CROSS-TALK IN CANCER-ASSOCIATED CACHEXIA:
DISSECTING SYSTEMIC EVENTS OF IMMUNE AND ENDOCRINE SYSTEM

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Summary
Cancer-Associated Cachexia (CAC) is a life-threatening syndrome characterized by involuntary loss of body weight mainly due to muscle and adipose tissue wasting. It has a high incidence and frequently appears concomitant to specific tumor types, such as pancreatic or lung cancer. CAC is related to poor prognosis, complications of cancer therapy and a general impaired quality of life. Currently, this disorder is not predictable and when it occurs the available therapies are just palliative and not able to revert the progression. CAC is a multifactorial syndrome, thus, understanding the cross-talk between the tumor and the rest of the organism is essential to improve the knowledge of CAC pathophysiology and most importantly, to find biomarkers useful for cachectic patients.

Pro-inflammatory mediators are important for CAC development, and although inflammation is a feature of CAC, the role of immune cells has not been studied. Increased number of granulocytes and decreased number of lymphocytes were consistently found across 3 different mouse models of CAC. In this context diverse mouse models of neutropenia, lymphopenia and knock-outs of neutrophil-related peptides were used to investigate the contribution of immune cells in CAC. No differences were found regarding cachexia hallmarks, suggesting that alterations in the immune populations are not essential for CAC development. Interestingly, Lipocalin-2 was identified as a potential new biomarker for CAC although its role is unknown.

Appetite is frequently reduced in CAC. Neuropeptide levels were analyzed by in situ hybridization in cachectic mice and showed an activation of the orexigenic pathway. Other hormones were measured in CAC. Corticosterone levels were increased in a pre-cachectic stage. Additionally, aldosterone mRNA expression levels were significantly upregulated in CAC. Intermediate peptides from the Renin-Angiotensin-Aldosterone System (RAAS) were also altered in CAC mouse models, being potential mediators of the disease. Human sera from cancer patient cohorts were also examined, and similar changes in RAAS were observed in cachectic pancreatic and lung cancer patients compared to non-cachectic.

RAAS activation together with anemia lead to the analysis of the heart function in mouse models of CAC. Interestingly, cachectic mice showed increased left ventricle volume and altered heart function.

Altogether, these data suggest that the mechanisms leading to CAC are independent of the alteration of immune populations despite the importance of some pro-inflammatory mediators. Moreover, solid data of RAAS activation are provided, which might be linked to heart functional changes in CAC.
Resumen
La Caquexia Asociada al Cáncer (CAC) es un síndrome potencialmente mortal, caracterizado por pérdida de peso corporal, músculo y grasa. Presenta una elevada incidencia y es más frecuente en algunos tipos de cáncer (páncreas, pulmón). La CAC se asocia a un pronóstico desfavorable, complicaciones en las terapias antitumorales y a un deterioro en la calidad de vida. Este síndrome no se puede predecir y, cuando aparece, los tratamientos son de paliativos, e incapaces de revertir su progresión. La CAC es multifactorial, y el estudio de la intercomunicación entre el tumor y el organismo es fundamental para mejorar el conocimiento de su fisiopatología y para encontrar biomarcadores útiles para los pacientes caquécticos.

Los mediadores pro-inflamatorios son importantes para el desarrollo de la CAC, y aunque la inflamación es un rasgo típico de la CAC, el papel que desempeñan las células inmunes no se ha estudiado. Observamos un incremento de granulocitos y un descenso de linfocitos en 3 modelos murinos de caquexia, de forma consistente. En este contexto, diversos modelos animales de neutropenia, linfopenia y knockouts de péptidos relacionados con neutrófilos fueron utilizados para investigar la contribución de las células inmunes en CAC. No se encontraron diferencias en cuanto al fenotipo caquéctico, lo que sugiere que las alteraciones de las poblaciones inmunes no son esenciales para el desarrollo de la CAC. Adicionalmente, se identificó la Lipocalina-2 como potencial biomarcador, aunque su función es desconocida.

En CAC el apetito está a menudo reducido. El análisis de los niveles de neuropéptidos por hibridación in situ indicó una activación de la vía orexigénica. Otras hormonas fueron estudiadas también. Los niveles de corticosterona aumentaron en pre-caquexia. Por otra parte, los niveles de mRNA de aldosterona eran significativamente mayores en CAC. Péptidos intermedios del Sistema Renina-Angiotensina-Aldosterona (RAAS) también se encontraron alterados en CAC, siendo posibles mediadores en esta enfermedad. Se examinaron muestras procedentes de pacientes y también se observó una alteración del RAAS en pacientes caquécticos de cáncer pancreático y pulmonar en comparación con pacientes sin caquexia. La activación del RAAS y la anemia condujeron al análisis de la función cardíaca en modelos animales de CAC. Los ratones mostraron incremento del volumen del ventrículo izquierdo y la alteración de la función cardíaca.

En su conjunto, estos datos muestran que los mecanismos que conducen al desarrollo de CAC son independientes de las alteraciones en las poblaciones inmunes a pesar de la importancia de los mediadores pro-inflamatorios. Además, se aportan datos sólidos de la activación del RAAS que podrían estar vinculados con cambios funcionales del corazón en CAC.
Table of contents
## Contents

Summary ........................................................................................................................................ 15
Resumen ......................................................................................................................................... 19
Table of contents ............................................................................................................................. 23
Abbreviations and nomenclature ..................................................................................................... 29
Introduction ..................................................................................................................................... 33
  Cancer-Associated Cachexia (CAC) ............................................................................................... 35
  CAC in the clinic: diagnosis and treatments .............................................................................. 35
  Diagnosis .................................................................................................................................... 35
  Management and therapies .......................................................................................................... 37
Pathophysiology and mechanisms leading to CAC ......................................................................... 38
  Loss of appetite ............................................................................................................................ 38
  Insulin resistance ........................................................................................................................ 39
  Loss of muscle ............................................................................................................................. 39
  Loss of adipose tissue .................................................................................................................. 40
MOLECULAR MEDIATORS ............................................................................................................ 41
  TNF-α ........................................................................................................................................ 41
  IL-6 ............................................................................................................................................ 41
  IL-1β .......................................................................................................................................... 42
Immune cells in CAC ...................................................................................................................... 42
  Neutrophils ............................................................................................................................... 42
  Lymphocytes ............................................................................................................................. 46
Neuroendocrine System in CAC ...................................................................................................... 46
  Hypothalamus as a center control for appetite ....................................................................... 47
  HPA axis in CAC ....................................................................................................................... 47
  Renin-Angiotensin-Aldosterone System (RAAS) ...................................................................... 48
Heart failure and cardiac muscle wasting in CAC ......................................................................... 49
Mouse models of CAC .................................................................................................................... 50
  Syngeneic mouse models .......................................................................................................... 50
  Genetic Engineered Mouse Models (GEMMs) ......................................................................... 50
  Mouse models used in this work ............................................................................................... 51
Objectives ....................................................................................................................................... 53
Material and methods ..................................................................................................................... 57
MICE .............................................................................................................................................. 59
Study approval ................................................................................................................................. 59
Results

The role of the central nervous system and neuroendocrine axis in CAC

The role of lymphocytes in CAC

The role granulocytes and neutrophils in CAC

Immune response in CAC

Statistical Analysis

Analytical Biochemical Technology

Cellular Biology Techniques

Molecular Biology Techniques

Mouse models

Animal experimental procedure

Reagents and devices

Mouse genotyping

RNA isolation from organs for reverse transcription

Reverse transcription

Semi-quantitative reverse transcription PCR (qRT-PCR)

Hypothalamic

Hypothalamic in CAC

Lymphocyte blockade

S100a9 in CAC

CSF in CAC

Reduced immune response correlates with reduced cachexia

Changes in White Blood Cells (WBC) is an early event in CAC

Distribution of immune cells

K5

K5-SOS Tg, C26 and CHX tumor-bearing mice develop CAC

Immune response in CAC

Immune-circulating cells in the blood are altered in CAC

Distribution of immune cells

Changes in White Blood Cells (WBC) is an early event in CAC

Reduced immune response correlates with reduced cachexia

The role granulocytes and neutrophils in CAC

Neutrophil blockade

CSF in CAC

S100a9 in CAC

Lcn-2 in CAC

The role of lymphocytes in CAC

Lymphocyte blockade

The role of the central nervous system and neuroendocrine axis in CAC

Hypothalamus in CAC

Hypothalamic-Pituitary-Axis (HPA)
Renin-Angiotensin-Aldosterone System (RAAS) ......................................................... 89
Cachectic patients: RAAS and cytokines ...................................................................... 92
Discussion .................................................................................................................. 95
Models: essential tool in CAC research ......................................................................... 97
CAC without the intervention of immune cells .............................................................. 98
Neutrophils .................................................................................................................. 98
Lymphocytes ............................................................................................................... 100
Is CAC orchestrated by neuro-endocrine system? ...................................................... 102
Appetite / Anorexia .................................................................................................... 102
Adrenal hormones ....................................................................................................... 103
The heart: another muscle in CAC ............................................................................ 104
CAC in human patients ............................................................................................... 105
Future directions ......................................................................................................... 105
Conclusions ................................................................................................................ 107
Conclusiones .............................................................................................................. 111
Bibliography ............................................................................................................... 115
Abbreviations and nomenclature
α-MSH -- Alpha-Melanocyte-Stimulating Hormone
ACE -- Angiotensin-Converting Enzyme
ACTH -- Adrenocorticotropic Hormone
AgRP -- Agouti-Related Protein
Ang I -- Angiotensin I
Ang II -- Angiotensin II
ANS -- Autonomous Nervous System
APP -- Acute Phase Proteins
APR -- Acute Phase Response
BBB -- Blood-Brain Barrier
BMI -- Body Mass Index
CAC -- Cancer-Associated Cachexia
CHF -- Chronic Heart Failure
CIC -- Circulating Immune Complexes
CNS -- Central Nervous System
CLP -- Common Lymphoid Progenitors
CMP -- Common Myeloid Progenitors
CRH -- Corticotropin-Releasing Hormone
CRP -- C Reactive Protein
DAMPs -- Damage-Associated Molecular Patterns
DEXA -- Dual-Energy X-Ray Absorptiometry
EMTs -- Epithelial-Mesenchymal Transition
G-CSF -- Granulocyte-Colony Stimulating Factor
GEMM -- Genetically Engineered Mouse Model
GPS -- Glasgow Prognostic Score
GHRELIN -- Growth Hormone Secretagogue Receptor type 1
HPA -- Hypothalamus-Pituitary-Adrenal axis
H-E -- Hematoxilin-Eosin
HPF -- High Power Field
iWAT -- Inguinal White Adipose Tissue
Lcn-2 -- Lipocalin-2
LC/MS -- Liquid Chromatography/Mass Spectometry
LLC -- Lewis Lung Cancer
MC4R -- Melanocortin 4 Receptor
MDSC -- Myeloid Derived Suppressor Cells
Nomenclature

Genes, proteins and mRNA

Mouse and genes are presented in italic and with the first letter capital e.g. Lcn-2

Proteins are presented non-italic with first letter capital e.g. Lcn-2

mRNA transcripts are presented italic in non-capital letters e.g. lcn-2
Introduction
Cancer-Associated Cachexia (CAC)

Cancer is a common disease with a high rate of morbidity and mortality worldwide, according to the World Heal Organization. It is estimated that almost 80% of cancer patients show a dramatic loss of body weight over the disease course (Porporato 2016). In fact, half of all cancer deaths worldwide are attributed to those cancers most frequently associated with a wasting syndrome, like pancreatic (0.33 million of deaths), pulmonary (1.59 million), or colorectal (0.69 million) cancers (Baracos et al. 2018). This decrease in the body mass is considered a prognostic factor in cancer and it is known as Cancer-Associated Cachexia (CAC). CAC usually appears in the last stages of the disease, although is often unrelated to tumor size or stage of the disease (Petruzzelli and Wagner 2016), and can be or not a side effect of the treatment. Additionally, cachexia is a wasting syndrome associated to other chronic diseases, frequently related with inflammation and the increased production of pro-inflammatory cytokines (Grossberg, Scarlett and Marks 2011), such as Chronic Obstructive Pulmonary Disease (COPD), Chronic Heart Failure (CHF), Rheumatoid Arthritis or AIDS (Tisdale 2002; Evans et al. 2008). In the case of CAC the primary stimulus is the tumor. Cachexia is defined as a multifactorial process secondary to an ongoing disease, characterized by an ongoing unintended decrease in body weight and skeletal muscle mass, often associated with loss of fat mass, that can be only minimally ameliorated by the administration of nutritional support (Fearon et al. 2011).

