study: Does hyperinsulinemia induce *per se* inflammation through macrophage stimulation and, consequently, insulin resistance in hepatocytes?

The role of insulin in inducing pro-inflammatory mediator expression in cultured macrophages has been the focus of some studies in the literature, where conflicting results can be found (124, 125). In this study we show that insulin augmented in a significant manner the expression of several pro-inflammatory mediators in U937 cultured differentiated macrophages. Despite that, insulin was not able to elicit monocyte differentiation into macrophages, indicating that the possible pro-inflammatory role of insulin does not relate to differentiation, but rather to inducing the production and release of pro-inflammatory mediators by immune cells. However, differentiation into M1 macrophages may probably be a prerequisite for insulin-dependent induction of cytokine gene expression, once this phenotype is recognized as pro-inflammatory due to its role in producing and releasing pro-inflammatory mediators.

Another feature of cachexia and of the Metabolic Syndrome is impaired intestinal permeability, which causes endotoxemia (126-128). Lipopolysaccharide, a component of gram-negative bacteria cell wall is a lipid derivative that contributes to the inflammation by the activation of pro-inflammatory signaling pathways by binding to Toll-like receptor 4 (127, 129, 130). U937 macrophages stimulated with insulin and LPS induced higher levels of key cytokine expression such as IL-1 β , IL-6 and TNF- α than either stimulus alone. Given that in both in MetS and CC a gut barrier dysfunction with increased LPS permeability is observed (126, 129), this study suggest a role of a synergism between insulin and LPS in the induction of pro-inflammatory cytokines that might drive the chronic low level inflammation observed in patients with MetS. Another molecule described to be overexpressed in MetS and CC is prostaglandin E₂ (131, 132), which, interestingly has two facets. It can be considered a pro-inflammatory factor, since it enhanced IL-1 β , IL-6, mPGES1 and COX2 expression. In the case of IL-1 β and the two prostaglandin synthesizing enzymes, this induction was further enhanced by the simultaneous presence of insulin. Thus, the hyperinsulinemia that accompanies MetS to cope with the insulin resistance may further aggravate the induction of an inflammatory response by other mediators like PGE₂. PGE₂, on the other hand could

also be considered an anti-inflammatory mediator, because it inhibited TNF- α and CCL2 gene expression in macrophages (133). However, the interaction of PGE₂ with insulin provoked a reversion of the inhibition in regard to TNF- α gene, restoring gene expression to basal levels, compared to cells treated with only PGE₂. This could also contribute to the pro-inflammatory role of insulin *per se*. Thus, insulin impaired anti-inflammatory PGE₂-dependent feedback inhibition loops and also elicited the release of pro-inflammatory mediators in synergism with other molecules capable of provoking inflammation, such as LPS and PGE₂.

The role of cytokines has been extensively studied both in CC and MetS (134-136). IL-1 β , TNF α , IL-6 and Interferon- γ have been named “classic cachexia cytokines” (38), as it has been robustly demonstrated that these cytokines are part of the modulation of the inflammation that accompanies cachexia (33, 137), and one or more is frequently altered in cachectic and MetS patients (138). Insulin elicited the high levels of chemokines and cytokines, such as IL-1 β , IL-8, CCL2, OSM and mPGE₂ in U937 macrophages and cachectic patients presented elevated plasma levels of IL-6 and TNF α , as IL-8. The cytokine IL-1 β is a mediator of the inflammatory response in Cachexia and MetS (134). This molecule is produced by a 30.6 kDa cytosolic precursor (pro-IL-1 β) from which the mature 17.4 kDa cytokine is cleaved by activated caspase I. The precursor is activated by the inflammasome and released subsequently (139). Gene and protein expression of IL-1 β was induced by insulin, as its levels were significantly higher in the supernatant of insulin-treated U937 macrophages. Induction was likely to be mediated by the NF κ B signaling pathway, since further experiments performed based on the results presented here (120) demonstrated that insulin activated the kinase upstream of the NF κ B activation (IKK β) by phosphorylation. When U937 cells were treated with an inhibitor of NF κ B, the insulin-dependent induction of IL-1 β was abolished (120). Inflammatory signaling pathways, such as the NF κ B, have been shown to be highly stimulated in peripheral tissues of cachectic and MetS patients (38, 140). Cytokines as IL-1 β , IL-6, TNF- α and IL-8 are all susceptible to NF κ B induction, once the promoter region of these pro-inflammatory mediators present sites for this transcription factor (141), stressing the importance of this pathway as a mediator of inflammatory conditions. The hypothesis that the AP1 transcription factor could also be part of the IL-1 β induction by insulin in macrophages was also assessed in a subsequent study, but rejected, since despite an increased phosphorylation in its upstream protein ERK 1/2, the downstream proteins c-Jun and c-Fos, essential components of AP1, were not altered (120).

The liver is an organ exposed to high insulin concentrations, as the pancreas releases insulin into the portal vein. Insulin was capable of inducing not only the synthesis and release of inflammatory mediators by macrophages, but the supernatant obtained from the culture of these macrophages caused impaired insulin signaling in hepatocytes. One of the main insulin stimulus “sensors” in hepatocytes is the enzyme glucokinase, as its induction is downstream to insulin signaling and this enzyme is highly regulated by insulin. Supernatant of insulin-treated U937 cells caused an almost 50% lower induction of glucokinase in hepatocytes, even when an acute insulin stimulus was provided, indicating that the supernatant of U937 macrophages released under insulin stimulus impaired substantially insulin signaling in hepatocytes. This is the same scenario found in CC and MetS, where macrophages are known to be the main contributor to local inflammation in the liver and peripheral organs (142, 143). The mechanisms behind the reduction in glucokinase expression involve several proteins of the insulin signaling pathway, such as STAT3. This protein transcription factor has a prominent role in the induction of pro-inflammatory factors and, at the same time in a negative feedback loop, suppressor of cytokine signaling (SOCS) molecules in different tissues as muscle, adipose and liver (22). This transcription factor is activated by phosphorylation through IL-6 cytokine family proteins. Once activated, STAT3 induces the expression of SOCS3, a protein that impairs directly the insulin signaling at its receptor level by directing binding to its substrate (54, 74). Cachectic patients exhibited higher IL-6 circulating levels compared to non-cachectic and controls, and the activation of STAT3 through phosphorylation was higher in hepatocytes stimulated with supernatant of insulin-treated macrophages, implying a possible role for the IL-6 cytokines family in the inflammatory process that culminates in diminished insulin signaling in hepatocytes. In cachexia, the role of STAT3 in systemic inflammation was reviewed by Zimmers and colleagues (22), in which IL-6 is linked to poor prognosis, and STAT3, linked to IL-6 action in several different compartments of the body. However, the link of STAT3 with poor prognosis needs to be further investigated.

The miRNA-21-5p is a non-coding RNA with high importance in inflammatory conditions, due to its capacity of modulating the magnitude of inflammatory responses. This miRNA interacts and regulates the activity of several proteins, including the transcription factor STAT3, which contains two binding sites in the 3'UTR for the miRNA-21-5p (101). A study has already focused on the interaction between miRNA-21-5p and immune cells pro-

inflammatory mediators production (144). MicroRNA-21-5p negatively correlates with IL-6 and TNF- α expression, acting as an anti-inflammatory molecule in cultured macrophages. Considering that plasma levels of miRNA-21-5p were diminished in the cachectic patients, and correlated negatively with the expression of IL-6, this pathway is certainly a candidate as contributor to the poor prognosis of both CC and MetS. Quality of life of cachectic patients was highly affected in this study, as verified by the QLC-C30 questionnaire, and could be partly derived from IL-6 modulation. Cachectic patients exhibited impaired Functional and Symptoms scales, and a negative correlation was found between IL-6 plasma levels and Global Health Status. Moreover, a positive correlation between IL-6 plasma levels and Symptoms Scales was found, reinforcing the role of IL-6 as a mediator of poor outcomes in CC, as published by Zimmers (22).