Nowadays, there are discrepancies across the literature about the diagnostic criteria of cachexia and also CAC, regarding the best prognostic factor comparing different systems (e.g. loss of body weight vs. changes in body composition) (Vanhoutte et al. 2016; Sadeghi et al. 2018). Although no agreement has been reached yet, there are fundamental and well-established features for the diagnosis of CAC.

CAC in the clinic: diagnosis and treatments

Diagnosis

Clinically, the most evident and easiest to measure parameter of cachexia in cancer patients is the involuntary weight loss. Loss of weight can be triggered by the aggressive anti-cancer treatment (chemotherapy and radiotherapy), but importantly, it can also start before any therapeutic agent has been administered to the patient. The decrease in body mass is mainly due to the loss of skeletal muscle, but additionally loss of adipose tissue frequently occurs. Remarkably, obese patients, which are highly prevalent in the Western countries, show a reduction in their body weight but rarely
reach low levels of BMI during CAC. Thus, obesity might mask the loss of weight (Baracos and Arribas 2018).

Cachexia is diagnosed when the patient loses more than 5% over the past 6 months or have a BMI less than 20 kg/m² according to Fearon et al. Other authors have proposed to evaluate additional parameters apart from the weight loss and BMI (weight in kilograms/height in square-metres) such as blood biochemistry, C-Reactive Protein (CRP) levels, hemoglobin, albumin and presence of anorexia, or weakness (Sadeghi et al. 2018).

The state before the appearance of cachexia is called “pre-cachexia”. At this time point the wasting is not yet dramatic, therefore preventive treatments could be still effective at this stage. This is a controversial concept and is not well defined in the literature due to lack of efficient biomarkers to predict CAC (Sadeghi et al. 2018).

Other measurements are evaluated for CAC diagnosis:

- **Loss of lean mass**: methods of muscularity monitoring are being slowly included in the protocols for the diagnosis of CAC, by either analyzing muscle mass or measuring muscle strength. Imaging techniques such as computed tomography (CT) or magnetic resonance imaging (MRI) are becoming popular (Fearon et al. 2011) (Jacquelin-Ravel and Pichard 2012).

- **Anorexia**: reduced food intake is a predictor of loss of weight and it is often seen in CAC. As it happens with weight, anorexia can also be influenced by anticancer therapies, but in many cases, it occurs independently.

- **Inflammation**: the most recognized serum components of CAC are mediators of systemic inflammation, notably the acute phase response proteins (APRPs) (Sadeghi et al. 2018). The Glasgow Prognostic Score (GPS) is a tool to quantify the systemic inflammation: it is a prognostic factor used in clinics both for the diagnosis and prognosis of CAC (McMillan 2008), and consists of a ratio between albumin and the C-Reactive Protein (CRP). This parameter strongly correlates with disease outcome: patients often suffer from hypoalbuminemia and CRP is frequently increased.

  Additionally, several reports in the literature show that Neutrophil-Lymphocyte Ratio (NLR), commonly used as prognostic factor in chronic diseases and cancer, also correlates with the loss of body weight in cancer patients (Onesti and Guttridge 2014; Derman et al. 2017).

- **Quality of life**: it is invariably reduced in CAC, and is closely related to all the aforementioned factors. Cachectic patients are usually weak, lacking energy, vigor and strength which causes stress. Although it can be considered a subjective parameter in CAC, the psychosocial factor is very relevant in the clinics as CAC provokes an additional distress to the patient and additionally to the caretakers.
**Management and therapies**

It is essential to keep in mind that CAC is always secondary to the tumor, therefore the anticancer therapy is the center of the treatment. Besides, the problems to consider for managing the disease are the weight loss, the caloric intake, the underlying catabolic drivers, the functional alterations and the psychosocial distress. The objective is to increase body weight, muscle mass and muscle strength or at least, to avoid further weight loss by using a multimodal approach.

**Nutritional support and appetite stimulators**

Oral nutritional supplements (vitamins, glutamine, aminoacids) are included as a first line used in the clinics, as well as orexigenic drugs such as glucocorticoids or megestrol acetate (progestogen). Some of these medications induce an increase in body weight, but do not stop the skeletal muscle atrophy, as the weight gain is mainly due to adipose tissue and water retention (Grossberg, Scarlett and Marks 2011; Dev et al. 2017).

New agents are now in clinical trials, like anamorelin (a ghrelin receptor agonist) or melanocortin receptor 4 antagonists, showing promising results. These drugs have a direct effect in the hypothalamus, which is the central regulator of appetite, and also induce systemic effects that promote protein anabolism and energy storage (Baracos et al. 2018).

**Muscle anabolism**

Anabolism of the skeletal muscle might also be increased by exercising, and so normally patients are encouraged to maintain or increase their physical activity according to their safe capacity and to clinical practice guidelines (Arends et al. 2017).

**Anti-inflammatory treatments**

Other consequences and symptoms of CAC also need to be treated in these patients: anti-inflammatory agents like nonsteroidal- anti-inflammatory drugs (NSAIDS), thalidomide, an immunomodulatory drug, are also part of the combined approach to CAC therapy, together with the anticancer therapy (Dev et al. 2017).

Importantly, part of the difficulty in treating CAC is that is its complexity as a multifactorial syndrome, with very general symptoms. Due to the lack of a unique and specific prognostic factor for the syndrome, CAC is often recognized at advanced stages, when the therapies available are not curable.
Pathophysiology and mechanisms leading to CAC

CAC is increasingly being considered as a prognostic factor for cancer patients. However, the elements involved in the development of CAC and the sequence of events leading to the final phenotype as well as the molecular mediators, remain obscure. Cancer alters the normal homeostatic control of energy balance. Loss of body weight frequently reflects a decrease in food intake, increase of catabolism in adipose tissue and especially muscle and dehydration.

Loss of appetite

Until recently, CAC was not considered an independent entity, and the loss of body weight in many cancer patients was attributed mainly to anorexia. However, anorexia itself cannot explain the weight loss and the profound metabolic changes occurring in CAC, as food supplementation does not revert the process (Tisdale 2002; Fearon et al. 2011). Furthermore, starvation produces a decrease in body weight due to lipolysis and loss of adipose tissue, but, unlike in CAC, the lean mass remains intact. In summary, CAC could occur in the absence, or concomitantly with anorexia, but they are considered two independent processes.

Cytokine expression levels in the brain are very low in homeostasis, but are dramatically increased upon peripheral inflammation. Indeed, many of the inflammatory mediators that are abundant in CAC, e.g TNF-α, IL-1β or IL-6, are able to cross the blood-brain barrier (BBB) and therefore, to induce changes in the hunger/satiety center allocated in the hypothalamus (Grossberg, Scarlett and Marks 2011). The most potent anorexigenic cytokine is IL-1β, which binds its receptor in the POMC neurons inducing the secretion of α-MSH (Ezeoke and Morley 2015). Importantly, this cytokine, among others, is also able to increase the metabolic rate through fever and thus the activation of thermogenesis in pathological conditions (Petruzzelli and Wagner 2016).

Besides anorexia and the intrinsic factors, many anti-cancer therapies (chemotherapy in particular) lead to decreased appetite as a side effect, because they can affect the hypothalamus, thus also causing nausea, vomits and even taste impairment. Eating patterns and routines might also be changed in cancer patients with cachexia due to psychological and social impact.
**Insulin resistance**

Insulin resistance might also be implicated in the low efficiency of nutrient uptake by the host cells, therefore participating in the development of CAC (Honors and Kinzig 2011). The importance of this mechanism still needs to be further investigated, but systemic inflammation might be involved. Furthermore, insulin is an essential hormone in the control of muscle proteolysis, as the availability and uptake of glucose suppresses proteolysis as a source of energy (Honors and Kinzig 2011). Thus, problems in insulin resistance or abnormal uptake from nutrients to the cells are important for muscle integrity.

**Loss of muscle**

Muscle atrophy is the main feature of CAC, and it is the main component of the body weight loss. In fact, a computational model showed that the proteolysis rate in the whole body is increased by 40% (Hall and Baracos 2009). Many of the pro-inflammatory cytokines that are common in CAC (TNFα, IL-1β, IL-6 or TWEAK) have a catabolic effect on skeletal muscle in CAC also in non-malignant condition, as they induce the reduction of the myofibrillar protein by reducing the expression of the nuclear transcription factor MyoD, thus preventing muscle repair (Acharyya et al. 2004). Other factors that produce a similar loss of muscle are Activin A and Myostatin. Eventually, the majority of these factors act through their respective receptors and in turn bind to promote the encoding component of the ubiquitin-proteasome pathway (UPP) (Baracos et al. 2018). UPP is the principal mechanism of proteolytic degradation pathway in the cell; when the UPP is activated, the protein that needs to be degraded is conjugated by a ubiquitin chain, that will bind to the proteasome and then, the tagged protein will be degraded. Autophagy, another cellular degradation system, is also an important mechanism in muscle wasting in CAC, particularly through the activation of the AKT-FoxO signaling axis; the inhibitory activity of FoxO1 and FoxO3 is often suppressed in cachectic conditions, leading to the transcription of atrophy genes (atrogenes) e.g. atrogin-1/MAFbx and Murf1 (Petruzzelli and Wagner 2016).

In addition to the loss of muscle mass, muscle strength is also dramatically reduced in patients suffering from CAC. Some mechanisms not related to the loss of protein but that affect the muscle functionality are also relevant in CAC. For instance, Transforming Growth Factor-β (TGFβ), which is frequently secreted by tumor cells, can induce dysfunction of the sarcomeres by altering Ca++ signaling, therefore affecting the homeostasis of muscle (Baracos et al. 2018).
Although catabolism is the most studied phenomena in skeletal muscle atrophy, loss of anabolism might also contribute to the sarcopenia phenotype. In particular, the loss of the regenerative capacity of the myocytes is related to the activation of NFkB and the expression of the self-renewing factor Pax7 (Petruzzelli and Wagner 2016).

**Loss of adipose tissue**

It is important to consider the adipose tissue as the most abundant metabolic tissue in the organism. Increased lipolysis is frequent in CAC patients, and the loss of adipose tissue precedes sarcopenia in both mice and human (Petruzzelli et al. 2014). During lipolysis, free fatty acids are released, providing a valuable source of energy for different cells, including tumor cells. Other changes take place in the fat depots, such as browning, that consists of a switch from white adipocytes to a beige/brown adipocyte-like phenotype (Mestas and Hughes 2004) (Kir et al. 2014; Petruzzelli et al. 2014). Ucp-1, as well as other secondary thermogenic genes and markers of browning (Pgc1α, Pparγ, Cidea, and Prdm16), are upregulated in those beige adipocytes, provoking changes at the mitochondrial level and releasing heat. Therefore, the function of these adipocytes changes upon CAC, from an anabolic energy storing to a catabolic thermogenic-like phenotype.

Browning normally takes place in cold conditions due to the activation of the β-adrenergic system, which also triggers lipolysis. This system is upregulated under stress conditions by the release of catecholamines and glucocorticoids in a physiological setting. In CAC, evidence of the activation of the β-adrenergic system has been reported (Tisdale 2002; Petruzzelli et al. 2014). Inflammation is also a pro-stress situation, therefore inflammation can trigger the activation of the β-adrenergic system, in particular factors like IL-6, and this could therefore induce lipolysis (Tsoli et al. 2012; Petruzzelli et al. 2014).

Importantly, due to the decrease of white adipocytes, leptin and other adipokines (adiponectin) are downregulated in CAC conditions. Those adipokines are essential for the regulation of appetite; however, in most CAC patients the hypothalamus does not react normally, therefore the cross-talk between the adipose tissue and the brain is profoundly altered. The pro-inflammatory status is postulated to be responsible for this disturbance, although little is known about the mechanism (Engineer and Garcia 2012).
MOLECULAR MEDIATORS

Essentially, the primary trigger of CAC is likely the tumor that secretes tumorkines which elicit changes directly in the target tissues (e.g. skeletal muscle, adipose tissue) or indirectly through the Central Nervous System (CNS) and the immune system, leading to the wasting syndrome. This process is only partially understood and the complex interactions between the tumor and the host, and the host itself remain elusive.

Numerous cytokines and hormones have been postulated to play a role in the etiology of cancer cachexia: these are able to modify the eating behavior, induce muscle wasting or elicit changes in the metabolism of the adipose tissue.

TNF-α

TNF-α, also named cachectin, was postulated as the main driver of CAC back in the 80s. Much literature is dedicated to the different roles that TNF-α plays in CAC. This cytokine has a direct catabolic effect on skeletal muscle as well as on adipose tissue, and it is one of the factors able to cross the blood-brain barrier (BBB) and induce anorexia (Porporato 2016). TNF-α is also associated with the synthesis of IL-1, also a pre-cachectic molecule. TNF-α might also play a role in decreasing insulin sensitivity and contributing to insulin resistance in cancer (Patel and Patel 2017). Although the presence and importance of TNF-α has been extensively studied in animal models, clinical trials failed to reduce the wasting of CAC by blocking TNF-α activity with an antibody (infliximab) (Patel and Patel 2017).