In this study, we not only confirm a role for the STAT3 pathway in hepatocytes, but also propose linking this regulation to the diminished miRNA-21-5p expression. This result was initially intriguing, as studies (100), correlate the high expression of plasma miRNA-21-5p as a biomarker for different types of cancer. Despite this, an interesting observation is that most of the studies, despite finding a significant increase in miRNA-21-5p expression in cancer compared to non-cancer patients, do not present uniform results among patients. Some of the patients actually present lower expression compared to control patients (145), and despite a significant difference in miRNA-21-5p expression between control and cancer patients, practically half of the patients showed miRNA-21-5p expression to be equal or even reduced in comparison to control patients. A review article (146), discussed this issue and raised another question about conflicting results in the literature on human levels of plasma miRNA-21-5p and cancer. Population ethnicity, choice of the endogenous controls, source of miRNAs and methods of detection would all have a chance to affect the results, confirming that studies with patients present several limitations that should be considered in the analysis. Confirming the conflicting results in the literature due to the miRNA-21-5p role in inflammatory conditions, a study by Zhang and colleagues (101) proposed a dual role for the miRNA-21-5p in cancer, once augmented expression is linked to its progression through STAT3 modulation, but in the same study it was shown that augmented miRNA-21-5p expression was linked to diminished STAT3 activation and phosphorylation, highlighting the importance for the broadening in the understanding on the mechanisms behind the interaction between miRNA-21-5p and its targets.

Liver insulin resistance is a common feature to Cancer cachexia and to the Metabolic Syndrome. Insulin-induced insulin resistance might be brought about in part by an insulin-dependent stimulation of cytokine production or enhancement of LPS-induced cytokine production in macrophages. Among these cytokines, IL-6 can elicit insulin resistance via a STAT-3-dependent induction of SOCS3. MicroRNA-21-5p is a posttranscriptional regulator of STAT3 expression. Reduced levels of this miRNA go along with increased STAT3 levels and consequently enhanced expression of STAT3 target genes like SOCS3. Thus, a reduction of miRNA-21-5p might further enhance IL-6 dependent SOCS3 production and insulin resistance. On the other hand, STAT3 mediated signaling pathway is also described as important for cell growth and survival (147), being the reduced expression of miRNA-21-5p in some cases possibly beneficial. Although, in specific metabolic disrupted conditions, in which low-grade inflammation is highly present, such as cancer cachexia and Metabolic Syndrome, miRNA-21-5p down-regulated expression exerts possibly detrimental effects.

6 CONCLUSION

Liver insulin resistance is a feature highly present in Cancer Cachexia and Metabolic Syndrome, impairing substantially quality of life of patients. Inflammation, through cytokines, appears as the common denominator in the development and maintenance of insulin resistance, with hyperinsulinemia and microRNAs as part of its modulation. Therapies that envisage counteracting the poor outcomes of insulin resistance in Cancer cachexia and Metabolic Syndrome should essentially target inflammation.

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APPENDIX

A - FREE AND INFORMED CONSENT FORM

Final Version 11.04.2014

FREE AND INFORMED CONSENT FORM (originally in portuguese)

Study: Role of adipose tissue microRNAs in the development and maintenance of cancer cachexia

You are invited (a) to participate in the research project mentioned above. The document below contains all the necessary information about the research we are doing. Your cooperation in this study will be of great importance to us, and if you want to quit at any time, this will not cause any harm to you.

I, (insert the name, sex, occupation, address and phone number)

_____, Identity Card, nº _____, and CPF / MF
_____ born (a) on ____ / ____ / _____, agree to the free will to participate as a volunteer (a) of the study "Role of adipose tissue microRNAs in the development and maintenance of cancer cachexia. " I declare that I obtained all necessary information as well as all possible clarification to my questions and that I will receive an identical copy of this Free Consent form.