IL-6

IL-6 is a pleiotropic interleukin secreted by many different cell types, such as T cells or macrophages that have multiple functions in the organism. Besides its chemoattractant and regulatory activity for immune cells in the context of CAC, IL-6 elicits many metabolic functions, most of them promoting energy mobilization: it can induce anorexia due to its ability to cross the BBB, it induces muscle wasting as well as lipolysis and browning in adipose tissue (Petruzzelli et al. 2014). In the liver, IL-6 induces the production of CRP, thereby increasing the production of a pro-inflammatory component. Also, IL-6 induces changes in the hepatic metabolism by reducing ketogenesis (Flint et al. 2016). Thus, IL-6 is a key cytokine in CAC development and maintenance. Clinical trials were done with Tocilizumab, an interleukin-6 (IL-6) receptor antagonist in lung cancer
patients showing positive results (Ando et al. 2014a, 2014b), although other authors were skeptical regarding the improvement of the phenotype (Berti et al. 2013).

**IL-1β**

IL-1β is also markedly associated with CAC. It has an increasingly recognized role in the regulation of feeding behavior and energy homeostasis. IL-1β is one of the most potent anorexigenic cytokines and its expression in the hypothalamus is strongly increased in response to disease and inflammation both in the periphery and within the CNS itself (Grossberg, Scarlett and Marks 2011).

Other pro-inflammatory cytokines (IFNγ, G-CSF, TWEAK, Leukemia Inhibitor Factor etc) and circulating molecules (prostaglandins, glucocorticoids) have been investigated in CAC. All of them seem to act in a synergistic manner leading to CAC, by regulating the immune system, or inducing direct or indirect changes in target organs.

Importantly, all these mediators are released from tumor cells, but also from host cells. As the stimulus is not eliminated (tumor/host response), tumorkines and pro-inflammatory mediators are constantly produced and elicit effects in peripheral tissues by chronic inflammation situation. In CAC and cancer, many other pro-inflammatory factors still unknown can be altered in the circulation. These could be used as biomarkers for the diagnostic of the disease or a potential therapeutic target.

**Immune cells in CAC**

As previously described, most of the mediators in CAC, are pro-inflammatory molecules, originated by the tumor and also by the host. However, if the immune cells are needed for CAC development remains unknown. Interestingly, cancer and also CAC show a paradoxical systemic inflammation-immunosuppression situation, reflecting dysfunction of lymphocytes and a dramatic increase in the myeloid population, particularly in neutrophils (e.g. NLR).

**Neutrophils**

Polymorphonuclear leucocytes (PMNs) are the most abundant white blood cells in human blood and represent the vast majority of circulating granulocytes. Neutrophils have a very short lifespan and therefore are constantly produced in the bone marrow from the most immature stem cells and common myeloid progenitors (CMP).
release and maturation are controlled by the Granulocyte-Colony Stimulating Factor (G-CSF) as well as by the balance between the retaining and releasing CXCR4 and CXCR2 axis (Soehnlein et al. 2017). Once in the circulation, neutrophils modify their phenotype and function, and, after about 12 hours, they leave the bloodstream and are eliminated in the bone marrow, liver, and spleen by apoptosis. Alternatively, neutrophils might infiltrate other tissues to execute their tissue-specific functions (Nicolás-Ávila, Adrover and Hidalgo 2017). The role of neutrophils in cancer is multifactorial and not completely understood. They can induce oncogenesis by the release of Reactive Oxygen Species (ROS) and nitrogen species, which can induce DNA damage in pre-neoplastic inflammatory sites (Ocana et al. 2017). The production of Matrix Metallopepdidase 9 (MMP9) might also contributes to tumor initiation.

In many cases, myeloid-derived cells including granulocytes (mainly neutrophils, but also eosinophils and basophils) and also monocytes, have an immature phenotype, a reduced phagocytic capacity and increased reactive oxygen species (ROS) and nitric oxide (NO) production (Veglia, Perego and Gabrilovich 2018). Besides, these cells can inhibit the adaptive immune responses, causing immunosuppression and supporting tumor growth and metastasis. Some of these populations are referred to as Myeloid Derived Suppressor Cells (MDSC), as there are very special group of cells with unique features and biological roles (Veglia, Perego and Gabrilovich 2018).

Neutrophils are able to produce and secrete Damage-Associated Molecular Patterns (DAMPs), and the interest in those has increased in the field of cancer. These DAMPs act as soluble mediators involved in modulating host immune responses and in promoting tumorigenesis and progression to malignancy (Srikrishna2012). They are also called “alarmins”, as they are released in response to stressors, especially after injury or cell death (Huang 2015).

In this thesis, S100a9 and Lipocalin-2 (Lcn-2) will be studied in the context of CAC as potential mediators.

S100a9

S100a9 is a DAMP named calgranulin B, constitutively expressed by myeloid cells, including neutrophils and monocytes, and is a biomarker in inflammatory diseases such as Rheumatoid Arthritis. In inflammation, the complex composed by S100a9 and S100a8, called calprotectin, modulates the inflammatory response by stimulating leukocyte recruitment and inducing cytokine secretion. Additionally, S100a8/a9 is also
important in tissue repair (Pandolfi et al. 2016). Expression is enhanced by stress response modulators such as norepinephrine and by glucocorticoids. S100a8 and S100a9 can also be found in a no-complex form. In a cancer context, calprotectin levels, and also S100a9 alone, are increased in tumor-bearing mice. As an alarmin, it can be secreted by tumor cells because of the stressful conditions and due to necrosis of malignant cells. In vitro studies showed that S100a8/a9 can promote cytotoxicity and apoptosis in tumor cell lines, but the antitumor effects are not observed in vivo (Shrikrishna et al. 2012). Whether S100a9 is upregulated in CAC, is unknown.

Lipocalin-2

Lipocalin-2 (Lcn-2) is a 25 kD DAMP involved in innate immunity and iron trafficking. It belongs to the lipocalin family, a group of small secreted proteins, which are transporters of small hydrophobic molecules, such as steroids or lipids among others. In particular, Lcn-2 is able to capture siderophores, low molecular-weight chelating agents released by certain bacteria that have a high affinity for ferric iron. Lcn-2 sequesters the iron and limits its availability for the bacteria, as a consequence Lcn-2 is considered an antimicrobial peptide. Regarding iron metabolism, Lcn-2 is also able to regulate the cellular iron status in an autocrine manner by inducing the overexpression of its own receptors (Schroll et al. 2012).

The expression of Lcn-2 is restricted to neutrophils at homeostasis. Specifically, Lcn-2 was localized in the secondary granules of the neutrophils, the same endosomal vesicles where chemotaxis-inducing factor are, such as Mac1 (CD11b/CD18), lactoferrin or calprotectin (S100a8/a9) (Kjeldsen et al. 1993). Lcn-2 can also be expressed in other cell types under specific stimulus. For instance, cytokines such as TNF-α and IL-1β induce the expression of Lcn-2 via STAT1 or NFkB respectively (Borkham-Kamphorst et al. 2013). Other cytokines such as IL-17 and IL-6 can also induce the secretion of Lcn-2, although this induction is not as strong as the others. Lcn-2 was found upregulated in solid tumors such as breast cancer and colorectal cancer (Rodvold, Mahadevan and Zanetti 2012) probably as a response from the cancer cells to a pro-inflammatory environment. It was hypothesized that Lcn-2 can play a tumorigenic role by promoting tumor cell survival through iron scavenging and even by promoting the induction of epithelial-mesenchymal transition (EMT). In some tumors, the upregulation of Lcn-2 correlates with HIF-1 levels and therefore, with a hypoxic microenvironment (Nakamura et al. 2014).
Lcn-2 has multiple functions in neutrophil trafficking, for example as a potent chemoattractant (Schroll et al. 2012) and also regulates the migration of neutrophils through the MAPK pathway. Lcn-2 binds Matrix Metalloproteinase-9 (MMP9) and thus, increases its stability and its activity over time. In fact, the complex Lcn-2/MMP9 is very important for neutrophil extravasation. Furthermore, Lcn-2 is part of the Acute Phase Response (APR), which main functions are to maintain homeostasis, to transport molecules and to defend against infections (Liu and Nilsen-Hamilton 1995). Lcn-2 might also be a relevant mediator in the self-control of the immune response through the activation of apoptosis of hematopoietic cells in a cell specific manner, affecting primary thymocytes and lymphocytes as well as neutrophils (Devireddy et al. 2001).

Recent publications postulate Lcn-2 as an important peptide not only involved in infections and sterile inflammation, but also a key regulator associated with important metabolic process. As an adipokine, Lcn-2 is also found in adipose tissue. The role of Lcn-2 in this context might be the regulation of insulin signaling, as other members of the lipocalin family such as the Retinol Binding Protein 4 (RBP4) (Mosialou et al. 2017). Lcn-2 expression is increased in obesity, consequently it could be related to an insulin resistance state.

It has been described that Lcn-2-deficient mice have an impaired adaptive thermogenesis under cold stimulation compared to wild-type mice (Zhang et al. 2014). Regarding bone metabolism, Lcn-2 is postulated as a mechanoresponding factor under mechanical unloading. Its overexpression causes osteoclastogenesis, because it diminishes the differentiation of osteoblasts and stimulates the production of both IL-6 and Receptor Activator of Nuclear factor Kappa B Ligand (RANKL) (Rucci et al. 2015). Importantly, Lcn-2, synthetized in bone, is able to cross the BBB and activate the melanocortin 4 receptor (MC4R) pathway, therefore controlling and reducing appetite, establishing that Lcn-2 is also controlling appetite (Mosialou et al. 2017).

In liver, Lcn-2 is an indicator of hepatic injury, also synthesized and secreted by hepatocytes under inflammation in mouse models of acute hepatitis, such as Concanavalin A and LPS (Borkham-Kamphorst et al. 2013). Lcn-2 was also proposed as an intermediate peptide in hepatic lipid metabolism by inducing and regulating the expression of perilipin 5 (PLIN5), a lipid droplet targeting protein that promotes association of lipid droplets with mitochondria; in that case, Lcn-2 would be able to regulate the formation of intracellular lipid droplets in hepatocytes in fatty liver conditions via PLIN5 (Asimakopoulou et al. 2014).
Lymphocytes

Lymphocytes are the adaptive immune cells and are essential for cell-mediated (T cells) and also antibody-mediated (B cells) immunity. Typically, the Common Lymphoid Progenitor (CLP) are the cells of origin of all lymphocytes, although other cells could also be T-cell progenitors (Bhandoola and Sambandam 2006). Importantly, inflammation can influence the balance between the lymphopoiesis and granulopoiesis by several factors such as TNF-α, promoting the production and release of myeloid cells (Ueda et al. 2004).

T cells develop in the thymus from a common progenitor (CD4- CD8-) and are defined by expression of a T cell receptor (TCR). Different lineages are generated from that common progenitor, including γδ T cells, CD8+ naive T cells, NK-T cells, regulatory T cells (Tregs) (CD4+ FoxP3+) and naïve CD4+ T cells, and eventually they will be released in the blood stream. The CD8+ T cells and CD4+ T cells need to get activated; in the case of the CD4+ T cells, they can polarize towards Th1, Th2, Th17 or Tregs, depending on the stimulus (Ruffel 2011).

T cell function of tumor infiltrating cells is impaired in cancer by the immunosuppressive mechanisms, as they are not able to efficiently recognize the cancer cells, for instance due to the abnormal functional state of PD-1+ T cells (“T cell exhaustion”) (Thommen and Schumacher 2018).

B cells mature from the CLP as well, and they are defined by the BCR receptor. Mature B cells leave the bone marrow and migrate to secondary lymphoid organs, where they can interact with exogenous antigen and/or T helper cells. B cells also play a dual role in cancer: B cell subsets are reported to promote anti-tumor immunity by producing antibodies, acting as antigen presenting cells and secreting cytokines or chemokines that regulate other leukocytes. On the contrary, those same functions would be detrimental for anti-tumor immunity e.g. secreting lymphotoxin which is a survival factor and induces angiogenesis. Also, the presence of circulating immune complexes (CICs) are in many cases a bad prognosis in cancer (Yuen, Demissie et Pillai 2016).

Neuroendocrine System in CAC

The neuroendocrine system is a complex mechanism that controls body homeostasis and involves the interactions between the nervous system and the endocrine system. The basic components of the neuroendocrine system are the Hypothalamus-Pituitary-Adrenal axis (HPA), the Autonomous Nervous System (ANS) and a functional
subgroup of the endocrine systems like thyroid, noradrenaline-adrenaline-producing cells of the adrenal medulla, etc.

In this thesis, some data from hypothalamus as the hunger-satiety master regulator will be discussed. Additionally, and related to the axis, adrenal hormones and the Renin-Angiotensin-Aldosterone System (RAAS) will also be studied as potential mechanisms of CAC.

**Hypothalamus as a center control for appetite**

The hypothalamus controls hunger and satiety, as a main part of the central melanocortin system. This system is integrated by different circuits involved in the control of the appetite (Ezeoke and Morley 2015):

- Neurons from the arcuate nucleus that express orexigenic peptides (appetite stimulant) like the Neuropeptide (NPY) or Agouti-Related Peptide (AgRP). NPY is a very potent stimulator of appetite, and its production is regulated by leptin, insulin (both inhibiting appetite), glucocorticoids, ghrelin (both stimulating the appetite) among others. Agouti-related protein (AgRP) neurons are distributed in the same of the hypothalamus. This factor is implicated in control of the energy balance as an antagonist of the melanocortin-3 and melanocortin-4 receptors (Austin et Marks 2009).
- Pro-opiomelanocortic (POMC) neurons in the arcuate nucleus of the hypothalamus and nucleus of the solitary tract in the brainstem. POMC is a precursor for many different active peptides such as the alpha-Melanocyte-stimulating hormone (α-MSH) which has anorexigenic functions. POMC has other functions, including regulation of lactation or the reproductive cycle (Millington 2007).
- Downstream targets expressing melanocortin-3 and melanocortin-4 receptors.

**HPA axis in CAC**

Together with the pituitary and adrenal glands, the hypothalamus integrates the HPA axis, which controls the reaction to stress, energy storage and expenditure, digestion, immune system etc. In fact, chronically elevated IL-6 levels in the brain have shown to activate the HPA axis and to cause adrenal gland hyperplasia. IL-1 β also activates HPA axis leading to elevated plasma levels of ACTH (Grossberg, Scarlett and Marks 2011; Petruzzelli and Wagner 2016). Additionally, in cardiac cachexia, increased serum levels of cortisol and catecholamines were detected in patients (Petruzzelli and Wagner 2016).
HPA axis is an essential regulator of many functions in the organism and the core of the axis are the hypothalamus, the pituitary gland and the adrenal glands. In summary, the hypothalamus secretes Corticotropic Releasing Hormone (CRH) and vasopressin, and they induce the pituitary gland to secrete the Adrenocorticotropic hormone (ACTH). ACTH then activates the cortex of the adrenal glands and glucocorticoids are released. The system counts on a feedback loop for regulating itself.

HPA has a close cross-talk with the immune system: pro-inflammatory cytokines like IL-1β are able to activate the HPA and, on the contrary, high levels of glucocorticoids suppress the immune and inflammatory responses. In CAC conditions, the levels of cortisol in cachectic patients is higher compared to the non-cachectic/pre-cachectic patients (Flint et al. 2016).

Importantly, cortisol, the main glucocorticoid in humans, regulates glucose homeostasis in the skeletal muscle by inhibiting the insulin-stimulated glucose uptake. Besides, glucocorticoids are also involved in protein synthesis and promote hepatic gluconeogenesis, which contributes to steroid-induced myopathy and impaired glucose tolerance (Kuo T, Harris CA 2013). Glucocorticoids also induce lipolysis (Tisdale 2002). In a similar manner, catecholamines (norepinephrine and epinephrine) have also a lipolytic effect in CAC, as part of the β-adrenergic system.

**Renin-Angiotensin-Aldosterone System (RAAS)**

The RAAS is a highly regulated system that controls the blood volume, arterial pressure and cardiac output as it is also involved in the systemic vascular resistance. Under particular stimuli (activation of the Sympathetic Nervous System, low blood pressure or a decrease in the sodium delivery to the distal tubules of the kidney), renin is released into the circulation by the juxtaglomerular cells of the kidney and initiates a proteolytic cleavage cascade from angiotensinogen, forming Angiotensin I (Ang I), and afterwards Angiotensin II (Ang II) by the effect of the Angiotensin Converting Enzyme (ACE) and other intermediates peptides.

Ang II is the best known of the peptides of RAAS as it acts as a potent vasoconstrictor effector, thus increases the systemic vascular resistance and arterial pressure. Ang II is involved in the water retention and it enhances sympathetic adrenergic function, stimulates cardiac and vascular hypertrophy.
Figure I.1: Simplified scheme from the Renin-Angiotensin-Aldosterone System (RAAS). The 2 main pathways are highlighted with colors: classical pathway (blue lines) Ang II-ACE-AT1R and the alternative pathway (red lines) Ang (1-7)-ACE2-MasR. ACE1: Angiotensin-Converting Enzyme 1; ACE2: Angiotensin-Converting Enzyme 2; Ang I, Ang II, Ang III, Ang IV: Angiotensin I, II, III, IV; Ang (2-10); Angiotensin (2-10); Ang (1-9); Angiotensin (1-9); Ang (1-7): Angiotensin (1-7); Ang (1-5): Angiotensin (1-5); AT1R: Angiotensin II type 1 receptor; AT2R: Angiotensin II type 2 receptor; AT4R: Angiotensin II type 4 receptor; Mas receptor: Ang (1-7) receptor type. Adapted from (Holappa 2015).

Interestingly, Ang II has an atrophic activity in the skeletal muscle through the induction of the UPP for protein degradation through its receptor in the myocytes (Cabello-Verrugio, Córdova and Salas 2012).

Aldosterone, which is the last product of the system, acts on the kidneys through the mineralocorticoid receptors. Mineralocorticoid receptors are also present in the skeletal muscle, and a potential effect from this hormone regarding muscle atrophy has been proposed (Murphy et al. 2013).

RAAS is also activated in other diseases associated to cachexia such as CHF or COPD, related to the high concentrations of Angiotensin Converting Enzyme (ACE) in the lungs in a chronic hypoxic conditions (Mascitelli, Pezzetta and Goldstein 2008; Toru et al. 2015) and that its blockade is beneficial in these patients (Shrikrishna et al. 2012). Aldosterone and RAAS activation are also important in AIDS and insulin resistance (Srinivasa et al. 2015).

**Heart failure and cardiac muscle wasting in CAC**

Several studies demonstrate cardiovasculard complications and cardiac muscle wasting in animal models (Springer et al. 2014) and also in human CAC (Barkhudaryan...
et al. 2017). These conditions impaired even more the cachectic patient health, quality of life, and affect the efficacy of anti-tumor therapy. In patients, cardiovascular diseases are prevalent and therefore might be established even before cancer appears. However, cardiac muscle wasting can occur directly by the effect of the tumor, as it is a muscle and might be affected as well by protein synthesis and degradation imbalance (Murphy 2016). Also, anti-cancer treatments are frequently cardiotoxic, which further impairs the cardiac wasting. Although currently there is no therapy for cardiac muscle wasting, some strategies are proposed similarly to other circulatory effects in derived from the tumors eg RAAS activation.

**Mouse models of CAC**

Being such a complex disease, it is unlikely that animal models are able to fully represent the disease. However, several mouse models have been proposed for modelling CAC and all of them should mimic the main features of CAC, which are the “loss of skeletal muscle mass” and a “progressive functional impairment” (Penna, Busquets and Argilés 2015).

**Syngeneic mouse models**

Most of the preclinical studies are based on transplantation mouse models, where cancer cell lines are injected in the recipient mice, typically via subcutaneous or orthotopic. Some features of CAC are exacerbated or lost, depending for example on the site of injection (Ballaró, Costelli and Penna 2016). The advantages of the transplantation models are that the studies can be carried out in immunocompetent mice, and the tumor growth and disease development are rapid, reproducible and easy to manage. The high tumor growth rate can be a limitation of the system at the same time, depending on the type of study. Another drawback is that these tumors are ectopic, which does not reflect what usually happens in cancer patients.

**Genetic Engineered Mouse Models (GEMMs)**

These strategies are based on genetically manipulated mice that develop spontaneous tumors. GEMMs better resemble the evolution of a tumor: the neoplasia is orthotopic, the tumor growth rate is generally lower and the process of carcinogenesis can be similar to the one in humans (Ballaró, Costelli and Penna 2016).
Mouse models used in this work

Bellow there is a list from the mouse models used in this work.

Transplantation mouse models

- **C26 colorectal carcinoma mouse model**: Originally from a chemically induced tumor (Balb/c). C26 cells are typically injected subcutaneously and induce CAC in few weeks.

- **CHX fibrosarcoma mouse model**: Derived from the MCA 207 fibrosarcoma cell line, these cells show a more aggressive phenotype. CHX can be injected subcutaneously or orthotopically and induce tumor and CAC in few weeks.

Genetic engineered mouse models

- **K5-SOSTg mice**: K5-SOS Tg mice carry the SOS transgene under the Keratin-5 promoter. These mice also have the wave allele in heterozygosity in the Egfr locus (Sibilia et al. 2000) and they spontaneously develop papillomas very early in age, as well as a rapid and dramatic loss of weight, including loss of skeletal muscle and adipose tissue as previously reported (Petruzzelli et al. 2014).

The next mouse models were injected with CHX in order to evaluate the effect of different peptides and cells in CAC.

- **S100A9 (-/-)**: Constitutive knock-out mice for S100A9 (S100a9\textsuperscript{tm1a(EUCOMM)Wtsi}). These mice show abnormalities in neutrophil morphology and physiology and altered cellular extravasation.

- **Csf3r (-/-)**: Constitutive knock-out mice for G-CSF R (Csf3r\textsuperscript{tm1Eur}). These mice show abnormal hematopoietic development and neutrophil differentiation and decreased cell number.

- **Lcn-2(-/-)**: Constitutive knock-out mice for Lcn-2 (Lcn2\textsuperscript{tm1Aade}). These mice tend to be heavier, have increased risk of infections and to be more prone to diet-induced obesity.

- **Rag1(-/-)**: Constitutive knock-out mice for Lcn-2 (Rag1\textsuperscript{tm1Mom}). These mice have thymus and spleen hypoplasia, defects in maturation of T and B cells, decrease production of immunoglobulins, decrease vascular permeability and an increased eosinophil number.
Objectives
CAC is a syndrome characterized by loss of weight, catabolism and inflammation among others. However, the role of immune cells, as well as specific hormonal systems is still unknown. The objectives of this thesis are:

1- Identify the changes in the immune system in CAC are. To characterize the modifications of circulating cells in CAC in different mouse models and to find potential mediators.

2- Investigate the role of the most common circulating populations in CAC, in particular granulocytes/neutrophils and lymphocytes by different strategies and mouse models of neutropenia and lymphopenia. Also, to study the role of neutrophil-associated molecules.

3- Determine the changes in specific neuropeptides and hormones in the brain and in the circulation in CAC. Specifically, to investigate peptides controlling appetite and pathways related to stress, adrenal hormones and the RAAS.
Material and methods
MICE

Study approval
All experiments were performed in accordance with institutional policies and national and European guidelines. Mice were housed in the CNIO animal facility and all animal experiments were approved by the Animal Experimental Ethics Committee of the Instituto de Salud Carlos III (Madrid, Spain).

Mouse models

*K5-SOS Tg*: GEMM prone to develop skin tumors (papilloma) mainly in the tail. This strain was generated in 2000 (Sibilia *et al.* 2000) and was later reported as a mouse model of CAC (Petruzzelli *et al.* 2014). *K5-SOS Tg* mice carry two mutated alleles in heterozygosity: *Egfr (wa+)/* and the *SOS* transgene under the Keratin-5 promoter (*K5-SOS +/-Tg*). No other tumors apart from the skin papilloma were detected. The *K5-SOS Tg* colony was managed by me. At 2 weeks, mutant mice showed a thicker skin in the tail area and a well-formed papilloma was observed at weaning. CAC is a very rapid process in these mice, as they become cachectic by week 5 age. Importantly, the pathogenesis of CAC accelerated.

*C26 syngeneic colorectal mouse model*: F1 mice from a Balb/c X DBA were purchased from Charles River at 8-10 weeks of age. After one week of adaptation, mice were anesthetized with 2% isoflurane and C26 tumor cells (1x10^6 cells) were injected subcutaneously in the medial area of the lumbar region. Mice overall condition and tumor growth were monitored 2 times a week. After 18-21 days, mice were sacrificed, and samples were collected for further analysis.

*CHX syngeneic fibrosarcoma mouse model*: At 9-13 weeks of age, C57BL/6 mice from the CNIO colony were anesthetized with 2% isoflurane and injected intramuscularly in the hind limb with 1x10^6 CHX cells. Mice overall condition and tumor growth were monitored 2 times a week. After 14-18 days, mice were sacrificed, and samples were collected for analysis.