I am aware that:

I) The study is necessary to investigate the possible causes of cachexia, characterized by severe weight loss, muscle atrophy, fatigue, weakness and decreased appetite; we have to verify whether cachexia affects the microstructures of the liver and also checking the hepatic metabolism;

II) If the patient agreed to participate in this research and depending on each surgical procedure performed, fragments may be removed about one gram per tissue, with a total collection time of about 5 minutes. This procedure has a minimum degree of risk and does not interfere with the standard operation procedures. But if there are complications with the research participant arising from the research, these will be treated at the HU / USP (secondary care hospital). The collected material is important for the understanding of the etiology of cachexia;

Patient or legal responsible _____

Study responsible _____

III) I agree that a collection of 20 mL of blood in control and cancer patients may be performed (except liver cancer), for plasma and serum parameters analysis. The collection will be performed by a qualified health professional.

Yes or no

IV) These samples (blood and tissue) will be held only for this study and or other projects (future research) and the procedures were authorized by the Ethics Committee of the Institute, with no influence on the treatment and not modifying the anesthetic and surgical procedure;

V) The procedures will not heal me, will not cause me any trouble or pain at the time of collection;

VI) Participation in this project has no order to submit me to any treatment and will not cause me any financial expenses in relation to medical, clinical and therapeutic procedures;

VII) I have the free will to give up or stop the cooperation in this study when I want, without any explanation;

VIII) The withdrawal will not cause any damage to my health or physical well-being and will not interfere with care or medical treatment;

IX) The results obtained during this study will be kept confidential, but I agree that they may be published in scientific journals, since my personal data are not mentioned;

X) I agree that the material and the data collected by me and the analysis results obtained by the researchers could be used in other projects (future research) as long as authorized by the Ethics Committee of the Institute;

Yes or no

XI) I agree that after the material is collected and stored in specific solutions for each technique, it will be packaged in freezer at -80 ° to maintain tissue integrity, for later use;

Yes or no

XII) I agree that after the material is used for research purposes and the objectives are achieved, the rest will be incinerated on adequate site for disposal of human biological material, as stated in the resolutions of the National Health Council (CNS);

Yes or no

XIII) If I want, I can personally take note of the results at the end of this research which will have a duration of 12-18 months;

I wish to be aware of the results of this research.

I did not wish to know the results of this research.

Patient or legal responsible _____
Study responsible _____

XIV) Concerning the special thematic area of this project to be developed at the Institute of Biomedical Sciences - ICB / USP / São Paulo, it has been signed an agreement between the universities and their researchers: Giorgio Trinchieri and Romina Goldzmid (National Institute of Health - NIH); JOSEP Argilés and Silvia Busquets (University of Barcelona); Alessandro Laviano and Maurizio Muscaritoli (University La Sapienza UniRoma); Gerhard Püschel and Tiziana Magaria (University of Potsdam); Stephen Farmer (Boston University); Marília Cerqueira Leite Seelaender, Alison Colquhoun and José Cezar Rosa Neto (ICB / USP); Paulo Sergio Alcantara, Linda Maximiniano, Oscar Fujita, Claudio Campi and Emerson Muller (HU / USP); José Piñata Otoch and Busatto Geraldo Filho (USP); Emerson Franchini (EEFE / USP); Renata Wassermann (IME / USP); Claudia Oller do Nascimento and Lila Missaie Oyama (UNIFESP); and Miguel Batista Junior (UMC - University of Mogi das Cruzes).

"I DECLARE THAT, AFTER CONVENIENTLY ENLIGHTENED BY THE RESEARCHER AND UNDERSTANDING WHAT HAVE BEEN EXPLAINED TO ME, I AGREE TO PARTICIPATE IN THIS RESEARCH."

Sao Paulo, _____ of _____ of 20____

() Patient / () responsible

Witness 1: _____
Name / RG / Phone:

Witness 2: _____
Name / RG / Phone:

Responsible for the Project: _____

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SUPPLEMENTARY MATERIAL

A - U937 cell culture experiment - buffers and Solutions

The buffers and solutions were, unless otherwise specified, sterile filtered through a 0.2-micron filter and stored in autoclaved glass bottles at 4°C. H₂O always refers to autoclaved ultrapure water.