All the data regarding weight in the mice were normalized by dividing the weight in grams by the tibiae length and multiplied by 100.
Animal experimental procedure

**Dual-Energy X-Ray Absorptiometry (DEXA)**

*K5-SOS* Tg mice were anesthetized with 2% isoflurane and allocated within the densitometer. The results include Bone Mineral Density (BMD) (mg/cm²), Bone Mineral Content (BMC) (mg), Bone area (cm²), Fat (%), Lean tissue (grams) and fat tissue (grams).

**Blockade of neutrophils with antiGr-1**

AntiGr-1 antibody (RB6-8C5), recognizing Ly6C and Ly6G, was used for *in vivo* depletion of neutrophils. It was purchased from the Monoclonal Antibodies Unit of CNIO. At weaning, antiGr-1 was injected in tumor-bearing *K5-SOS* Tg mice, while mice with same genotype were injected with PBS. The antibody was intraperitoneally administered 2 times a week for 2 weeks. Mice were sacrificed at 5 weeks of age.

**Echocardiography**

By echocardiography, heart function of *K5-SOS* Tg and CHX mice was evaluated. Ultrasounds were carried out by the Molecular Imaging Unit from CNIO. Mice were anesthetized with isoflurane (initial dose 4%, reduced to 2% as maintenance dose) and had the thoracic area shaved for the procedure. In order to avoid hypothermia during the procedure, the surface of the table was heated. The images were obtained by B-mode and pulse-waved Doppler mode.

**Reagents and devices**

Agarose was purchased from Lonza. PBS and TAE buffer were purchased from Alaos. All the other reagents/kits were purchased from indicated manufacturers. Trizol and Trizol LS (used for isolating RNA from sorted cells) were purchased from Panreac AppliChem and Life Technologies S.A. respectively. Oligonucleotides were purchased from Sigma-Aldrich.

Genotyping analysis was performed with QuantityOne 1-D Analysis Software.

Data analysis was performed with Windows 10 (Microsoft), Microsoft Office (2013), WPC Office, Flowjo V10, GraphPad Prism, Photoshop CS4 (Adobe), Image J and the figures were prepared using Illustrator CS4 (Adobe) and PowerPoint (Microsoft).
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<th>DEVICE</th>
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**Molecular Biology Techniques**

**Mouse genotyping**

Genomic DNA was obtained from finger biopsies. The biopsies were digested in 500µl of a mix of lysis buffer (named “tail buffer”) and proteinase K (at 1%) at 56°C overnight. The tail buffer is composed by (Tris 1.5M pH8.8) EDTA (0.5M), NaCl (5M) SDS (10%), and proteinase K. Once the samples were completely dissolved, 300µl of NaCl 6M were added. After vortexing, samples were centrifuged at full speed (13200G), for 10
minutes at 4°C. Supernatants were transferred to another tube and 500µl of isopropanol were added for DNA precipitation. After vigorously shaken, the samples were centrifuged at 13200G at 4ºC for 10min. Supernatants were then discarded, and 75% ethanol was added to the pellet in order to purify the DNA, and re-centrifuged in the same conditions. Supernatants were again discarded. Once the ethanol was dry, 200µl of distilled water were added for the DNA to dissolve and put in the incubator at 37ºC for 1 hour.

All PCR reactions were performed in a reaction volume of 25µl. Genotyping reaction of rag1 and lcn-2 were composed of 12.5 µl of Taq Polymerase (REDTaq® ReadyMix™ PCR Reaction Mix), primers (10µM of each primer), 8.3 or 9µl of water and 1 µl of the genomic DNA. PCR program: 3 min 94ºC, (30 sec 94ºC, 45 sec 60ºC, 60sec 72ºC)30x, 10min 72ºC. PCR products were analyzed by gel electrophoresis using 2% agarose gels and the DNA was visualized using Gel Red stain (Biogen).

Following primers were used (depicted in 5´- 3´orientation).

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**RNA isolation from organs for reverse transcription**

Frozen tissue samples were lysed in 1 ml of Trizol (Sigma) using a Precellys® tissue homogenizer. 200µl of chloroform were added and the samples were centrifuged for 15 minutes at 13,200 rpm at 4ºC. The aqueous phases containing the RNA were carefully transferred to new tubes, and 500µl of isopropanol were added for precipitation of the RNA. After vigorously shaking, the samples were centrifuged for 30 minutes at 13,200 rpm at 4ºC. Supernatants were removed, and the samples were
washed by adding 1000 µl of cold 80% ethanol followed by a centrifugation at 13,200 rpm for 10 minutes at 4°C. Once dries, the pellets were dissolved in nuclease-free water; the added volume depended on the pellet size. Then the samples were stored at -80 until they are used. In the particular case of the adrenal glands, or equivalent, half of the volumes were used.

**Reverse transcription**

In order to purify the sample, DNAse treatment was performed. 1-2 µg of RNA were diluted in a total volume of 10 µl, including 1 µl of Reaction Buffer and 1 µl of DNAse I (1U/ µl) and nuclease-free water. After incubating for 15 min at room temperature, 1µl of 25mM EDTA was added for inactivating the reaction. 10µl of GoScript™ Reverse Transcription Mix was prepared for each cDNA reaction (4µl of nuclease-free water, 4µl of GoScript™ Reaction Buffer, Oligo (dT) and 2µl of GoScript™ Enzyme Mix). This mastermix was combined with the RNA sample (final volume of 20µl) and, after mixing well, the reaction was incubated following these steps: 1- anneal primer (5 min 25ºC), 2- extension (60 min 42ºC), and 3- inactivation (15 min 70 º C). A dilution of 1:6 is made in nuclease-free water and the samples were stored at -20ºC until qPCR was performed.

**Semi-quantitative reverse transcription PCR (qRT-PCR)**

Quantitative PCR Primer Database (QPPD) was used for searching for primers that were previously validated in the literature ([https://pga.mgh.harvard.edu/primerbank/](https://pga.mgh.harvard.edu/primerbank/)). NCBI Standard Nucleotide BLAST online tool was used in order to verify the primers ([https://blast.ncbi.nlm.nih.gov/Blastcg?PAGE_TYPE=BlastSearch](https://blast.ncbi.nlm.nih.gov/Blastcg?PAGE_TYPE=BlastSearch)). 2µl of cDNA, 5µl of Sybr Green qPCR Master mix, 3µl of nuclease-free water and 0.2 µl of primers (0.1 µM) were used per reaction. The comparative cycle threshold method was used for quantification and expression levels were normalized using the housekeeping gen rpl4. All samples were run in technical duplicates. The standard program was: 2min at 95ºC, (15 sec 95ºC, 15 sec 55ºC, 20 sec 68ºC)40x. ddCt method for qRT–PCR data analysis. Following primers were used (depicted in 5´to 3´orientation):
Cellular Biology Techniques

Paraffin blocks

Tissues were fixed in 4% neutral-buffered formalin for 24-48h at RT and embedded in paraffin blocks by the CNIO Histopathology Unit. Sections of 3µm were cut for further staining or immunohistochemistry using the microtome (Leica).

Hematoxilin- Eosin (H-E) staining

This staining was performed in the autostainer (Sakura). Paraffin sections were deparaffinized (sequential immersion in 2 x 2 min in xylol, 2 x 2 min in 100% ethanol, 2 x 2 min in 90% ethanol, 5 min in 75% ethanol). Slides were stained in Hematoxylin (Panread) for 15 min, washed in water for 1 minutes and stained in Eosin solution (Sigma) for 2 min. Slides were dehydrated in 100% ethanol for 30 minutes, immersed in xylol for 30 seconds and mounted in xylol-based mounting media (Merck).

Immunohistochemistry

Deparaffination was performed in an autostainer. After an incubation of 10 min at 56 °C, paraffin sections were deparaffinized (sequential immersion in 2 x 2 min in xylol, 2 min in 100% ethanol, 2 min in 96% ethanol and 2 min in water) and boiled for 20 min in antigen retrieval buffer (10 mM Sodium Citrate, 0.05% of Tween®20, adjusted to pH 6) using a pressure cooker. Then, tissues were permeabilized and blocked with a blocking buffer (0.5% Triton X-100 in 2% BSA in PBS) and 1% serum of the secondary antibody host for 1 hour at RT. Right after, samples were incubated with the primary antibody diluted in the blocking buffer overnight at 4°C. The slides were then washed 3 x 5 minutes in PBS and incubated with the secondary antibody diluted in 1% BSA in PBS for 1 hour at RT and washed afterwards 3 x 5 minutes each in PBS. The samples were incubated for 30 minutes with the ABComplex prepared within half an hour in advance. After washed, DAB (from VECTASTAIN Elite

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<tr>
<th>Transcript</th>
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<tr>
<td>rpl4</td>
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<td>TCTTGGGCAACCACCTTTTTC</td>
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<tr>
<td>lcn2</td>
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</tr>
<tr>
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<td>CYP11b2</td>
<td>ACTCGGGTGTTGGAAGAACAT</td>
<td>GCCACTGTAGTGCTGAGAGC</td>
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ABV Kit, Vector Labs) was added for developing the staining. The reaction was stopped by immersing the slides in distilled water and they were counterstained with Hematoxylin (Panreac), washed for 4 minutes, and dehydrated by sequential immersion in alcohol (70% ethanol 2min, 96% ethanol 2 min, 100% ethanol 2 min, xylol 2 x 2 min). The slides were mounted with xylol-based mounting media (Merck).

Following primary antibodies were used at the indicated concentration:
- CD45: Purified Rat Anti-Mouse CD45, Clone 30-F11, BD Pharmingen (550539)
- Lcn-2: Polyclonal Goat Anti-Mouse Lcn-2, R&D (AF1857)

Vecstain ABCkits were used for the secondary antibody and the AB complex (anti-rat IgG: PK-6104; anti-goat IgGG: PK-6105) from PALEX MEDICAL, S.A.

**ELISAs**

Circulating levels of cytokines and adipokines were determined with the following ELISA kits:
- IL-6 (mouse): Mouse IL-6 Quantikine ELISA, R&D (M6000B)
- TNF-α (mouse): Mouse TNF-alpha Quantikine ELISA, R&D (MTA00B)
- IL-1 β (mouse): Mouse IL-1beta/IL-1F2 Quantikine ELISA, R&D (MLB00C)
- S100A9 (mouse): Mouse S100A9 Duoset ELISA, R&D (DY2065)
- Lcn-2 (mouse): Mouse Lcn-2 Quantikine ELISA R&S (MLCN20)
- Leptin (mouse): Mouse Leptine Quantikine ELISA, R&D (MOB00)
- Adiponectin (mouse): Mouse Adiponection/Acrp30 Quantikine ELISA (MRP300)

**Flow cytometry**

**Blood**

Blood samples were collected in EDTA tubes (0.5ml maximum per tube) for avoiding clots. Depending on the volume of blood, the samples were mixed with 3-6ml of Red Lysis Buffer (Sigma-Aldrich) and incubated for 10 minutes at RT. Then, the samples were transferred to a 96 wells plate and blocked with wash buffer (PBS + 5% FBS + 0.5% EDTA 0.5M) and mouse Fc block CD16/32 at 1:200 (BD Pharmingen) for 20-30 minutes. After 300G 10 minutes centrifugation at 4ºC, the samples were stained with the antibodies for 45-60 minutes, washed with PBS 2 x 5 min and filtered through a filtering mesh before adding DAPI and running the samples in the analyzer/sorter.

Compensation beads were prepared (OneComp eBeads, Invitrogen-Thermo Fisher Scientific) and the same antibodies used for the panel.

Following antibodies and colors were used at 1:200 (B220 APC-Cy7 1:100):
Papilloma

After their isolation, a small piece of the tumor (1 gram) was cut into small pieces and put at 37ºC with shaker for 1 hour in digestion buffer (serum-free RPMI Medium 1640 containing Liberase TL (Roche) at 200µg/ml). To improve the efficiency of the isolation, mechanical digestion was also performed with a 1ml pipette. Then, the single-cell suspension was filtered through a 70µm strainer with Wash Buffer. Afterwards, the samples were stained with the antibodies.

In situ hybridization

In situ hybridization analysis of critical hypothalamic factors were analyzed as previously described (García et al. 2003). Brains were isolated from calvarias and carefully posed upside down on a piece of foil in a dry ice box. The rest of the procedure was performed in Dr. Señarís lab (University of Santiago de Compostela).