Cell culture Medium I:

Inactivated FCS	10%	100 ml
Penicilin / Streptomycin	1%	1 ml
RPMI 1640		add to 1000 ml

Cell culture Medium II ("Serum free medium")

Inactivated FCS	0.5%	5 ml
Penicilin / Streptomycin	1%	1 ml
RPMI 1640		add to 1000 ml

Penicillin / streptomycin

The solution was already obtained as a mixture of penicillin (10,000 U) and streptomycin (10,000 ug/ml). It was then stored in 10 ml-aliquots at -20 °C. The cell culture media were always prepared with 1% of this mixture, by adding 100 U penicillin and 100 ug/ml streptomycin.

Insulin stock solution (10 µM)

Insulin	10 uM	2.9 mg
BSA	0.1% (w/v)	50 mg
0.9% (w / v) NaCl solution		add to 50 ml

Insulin was dissolved at pH 3.0 in 20 ml of 0.9% (w/v) NaCl solution and the pH adjusted with NaOH to pH 7.4 and then BSA was added. When filling the solution up to 50ml, the pH was controlled. The solution was stored in aliquots at -20 °C.

LPS stock solution (5mg/ml)E.coli Serotype 0127:B8Stock 5 mg/ml in H₂O

In medium diluted to 100 ng/ml

PMA stock solution (100 µg/ml in 100% ethanol)

In medium diluted to 100 ng/ml

B - Rat primary hepatocytes experiment - buffers and Solutions

The buffers and solutions were, unless otherwise specified, sterile filtered through a 0.2-micron-filter and stored in autoclaved glass bottles at 4 °C. H₂O always refers to autoclaved ultrapure water.

Perfusion buffer stock solution (10x)

NaCl	1400 mM	81.820g
KCl	50 mM	3.728 g
MgCl ₂ x 6 H ₂ O	8 mM	1.626 g
Na ₂ HPO ₄ x 2 H ₂ O	16 mM	2.848 g
KH ₂ PO ₄	4 mM	0.544 g
		add H ₂ O 1000 ml

The pH of the solution was adjusted with HCl to pH 7.4.

NaHCO₃ solution (1 M)

NaHCO ₃	1 M	8.4 g
		add H ₂ O to 100 ml

The solution was stored at room temperature.

EDTA solution (0.5 M)

EDTA	0.5 M	14.61 g
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add H₂O to 100 ml

The pH of the solution was adjusted with NaOH to pH 8.0.

Glucose stock solution (2 M)

D-glucose x H ₂ O	2 M	39.63g
		add H ₂ O to 100ml

Lactate solution (0.5 M)

Lactate syrup (60% (w/w))	0.5 M	7 ml
		add H ₂ O to 100 ml

Pyruvate stock solution (200 mM)

Pyruvate	200 mM	2.2 g
		add H ₂ O to 100 ml

Calcium chloride solution (2 M)

CaCl ₂	2 M	29.4 g
		add H ₂ O to 100 ml

Perfusion's Buffer

Perfusion-Standard solution (Stocksol - 10x)	1x	100 ml
NaHCO ₃	25 mM	25 ml 1 M Stock
EDTA	2 mM	4 ml 0.5 M Stock
Glucose	15 mM	7.5 ml 2 M Stock
Lactate	2 mM	4 ml 0.5 M Stock
Pyruvate	0.2 mM	1 ml 200 mM Stock
		add H ₂ O 1000 ml

The medium was incubated at 37 °C for 45 min with carbogen [95% O₂, 5% CO₂, (19: 1)].

Then, the pH was adjusted with 1 N HCl to pH 7.4.

Wash buffer

Perfusion- Stdsol. (10x)	1x	50 ml
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CaCl ₂ x H ₂ O	1 mM	250 ul	2 M
			add H ₂ O to 500 ml

The medium was heated at 37 °C for 45 min. Then, the pH was adjusted with 1N HCl to pH 7.4.

Percoll density gradient

Perfusion-Standard solution (Stocksol - 10x)	1x	4.2 ml
Percoll		27.8 ml

The Percoll density gradient was heated to 37 °C.

Physiological sodium chloride solution

NaCl	0.9% (w/v)	9 g
		add H ₂ O to 1000 ml

The solution was not sterile filtered.

Medium 199 (M199)

Powder medium M199 with Earle's salts without NaHCO ₃		9.77 g
D-glucose x H ₂ O	5.5 mM	1.10 g
HEPES	15 mM	3.57 g
NaHCO ₃	18 mM	1.51 g
BSA	0.2% (w/v)	2.00 g
		add H ₂ O to 1000 ml

The medium was equilibrated with carbogen [95% O₂, 5% CO₂, (19: 1)] and the pH adjusted with HCl to pH 7.4. Medium 199 contained a total of 10 mM glucose.

Insulin stock solution (10 μM)

Insulin	10 μM	2.9 mg
BSA	0.1% (w/v)	50.0 mg
0.9% (w/v) NaCl solution		add to 50 ml

Insulin was dissolved at pH 3.0 in 20 ml of 0.9% (w/v) NaCl solution and the pH adjusted with

NaOH to 7.4 and then BSA was added. When filling the solution up to 50 ml, the pH was controlled. The solution was stored in aliquots at -20 °C.

Dexamethasone stock solution (100 µM)

Dexamethasone	100 µM	2.0 mg
Ethanol p.a.	0.3% (v/v)	150 µl
0.9% (w/v) NaCl solution		add to 50 ml

Dexamethasone was initially dissolved in ethanol p.a. and then treated with 0.9% (w/v) NaCl diluted solution. The solution was stored in aliquots at -20 °C.

Penicillin / streptomycin

The solution was already obtained as a mixture of penicillin (10,000 U) and streptomycin (10,000 µg/ml). It was then stored in 10 ml-aliquots at -20 °C. The cell culture media were always prepared with 1% of this mixture, by adding 100U penicillin and 100 µg/ml streptomycin.

M199 culture medium I

NCS	4% (v/v)	4 ml	
Penicillin / Streptomycin	1% (v/v)	1 ml	
Dexamethasone	100 nM	100 µl	100 µM Stock
Insulin	0.5 nM	5 µl	10 µM Stock
Medium 199			add to 100 ml

The medium was heated to 37 °C.

M199 culture medium II

Penicillin / Streptomycin	1% (v/v)	1 ml	
Dexamethasone	100 nM	100 µl	100 µM Stock
Insulin	0.5 nM	5 µl	10 µM Stock
Medium 199			add to 100 ml

The medium was heated to 37 °C.

C - U937 macrophages and rat primary hepatocytes protein expression analysis by Western blot - Buffers and solutions

If not otherwise stated, the solutions were stored at room temperature.

Protease inhibitors (x 1000)

Pefablock	200 mM	47.9 mg
Leupeptin	1% (w/v)	10 mg
Trypsin inhibitor	1% (w/v)	10 mg

Each protease inhibitor was dissolved in 1 ml H₂O and aliquots stored at -20 °C.

Sodium orthovanadate (tyrosine phosphatase inhibitor)

Na ₃ VO ₄	200 mM	36.8 mg
		add H ₂ O to 1 ml

The pH of the solution was adjusted with NaOH to pH 10.0. The activation was performed by heating the solution to boiling temperature and cooled until its color changed from yellow to colorless. The solution was aliquoted and stored at -20 °C.

Lysis buffer for Western Blot

Tris / HCl (pH 7.5)	20 mM	4 ml of 1 M Stock
NaCl	150 mM	1.752 g
EDTA	1 mM	400 µl 0.5 M Stock
EGTA	1 mM	76 mg
Triton X-100	1% (v/v)	2 ml
Sodium pyrophosphate	2.5 mM	233 mg
β-glycerol phosphate	1 mM	43 mg
NaF	50 mM	420 mg
		add H ₂ O to 200 ml

The pH of the solution was adjusted with 1 N HCl or 1 M NaOH to pH 7.5. The solution was stored at 4 °C.