Briefly, coronal brain sections (16 µm thick) were cut on a cryostat, mounted onto gelatin-coated slides, and immediately stored at -80 ºC until hybridization. AgRP, NPY and POMC mRNA levels were determined in the arcuate nucleus, whereas prepro-OX and CRH mRNA levels were determined in the lateral hypothalamus and the paraventricular nucleus, respectively, using specific antisense oligodeoxynucleotide probes. These probes were 3’-end labelled with 35S-dATP using terminal deoxynucleotidyl transferase. The slides from the different genotypes were always exposed to the same auto-radiographic film and were analysed using ImageJ software. Due to AgRP and NPY co-expression in the same cells, the study was performed in serial sections of each brain. 2-4 slides (four sections each) were used per animal.
The probes used for the detection of NPY, AgRP, POMC, OX, CRH and TRH are listed below:

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<tr>
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</tr>
<tr>
<td>POMC</td>
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<td>Prepr o-OX</td>
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</tr>
<tr>
<td>CRH</td>
<td>5'AGGTTGGAAGGTGAGATCCAGAGAGATGGGCGGCTCTCGGACCGCCT3'</td>
</tr>
<tr>
<td>TRH</td>
<td>5'-ATACCAGTTAGCAGAAAGATCAAGCCAGAGCCATCATCAGCCAA-3'</td>
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**Analytical Biochemical Technology**

**Liquid Chromatography/Mass Spectometry (LC/MS)**

Serum samples were collected from mice at end time-point in tubes with no anticoagulant. After 20 min centrifugation at 12000G at 4ºC, sera were transferred to a new tube and immediately freeze and stored at -80. The rest of the process was performed in “Attoquant Diagnostics”.

**Statistical Analysis**

If not indicated differently, data are mean ± sem (standard error of the mean). In the tables of the results, data are median ± sem.

When comparing 2 groups, two-tailed ttest was used. If the statistics included 3 groups, one-way ANOVA with Bonferroni correction and a comparison between all the three groups was used.

All the quantitative data were analyzed using GraphPad Prism5. Prism data graphs were imported to Adobe Illustrator CS4 (Adobe) for figure preparation. Pictures were analyzed using Photoshop CS4 (Adobe) and ImageJ software.
Results
Characterization of mouse models of CAC

K5-SOS Tg, C26 and CHX tumor-bearing mice develop CAC

The mouse models proposed for this study were validated as CAC models. Around 5 weeks of age the K5-SOS Tg mice, and 15 to 21 days post-injection the C26 and CHX transplantation models, cachectic mice had lost more than 15% of the body weight compared to controls (Figure R.1A). Loss of body weight involved a reduction in adipose tissue (subcutaneous and visceral depots) as well as in lean mass (Figure R1.D-F). Other organs were also measured; no significant differences in liver weight were detected (Figure R.1B), whereas spleens from cachectic mice were significantly enlarged in the three mouse models (Figure R.1C).

Figure R.1. Cachexia in K5-SOS Tg, C26 and CHX tumor-bearing mice. The three mouse models develop CAC in terms of body weight loss, adipose tissue atrophy and decrease in muscle mass. All weights were measured in grams and normalized to tibiae length (mm); n≥5.

Dual-Energy X-Ray Absorptiometry (DEXA) was used in K5-SOS Tg mice to determine if this imaging technique was sensitive enough to detect the loss of muscle and adipose tissue. Significant reduction of lean and fat mass was observed in cachectic mice compared to non-cachectic mice (Figure R.2A-B). Additionally, Bone Mineral Density (BMD) and Bone Mineral Concentration (BMC) were measured and both parameters were significantly decreased in mice with CAC (Figure R.2C-D).
Figure R.2. Decrease in lean and fat mass, BMD and BMC in K5-SOS Tg. Significant decrease in lean mass and adipose tissue was detected by DEXA in K5-SOS Tg mice compared to controls. Additional data from DEXA showed significant reduction in BMD and BMC in CAC; n≥10.

Anemia is frequent in cachectic patients and therefore some blood parameters were measured in the studied mouse models of CAC. Hematocrit was remarkably decreased in cachexia in all cases (Figure R.3A), and the number of erythrocytes was significantly reduced in the genetic model and in C26 tumor-bearing mice (Figure R.3B). Likewise, hemoglobin levels were significantly decreased in mice from K5-SOS Tg and C26 mouse models, but not in CHX mice (Figure R.3C).

According to the literature, an increase of pro-inflammatory circulating mediators such as TNF-α or IL-6 is a common feature in many models of CAC. In the mouse models analyzed here, IL-6 was significantly increased in CHX mice and tended to be increased in the other models as well (Figure R.4B). TNF-α levels were very low in controls and cachectic tumor-bearing mice, and only significantly increased in the genetic model. Other circulatory mediators, including G-CSF and Lcn-2, were measured by ELISA. Both peptides were significantly upregulated in sera from K5-SOS Tg GEMM and in the CHX transplantation mouse model (Figure R.4D,E).

Since adipose tissue loss is often accompanied by a decrease in circulating adipokines, leptin and adiponectin levels were measured. As expected, the results of the ELISA revealed a significant reduction in plasma leptin and adiponectin levels (Figure R.4CD).
Figure R.4. Levels of circulating mediators in three models of CAC. Circulating mediators including cytokines and adipokines were measured by ELISA in sera from the three models of CAC; n≥3.

All these data confirmed the development of CAC in all the three proposed mouse models in terms of loss of weight, adipose and muscle mass, together with some typical features of the syndrome such as anemia. Additionally, levels of adipokines were significantly reduced whereas pro-inflammatory markers were strongly increased. Next, we used these three mouse models to identify systemic hallmarks in CAC.

**Immune response in CAC**

Immune-circulating cells in the blood are altered in CAC

As pro-inflammatory cytokine levels were found higher in the circulation of CAC mice, analysis of White Blood Cells (WBC) was performed at the end point (Figure R.5A). The proportion of neutrophils in the blood, identified as CD11b+; Ly6Ghi cells by flow cytometry, was dramatically higher in CAC at the expense of other cell types like lymphocytes (Figure R.5B). Additionally, cachectic mice showed more variable expression levels of Ly6G and CD11b, suggesting that neutrophils are more heterogeneous in CAC (Figure R.5C).

Neutrophils are the vast majority granulocytic population. By the hematology analyzer, an increase in total number of granulocytes was found, as well as a decrease in lymphocytes (Figure R.5D-E). Granulocyte-Lymphocyte Ratio (GLR), the ratio between granulocytes and lymphocytes, is a good indicator to analyze the condition of
a tumor-bearing host and was significantly increased in mice with CAC in all three models (Figure R.5F). Importantly, high levels of GLR correlated with low carcass weight (Figure R.5G-I).

Figure R.5. Immune populations from the blood are altered in CAC. Total WBC, granulocyte and lymphocyte numbers were measured with the hematology analyzer and the GLR was calculated with those parameters. (B-C) Flow cytometry analysis in the blood allowed the identification of different subtypes of lymphocytes (T and B cells) and neutrophils; data showed in percentage. (G-I) Correlation between carcass weight (body weight minus tumor weight) and GLR; n>4.

Distribution of immune cells

Other tissues apart from blood were analyzed to determine the inflammatory status. As previously mentioned, cachectic mice develop splenomegaly, which is potentially a sign of systemic inflammation. By Hematoxylin-Eosin staining (H-E), differences in histology were detected. White pulp and follicles were reduced in size and number, and more megakaryocytes were identified in CAC (Figure R.6A). By flow cytometry analysis of the immune cell numbers in the spleen, we found similar results as in blood (data not shown).

Tumor formation is the primary event in CAC triggering the inflammatory reaction by secreting molecular mediators. By H-E staining, the infiltration of immune cells was not evident but occurred in some necrotic areas in the transplantation tumor models (Figure R.6B).
Livers from the *K5*-SOS Tg mice showed a dramatic tissue infiltration of CD45 positive cells arising in the portal areas at the last time-point. Although a mild increase was observed in the transplantation models, that was not significant, suggesting this might be an exacerbation of the system in the *K5*-SOS Tg model (Figure R.6C).

**Figure R.6. Spleen, tumor and liver at histological level.** H-E (X4) from spleen and tumors in the three mouse models of CAC. (C) Percentage of CD45+ cells in livers from the three mouse models calculated by referring positive to negative CD45 cells (IHC staining) in 10 high-power fields (HPF).

**Changes in White Blood Cells (WBC) is an early event in CAC**

In order to know if the imbalance in immune populations was an early event in CAC development, immune cells were also measured at earlier time-points. In particular, a “pre-cachectic” stage was established when less than 50% of gonadal adipose tissue was lost and no changes in skeletal muscle and total body weight were detected at necropsy (Figure R.7A-C).

Importantly, the data showed that at pre-cachexia, granulocytes were already upregulated (Figure R.7G). This finding is extremely important as the granulocytes increase in the blood preceded loss of muscle, suggesting that immune system alteration is an early event in CAC. GLR tended to be increased in the transplantation mouse models (Figure R.7F). GLR did not increase in *K5*-SOS Tg mice because lymphocyte levels were significantly higher (Figure R.7H).
Figure R.7. Immune cells in early CAC. Cachectic phenotype was not established yet but changes in immune cells were already detected in pre-cachexia, as measured by the hematology analyzer; n≥6.

Reduced immune response correlates with reduced cachexia

To know if the observed changes in inflammation and immune cells were due to CAC or the tumor itself, C26 cells knockdown for IL-6 were used. In this experiment, F1 (Balb/c X DBA) mice were injected with C26 wild-type cells and with C26 cells deficient in IL-6 production (shIL-6).

At the endpoint of the experiment, the levels of IL-6 detected in serum from shIL-6 C26 injected mice were significantly lower compared to those from mice injected the wild-type cells (Figure R.8I). shIL-6 C26 cells tended to induce less CAC than wild-type C26 cells, in terms of decrease body weight, adipose tissue and skeletal muscle mass loss, as previously described (Petruzelli et al. 2014) (Figure R.8A-H). Interestingly, GLR was reduced as well due to a decrease in granulocytes.
Figure R.8. Milder inflammation in milder CAC. Mice injected with shIL-6 C26 cells improved loss of weight compared to wild-type C26 tumor-bearing mice. Levels of IL-6 in sera of all 3 groups indicate that shIL-6 C26 cells do not induce IL-6 production as high as in the cachectic group; n≥2.

Overall, we can conclude that granulocytes are increased while lymphocytes are decreased in the blood of mice with CAC. This correlates with cachexia degree since the mouse blood GLR is reduced in pre-cachexia and after injection of the IL-6-silenced non-cachectic version of the C26 cancer cell line. Since GLR is consistently higher in CAC, we wondered whether the proportion of leukocytes might be important. To investigate the potential relevance of granulocytes (neutrophils, as the principal component) and lymphocytes as in CAC development, different strategies were used.

The role granulocytes and neutrophils in CAC

Granulocytes are consistently increased in the three mouse models of CAC and, as shown in the figure R.5B, the proportion of neutrophils (CD11b+; Ly6G+) were dramatically increase in blood of cachectic mice. By using two models of neutropenia, we investigated the potential role of neutrophils in CAC.

Neutrophil blockade

K5-SOS Tg mice were treated with an anti-Gr-1 antibody for 2 weeks (i.p. administration), while PBS was administered to control tumor-bearing mice. After several pilot experiments, the most accurate dosage for these mice was established.
with 2 injections per week. Neutrophil depletion was validated by blood test and by flow cytometry analysis in the spleen (Figure R.9E-F). Ly6B.2 was used for the identification of neutrophils, as anti-Gr-1 targets the cell membrane molecules Ly6G and Ly6C.

Figure R.9. AntiGr-1 treatment induces a reduction in lymphocytes. (A-D). Cell blood count determined by hematology analyzer showed decrease number of total granulocytes and lymphocytes. Flow cytometry from the spleen revealed the anti-Gr-1 induced reduction in the percentage of CD11b+Ly6B.2+ cells and also the significant decrease in T cells, in particular CD8 T cells; n≥4.

Surprisingly, and although there were significantly fewer circulating leukocytes in antiGr1 treated mice compared to the vehicle group, (Figure R.9A), GLR was not decreased in blood of those mice. This can be explained by the significant reduction in lymphocytes (Figure R.9H-J), suggesting that there might be a collateral effect of the treatment in the lymphoid population. Circulating mediators (Tnf-α, IL-6, Lcn-2) show similar levels between the groups (Figure R.10).
No differences in the circulating mediators were detected in anti-Gr-1 treated mice. TNF-α, IL-6 and Lcn-2 levels measured by ELISA in sera; n=4.

No differences regarding total body weight, spleen size, adipose tissue or muscle mass were found regarding changes in weight (Figure R.11). Interestingly, tumors were smaller in mice treated with antiGr1 compared to the vehicle-K5-SOS Tg mice at end stage, and the appearance of the tumor was also different (Figure R.11B).