Immediately before use, per ml of lysis buffer was freshly added:

Pefablock	200 μ M	1 μ l	200 mM Stock
Leupeptin	10 μ g/ml	1 μ l	10 mg/ml Stock
Trypsin inhibitor	10 μ g/ml	1 μ l	10 mg/ml Stock
Na ₃ VO ₄	1 mM	5 μ l	200 mM Stock

Bradford reagent

Serva Blue (Nr.35050)	0.01% (w/v)	100 mg
Ethanol (95%)	4.75% (v/v)	50 ml
Phosphoric acid (85%)	8.5% (v/v)	100 ml
		add H ₂ O to 1000 ml

The solution was filtered through a filter and stored at room temperature.

BSA standard solution (1 mg/ml)

BSA	1 mg/ml	10 mg
		add H ₂ O to 10 ml

The solution was aliquoted and stored at -20 °C.

5x SDS sample buffer

Tris / HCl (pH 7.5)	400 mM	2.5 ml 1 M Stock
SDS	10% (w/v)	50 ml 20% (w/v) Stock
Glycerol	25% (w/v)	28.74 ml 87% (w/v) Stock
Bromophenol blue	0.0125% (w/v)	125 mg
		add H ₂ O to 100 ml

Sample buffer

5 x SDS sample buffer	4 x	800 μ l
β -mercaptoethanol	20%	200 μ l

The solution was freshly prepared.

Acrylamide solution (30% (w/v))

The solution was purchased ready-to-use by the company Roth and stored at 4 °C.

Resolving gel buffer

Tris	1.5 M	18.17 g
SDS	0.4% (w/v)	4 ml of 10% (w/v) Stock
H ₂ O to 100 ml		

The pH of the solution was adjusted with HCl to pH 8.8. The solution was stored at 4 °C.

Stacking gel buffer

Tris	0.5 M	50 ml 1 M Stock
SDS	0.4% (w/v)	4 ml of 10% (w/v) Stock
add H ₂ O to 100 ml		

The pH of the solution was adjusted with HCl to pH 6.8. The solution was stored at 4 °C.

APS solution (10% (w/v))

APS	10% (w/v)	10 g
add H ₂ O to 100 ml		

The solution was stored at 4°C.

10 x SDS electrophoresis running buffer

Tris	250 mM	30.3 g
Glycine	1.91 M	144.1 g
SDS	1% (w/v)	100 ml 10% (w/v) Stock
add H ₂ O to 1000 ml		

1x SDS electrophoresis running buffer

10x SDS electrophoresis running buffer	1x	100 ml
add H ₂ O to 1000 ml		

Transferbuffer A

Tris	300 mM	300 ml 1 M Stock
SDS	0.1% (w/v)	10 ml 10% (w/v) Stock
Methanol	20% (v/v)	200 ml
add H ₂ O to 1000 ml		

The pH of the solution was adjusted with NaOH to pH 11.5.

Transferbuffer B

Tris	2 mM	25 ml 1 M Stock
SDS	0.1% (w/v)	10 ml 10% (w/v) Stock
Methanol	20% (v/v)	200 ml
		add H ₂ O to 100 ml

The pH of the solution was adjusted with NaOH to pH 10.5.

Transferbuffer C

Tris	25 mM	25 ml 1 M Stock
SDS	0.1% (w/v)	10 ml 10% (w/v) Stock
Methanol	20% (v/v)	200 ml
		add H ₂ O to 1000 ml

The pH of the solution was adjusted with 1 M boric acid to pH 9.0.

10x TBS buffer

Tris	0.20 M	24.2 g
NaCl	1.36 M	80.0 g
		add H ₂ O to 1000 ml

The pH of the solution was adjusted with HCl to pH 7.6.