Regarding the immune cells infiltrating the tumor, no differences in percentage were detected in Ly6B.2+ cells (Figure R.11E) and myeloid CD11b+ cells (Figure R.11D), while no myeloid (CD11b-) cells tended to be higher (Figure R.11B). Total CD45+ cells were significantly lower in tumors from antiGr1 treated mice (Figure R.11A), indicating that the tumor phenotype, including the immune microenvironment, was affected by the systemic treatment against Ly6G+ and Ly6C+ cells.
Figure R.11. Infiltration of non-myeloid cells are increased in anti-Gr-1 treated mice. Macroscopic pictures of tumors from anti-Gr-1 treated and vehicle treated K5-SOS Tg mice. Immune population in the tumor was analyzed by flow cytometry; n≥4.

CSF in CAC

G-CSF levels were higher in cachectic mice compared to controls, which might explain the increase in neutrophils, and granulocytes. In order to know if G-CSF signaling was important in CAC in Csf3r (-/-) mice were injected with CHX cells. Levels of granulocytes were decreased as expected, compared to cachectic wild-type mice (Figure R.12D). Regarding CAC features, no differences in body weight loss was detected in the Csf3r (-/-) (Figure R.12A). Further analysis of necropsy data and others need to be performed to correctly interpret the data.

Figure R.12. Csf3r (-/-) tumor-bearing develop CAC. By the hematology analyzer the granulocyte levels were lower than control cachectic mice, as expected. Loss of body weight (carcass-initial body weight) did not change in knock-out mice. Statistical analysis between tumor bearing mice; n≥6.

Overall, the experiments with the two neutropenic mouse models suggested that the number of neutrophils is not causative of CAC. However, as neutrophils become the most prominent population of WBC in the blood in CAC, neutrophil-derived molecules...
might play a role in CAC. To assess the contribution of neutrophil-related circulating mediators in CAC, we explored the role of S100a9 and Lcn-2.

**S100a9 in CAC**

S100a9 is an alarmin constitutively expressed in neutrophils and monocytes that plays an important role in modulating inflammatory response by stimulating leukocyte recruitment and inducing cytokine secretion. S100a9 was measured by ELISA in sera of GEMM and transplantation cachectic mice. However, the obtained measurements were under detection level of the kit used in both controls and mice with CAC (data not shown). As granulocytosis and neutrophilia were consistent findings in the mouse models of CAC, we assessed the role of S100a9 in cachexia progression by injecting CHX cells in S100a9 (-/-) mice. Interestingly, S100a9 (-/-) mice showed an increase in immune cells in the blood and the lymphocytic and granulocytic populations tended to be enlarged (Figure R.13H). GLR did not change (Figure R.13D).

![Figure R.13: Total leukocytes and lymphocytes tended to increase in circulation of cachectic S100a9 (-/-) mice compared to wild-type tumor-bearing mice by hematology analyzer; n≥3.](image)

No significant differences in body weight loss were detected between control and S100a9 (-/-)-injected mice (Figure R.14A). Spleens of the S100a9 (-/-) cachectic mice tended to be smaller (Figure R.14B) while gonadal adipose tissue weight tended to be higher (Figure R.14D,E) at end time-point.
This experiment suggests that S100a9 is not relevant in CAC. Due to the lack of major differences and to sample limitations, \( S100a9 \) was not measured in these experiments.

**Lcn-2 in CAC**

As shown in Figure R.4E, circulating Lcn-2 levels were increase in CAC. Lcn-2 is an alarmin and also an adipokine with numerous functions, mainly related to iron, innate immunity, and metabolism. Therefore, Lcn-2 might represent another circulating inflammation-related mediator relevant for CAC.

In pre-cachexia there were no significant changes in circulating Lcn-2, although the levels were already higher in the majority of mice in \( K5\text{-SOS Tg} \) and CHX tumor-bearing mice at this early time-point (Figure R.15A). Interestingly, in mice injected with shIL-6 C26 cells Lcn-2 levels were intermediate between cachectic C26 tumor-bearing mice and controls (Figure R.15B).
Figure R.15. Lcn-2 is upregulated in CAC. Lcn-2 levels were higher in pre-cachexia compared to controls and was diminished in shIL-6 C26 tumor-bearing mice, as detected by ELISA. In the liver, IHC staining showed a massive upregulation in Lcn-2 levels mainly in hepatocytes of cachectic mice of the three models, compared to control mice (F-I). Lcn-2 expression levels were confirmed by qPCR (D-E).

Interestingly, Lcn-2 expression was particularly upregulated in the liver of CAC mice as detected by qPCR (Figure R.15C-D) and IHC (Figure R.15E-H). Thus, Lcn-2 is highly upregulated in CAC. In order to understand if Lcn-2 is necessary for CAC development, transplantation experiments in Lcn-2 (-/-) mice were carried out.

Lcn-2 (-/-) mice were injected with CHX fibrosarcoma cells at 9-10 weeks of age and sacrificed 18 days post-injection. Some experimental mice reached the humane endpoint at 15-16 days post-injection, thus they were sacrificed earlier. Lcn-2 (+/+) and Lcn-2 (-/-) tumor-bearing mice showed similar values concerning body weight loss, tumor size, spleen or muscle weight (Figure R.16 A-C, F). Regarding fat mass, big variability was detected in iWAT and gWAT weights from knock-out mice compared to controls (Figure R.16D-E).
Figure R.16. Lcn2 (-/-) develop CAC. No differences in any of the parameters of CAC at necropsy and no changes in the immune cell proportion in the blood measured by the hematology analyzer were detected in Lcn2 (-/-) cachectic mice compared to controls; n≥3.

The levels of IL-6 and TNF-α remained the same in mice from the two genotypes (Figure R.17A-B). By ELISA, circulating Lcn-2 levels detected in the sera of Lcn-2 (-/-) mice were significantly lower compared to Lcn-2 (+/+). (Figure R.17C). This suggests that most of Lcn-2 in CHX-mediated CAC model is produced by the host.

All these data suggest that Lcn-2 and S100A9 are not necessary for CAC.

Figure R.17. Circulating mediators in sera of cachectic Lcn-2 (-/-) mice and wild-type tumor bearing mice. Similar levels in circulating pro-inflammatory mediators were detected by ELISA; n≥3.
The role of lymphocytes in CAC

Lymphocyte blockade

As previously shown, lymphocytes are dramatically decreased in both transplantation mouse models and tend to decrease also in the K5-SOSTg mice. This finding, together with the increase in the population of granulocytes, ended up in an increase GLR under CAC conditions. Since the decrease in lymphocytes was associated with the cachectic phenotype, we sought to determine whether CAC might be modified in the absence of mature lymphocytes. To answer this question, CHX fibrosarcoma cells were injected in Rag1 (-/-) mice. Mice were injected at 9-12 weeks of age, and sacrificed 18 days later, although some mice were euthanized earlier for humane reasons.

Figure R.18. GLR is significantly reduced in cachectic Rag1 (-/-) mice compared to cachectic wild-types. Lymphocytes were significantly reduced as detected by the hematology analyzer; n≥4.

Regarding CAC, Rag1 (-/-) mice and wild-type tumor-bearing mice showed a similar result in terms of body weight, tumor size, inguinal adipose tissue weight or skeletal muscle weight at the end time-point (Figure R.19A, C, E, F). gWAT weight tended to be preserved in the Rag1 (-/-) tumor-bearing mice but no significant difference was found (Figure R.19E). The spleens from the cachectic Rag1 mutant mice were lighter than controls with CAC (Figure R.19B) but this phenotype is inherent to the absence of B and T cells also in non-pathological conditions.
Figure R.19. *Rag1* -/- develop CAC in similarly to wild-type controls. Body weight loss was calculated considering the initial body weight of each mouse. At necropsy no differences in CAC parameters were detected; n≥4.

No changes in IL-6, TNF-α or G-CSF circulating serum levels were detected between *rag1* (-/-) and wild-type mice with CAC (Figure R.20). These data suggested that low levels of lymphocytes are neither harmful nor beneficial for CAC, and that mature B and T cells do not represent a significant source of pro-cachectic inflammatory mediators.

Figure R.20. Classical cachectic mediators showed no significant differences in *rag1* (-/-) cachectic mice. IL-6 and TNF-α levels, measured by ELISA, were similar in cachectic mice with or without mature lymphocytes; n≥3.
The role of the central nervous system and neuroendocrine axis in CAC

Hypothalamus in CAC

Hypothalamus is the center of the appetite regulation and it is involved in the main hormonal pathways of stress and metabolic system. Brains and pituitary glands from K5-SOS Tg mice and CHX tumor-bearing mice with their respective controls were analyzed in order to understand the role of the Central Nervous System in CAC. The data showed an increase in expression of orexigenic neuropeptides NPY and AgRP as well as significant decrease levels of the anorexigenic POMC in K5-SOS Tg (Figure R.21A), while POMC and AgRP are already altered at early time-points in the K5-SOS Tg mice (Figure R.21B). Importantly, the same patter was reproduced in CHX cachectic mice (Figure R.21C). Together with the low levels of leptin and adiponectin, these data suggested that the hypothalamus is receiving hunger signals in CAC, although this activation does not lead to an increase in the total body weight. Experiments with metabolic cages for measuring food etc would be needed for study feeding behavior.

Figure R.21. Orexigenic signals in the hypothalamus of the K5-SOS Tg and CHX. NPY and AgRP were increased in K5-SOS Tg by in situ hybridization, and POMC was decreased, which indicate an activation of the hunger pathway; n≥3.
Hypothalamic-Pituitary-Axis (HPA)

Corticotropic-Release Hormone (CRH) did not change in CHX mouse model but was mildly elevated in K5-SOS Tg mice (Figure R.21AB). This made us analyze more into detail the pathway leading to corticosterone production.

Expression levels of the corticosterone synthase (CYP11b1) were significantly increased in adrenal glands from K5-SOS Tg and tended to be upregulated in CHX-injected mice (Figure R.22A). Additionally, blood samples obtained at different time-points in both mouse models were analyzed by Liquid Chromatography/Mass Spectrometry (LC/MS). Corticosterone levels showed a significant upregulation in sera of K5-SOS Tg cachectic mice at 5 weeks, but not at 8 weeks, and also in the CHX transplantation mouse model in the cachectic and pre-cachectic time point (Figure R.22C). The increased levels of corticosterone reflect an activation of the stress pathway. Since the high levels of the corticosterone in the blood at pre-cachexia might act as a negative loop, this might explain the lower levels at the cachexia stage thus the levels in cachexia are lower.

Figure R.23. Activation of the adrenal glands in CAC. In K5-SOS Tg model the data relative to adrenal glands were measured at three different time-points (3, 5 and 8 weeks); qPCR CHX Cx CYP11b1 n≥2. Rest n≥5.

For these experiments, and since adrenal glands are not completely mature until the week 7 (Bielohuby et al. 2007), different time points were used: 3 weeks, considered as pre-cachexia, and 5 and 8 weeks were chosen as different points of cachexia. Additionally, due to the potential differences regarding the gender, only the data from males will be shown in this chapter.
Renin-Angiotensin-Aldosterone System (RAAS)

Data on the previous block of results indicate an increased activity of the fasciculate layer of the adrenal gland cortex. Aldosterone is another steroid also synthetized in adrenal glands and it is upregulated in cardiac cachexia. Blood LC/MS analysis revealed that aldosterone was significantly higher in sera from cachectic mice at 5 and 8 weeks compared to age-matched control mice. Those data were validated by qPCR analysis of aldosterone synthase expression levels (Figure R.22B, D). Likewise, CHX adrenal glands and sera were analyzed but no significant differences were detected. As aldosterone is the last peptide of the very complex Renin-Angiotensin-Aldosterone System, intermediate peptides of RAAS were analyzed in blood of those two mouse models by using same methodology (LC/MS). The RAAS system, which controls a plethora of physiological systemic events such as vasodilation/vasoconstriction in response to internal or external stimuli, is extremely sensitive and difficult to measure, therefore the variability was remarkable in some cases.

At early timepoints (3 and 5 weeks), no differences in Ang I were detected in the K5-SOS Tg mice. However, CHX tumor-bearing mice showed significant differences not only in Ang I, and Ang IV, as well as in Ang (1-5) in pre-cachexia compared to controls (see Table I1, Introduction). At end-stage, Ang (1-7) was significantly upregulated in both mouse models; Ang I and Ang II were specifically increased in CHX transplantation model (Figure R.23).
Figure R.23. Increased intermediate peptides of RAAS in mouse models of CAC. Ang II tended to be upregulated in K5-SOS Tg (A, B, C), and it is significantly upregulated in the CHX mouse model as well as Ang I, also upregulated in pre-cachexia (G). Other intermediate peptides tend to be also upregulated.