Tween-Stocksolution (20% (v/v))

Tween	20% (v/v)	20 ml
		add H ₂ O to 100 ml

TBS / Tween buffer

10x TBS buffer	1x	100 ml
Tween	0.1% (v/v)	5 ml 20% (v/v) Stock
		add H ₂ O to 1000 ml

Ponceau S staining solution

Ponceau S	0.25%	2.5 g
Methanol	40% (v/v)	400 ml
Acetic acid	15% (v/v)	150 ml
		add H ₂ O to 1000 ml

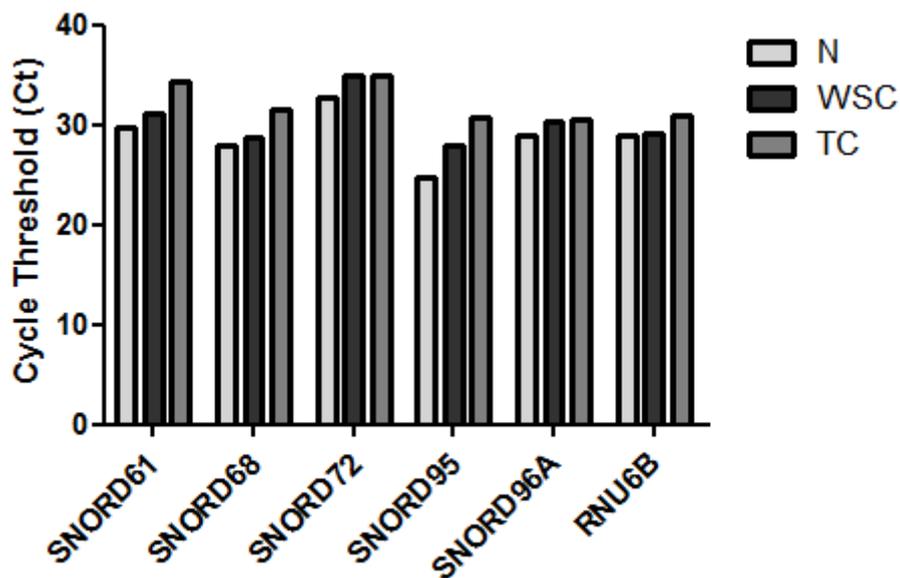
The solution was filtered through a filter.

Blocking solution

Skimmed milk powder	5% (w/v)	5 g
		add TBS / Tween buffer to 100 ml

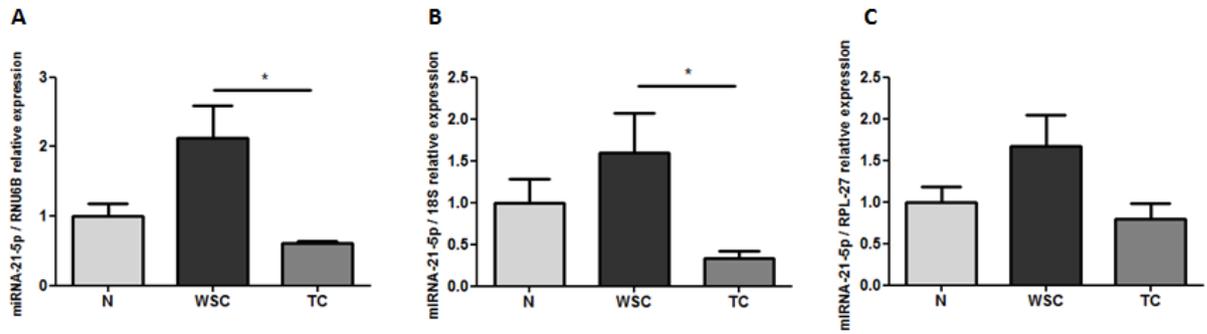
The solution was freshly prepared and stored at 4 °C.

Figure S1 – MicroRNA ARRAY for endogenous control test



The endogenous controls SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A and RNU6B were amplified by Realtime PCR in a pool of patients plasma samples in the group control (N), non-cachectic cancer (WSC) and cachectic cancer patients (TC).

Figure S2 - MicroRNA-21-5p plasma expression using three different endogenous controls



The miRNA-21-5p plasma expression was calculated after corrected for outliers in control (N), non-cachectic cancer (WSC) and cachectic cancer patients (TC), using RNU6B (A), 18S (B) or RPL-27 (C) as reference genes. One-way ANOVA followed by Tukey's post-test for parametric data or Kruskal Wallis followed by Dunn's post-test for non-parametric data; * p < 0.05.