The activity of the main enzymes involved in this pathway can be determined by calculating the ratio between specific peptides. The ratio between Ang II/ Ang I, which indicates the activity of Angiotensin-Converting Enzyme (ACE), was significantly higher in K5-SOS Tg cachectic mice but not in CHX mice; another ratio, between Ang (1-5)/ Ang (1-7), was increased in cachectic CHX mice (Table R.1). Additionally, Plasma-Renin Activity (PRA) was also increased in those CHX mice, even at pre-cachexia.

Together with the levels of the intermediate peptides of the RAAS and the high levels of aldosterone, these data indicate that RAAS is activated in CAC. RAAS activation causes an increase in blood volume and vasoconstriction. It is the basic system for regulating blood pressure and thus, it is closely related to heart physiology.

Table R.1. ACE activity is increased in mouse CAC. Enzyme activity was calculated as the ratio of intermediate peptides of RAAS. PRA: Plasma Renin Activity; ACE1-S: Angiotensin-
Converting Enzyme1 activity; ACE2: Angiotensin-Converting Enzyme1 activity; AA2Ratio: Aldosterone/Angiotensin II ratio.

<table>
<thead>
<tr>
<th></th>
<th>PRA-S</th>
<th>ACE-S</th>
<th>ACE-S</th>
<th>AA2 Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(AngI+ Ang II)</td>
<td>(AngII/ Ang I)</td>
<td>(Ang 1-5/ Ang 1-7)</td>
<td>(Aldo/Ang II)</td>
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<tr>
<td><strong>control</strong></td>
<td></td>
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<tr>
<td><strong>3 weeks</strong></td>
<td></td>
<td></td>
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<tr>
<td>K5-SOS Tg</td>
<td>377.8 ± 74.09</td>
<td>1.54 ± 0.51</td>
<td>2.86 ± 0.64</td>
<td>0.85 ± 0.45</td>
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<tr>
<td>p value</td>
<td>0.349</td>
<td>0.914</td>
<td>0.565</td>
<td>0.084</td>
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<td><strong>5 weeks</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>K5-SOS Tg</td>
<td>602 ± 76.83</td>
<td>1.71 ± 0.22</td>
<td>5.58 ± 1</td>
<td>0.72 ± 0.14</td>
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<tr>
<td>p value</td>
<td>0.108</td>
<td>0.018</td>
<td>0.494</td>
<td>0.371</td>
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<td><strong>8 weeks</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>K5-SOS Tg</td>
<td>1184.8 ± 164.73</td>
<td>2.38 ± 0.26</td>
<td>8.43 ± 1.18</td>
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<tr>
<td>p value</td>
<td>0.129</td>
<td>0.043</td>
<td>0.998</td>
<td>0.002</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>PRA-S</th>
<th>ACE-S</th>
<th>ACE-S</th>
<th>AA2 Ratio</th>
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<tr>
<td></td>
<td>(AngI+ Ang II)</td>
<td>(AngII/ Ang I)</td>
<td>(Ang 1-5/ Ang 1-7)</td>
<td>(Aldo/Ang II)</td>
</tr>
<tr>
<td><strong>control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>preCx</strong></td>
<td>1917.7 ± 448.63</td>
<td>0.2 ± 0.05</td>
<td>7.9 ± 1.21</td>
<td>0.5 ± 0.14</td>
</tr>
<tr>
<td><strong>preCx - CHX</strong></td>
<td>8565.6 ± 1116.93</td>
<td>0.1 ± 0.04</td>
<td>7.5 ± 1.57</td>
<td>0.3 ± 0.03</td>
</tr>
<tr>
<td>p value preCx - CHX</td>
<td>6.7 * 10^{-7}</td>
<td>0.47</td>
<td>0.914</td>
<td>0.054</td>
</tr>
</tbody>
</table>

In order to know if the differences in RAAS peptides between cachectic and control mice were a consequence of a heart phenotype, echocardiography of the K5-SOS Tg and CHX mouse models was performed.

Hearts from cachectic mice had increased systolic and diastolic areas as well as increased left ventricle volume (Figure R.23B, D, E). The shortening fraction was decreased in the K5-SOS Tg mice (Figure R.23C), which also had heavier hearts (Figure R.23A) and a decreased ejection fraction (Figure R.23I) indicating heart dysfunction.
Figure R.23. Heart function is impaired in CAC. Cardiac functionality was analyzed by ultrasounds. Left ventricle measurements, including systolic and diastolic areas and volume, were increased in cachectic mice. The ejection fraction was preserved in CHX but dramatically reduced in K5-SOS Tg mice; n≥2.

Cachectic patients: RAAS and cytokines

In collaboration with the University of Vienna and Attoquant Diagnosis, sera from human patients were collected and analyzed by LC/MS to determine the relevance of those findings in human cancer samples. The available samples corresponded to patients diagnosed with “tumor and no cachexia”, “pre-cachexia” and “cachexia”, according to the clinical criteria regarding body mass. The patients had pancreatic, pulmonary and kidney adenocarcinomas. 5 patients per group were analyzed for proof of concept. The main limitation of this experiment with human samples was the high variability, which is somehow inherent to the system, due to its high sensitivity. Besides, the different values observed might be also due to the sampling, as the groups were very heterogeneous in many parameters such as gender, age, treatment etc. However, valuable results were obtained from the analysis of these samples.

When analyzed together by pooling the three tumor types at different time-points, no differences were detected in the main peptides of the system. The data were analyzed separately by organ-tumor type and stage of the disease according to clinical criteria.
Ang I and Ang II circulating levels were increased in from pancreatic and lung cancer patients with were increased in CAC compared to those without cachexia. Group heterogeneity and sensitivity of the system resulted in a remarkable variability, so these promising results should be further validated in bigger cohorts (Figure R.24AB).

**Figure R.24.** Very high variability in the RAAS in different tumor types. (A) Angiotensin I showed no differences referred to the timing of the condition; n=5. (B) Angiotensin II levels tended to be higher in the last time-point of the disease, although no significance was detected; n=5. (C) Regarding aldosterone levels, no differences were detected in the sera from the three tumor types; n=5.

**Table 2.** Ratios from of RAAS in cachectic cancer patients. PRA: Plasma Renin Activity; ACE1-S: Angiotensin-Converting Enzyme1 activity; ACE-S: Angiotensin-Converting Enzyme1 activity; AA2Ratio: Aldosterone/Angiotensin II ratio.
Sera from patients with kidney cancer showed completely different pattern regarding most of the peptides, which could be easily explained by the essential role of kidney in the systemic RAAS (Figure R.25C, F). No differences in aldosterone were detected (Figure R.25G).

Add IL-6 and G-CSF were analyzed by ELISA in those patients. No differences in G-CSF were detected in CAC compared to tumor-bearing patients with no cachexia; on the contrary, IL-6 was upregulated in of the groups regarding tumor type (Figure R.25).

<table>
<thead>
<tr>
<th></th>
<th>PRA</th>
<th>ACE (II/I)</th>
<th>ACE</th>
<th>AA2 ratio</th>
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</thead>
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<tr>
<td></td>
<td>(Ang II+ Ang I)</td>
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<td>(Ang 1-5/Ang 1-7)</td>
<td>(Aldo/AngII)</td>
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<tr>
<td>Pancreas</td>
<td>NCx</td>
<td>89,4 ± 40,59</td>
<td>1,025 ± 0,48</td>
<td>0,74 ± 0,06</td>
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<td></td>
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<td>0,3679</td>
<td>0,5807</td>
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<td>Lung</td>
<td>NCx</td>
<td>197,19 ± 49,24</td>
<td>1,25 ± 0,3</td>
<td>0,88 ± 0,16</td>
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<td></td>
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<td>69,63 ± 15,93</td>
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<td>0,67 ± 0,07</td>
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<td></td>
<td>Cx</td>
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<td>0,647 ± 0,14</td>
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<tr>
<td><strong>p value</strong></td>
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<td>0,0255</td>
<td>0,5655</td>
<td>0,6648</td>
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<tr>
<td>Kidney</td>
<td>NCx</td>
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<td>1,56 ± 0,25</td>
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<td>314,03 ± 76,75</td>
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<td>1,42 ± 0,25</td>
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<td>0,0638</td>
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</tbody>
</table>

*PreCx - Cx*

**p value**

0,0498 0,0255 0,0324

**Figure R.25. Cytokines in human cancer patients.** Analyzed by ELISA, levels of IL-6 were significantly increased in cachectic patients of pancreatic and kidney tumors. No significant differences of G-CSG were detected between those groups n=
Discussion
CAC is an intricate metabolic syndrome secondary to tumors that impairs the quality of life of cancer patients and hinder the success of anticancer therapies. Sarcopenia and loss of muscle strength is the most evident feature of CAC. However, CAC affects the whole organism and thus other systems need to be studied.

**Models: essential tool in CAC research**

There is an urgent need of understanding the pathophysiology of CAC and to find accurate biomarkers in order to predict which patients will develop the syndrome. Few large-scale human trials are ongoing because patient recruitment and follow-up is extremely difficult, due to the severity of the disease. In addition to these limitations, CAC is a very heterogeneous entity, which implies large enrolment of patients in order to achieve adequate power and statistical meaning (DeBoer 2009).

As a systemic syndrome, cachexia research shall be conducted in whole organisms, and mouse models are excellent tools for studying CAC in the absence of large-scale human trials. The biological variability can be attenuated with higher numbers of mice and by using more specific strategies. To minimize the variability, several mouse models were analyzed in this work.

*K5-SOSTg* is a very fast mouse model, thus we can obtain big number of mice in short periods of time. *K5-SOSTg* show very dramatic CAC; this is an advantage of the model as it allows to identify new potential features of CAC that are not evident in other models. For instance, while there was a slight increase CD45+ cells in livers from transplantation models, the percentage of the immune population was more than double in the *K5-SOS* Tg.

*C26* transplantation mouse model is widely accepted as mouse model of CAC in the field. It is well documented that the model is IL-6 dependent, as shIL-6 cells induce milder phenotype (Petruzzelli et al. 2014). C26 colorectal cancer cell line was originated in mice from Balb/c strain, therefore it could not be used in most of the GEMMs, which have C57BL/6 background. Several experiments were performed in B16 melanoma mouse model (Petruzzelli et al. 2014; Voltarelli et al. 2017); unfortunately, tumors overgrew too fast and it was not possible to describe the cachectic phenotype before mouse humane end-point. Thanks to our collaborators, CHX fibrosarcoma model was used in this work to induce CAC in GEMMs. This new xenograft model of cachexia provided me with new alternatives since I can implant these cells in C57BL/6 mice such as the knockout mice used in this thesis.
CAC without the intervention of immune cells

Systemic inflammation with certain degree of immunosuppression (adaptive immunity) is common in both human and murine CAC (Argilés et al. 2011), and might be comparable up to some extent. It is widely accepted that inflammation is an underlying common factor shared by diverse cachetogenic diseases, and that pro-inflammatory cytokines and peptides are relevant in the development of the wasting syndrome. Besides, cancer and inflammation are closely linked, as some cancers arise from inflammatory sites and tumor microenvironment is substantially orchestrated by inflammatory cells (Coussens and Werb 2002). This and the negative correlation between the GLR and body weights observed in my experiments which are consistent with published work on NLR (Derman et al. 2017), reinforced the hypothesis that inflammatory status and also immune system were critical for CAC development. To my knowledge there are no publications of CAC in immunodeficient mice excluding one paper inducing CAC in nude (Jones-Bolin and Ruggeri 2007) and another one inducing CAC in SCID mice (Murraya et al. 1997). Besides, pro-inflammatory mediators are known to be involved in CAC and to cause direct effects in target organs (eg muscle, adipose tissue), but very limited publications further investigate the potential role of immune cells in CAC.

Neutrophils

It is important to know that neutrophils are around 50-60% of all blood leukocytes in humans, while in mice this percentage is reduced to 15-20%. Still, neutrophils are the big majority of the total granulocytic population. According to the data obtained in the anti-Gr-1 experiment in K5-SOS Tg, neutrophil upregulation in circulation is rather a readout than a cause in CAC: neutrophil depletion (Ly6G+; Ly6C+), and additionally depletion of other Ly6C+ cells, demonstrated not to modify the cachectic phenotype. Considering the 50% of reduction of neutrophils (not complete depletion), an important observation needs to be noted though, regarding the significant reduction of the size and the different appearance/morphology of tumors. In the absence of Ly6G, neutrophil migration is impaired (Lee et al. 2013), and that might consequently impair angiogenesis (Tazzyman, Lewis and Murdoch 2009), which would explain tumor shrinking. This is a limitation for stating a robust conclusion out of the experiment and reflects, once again, the complexity of this syndrome; the treatment induces differences in the tumor but no differences in CAC. The data on the lack of effect over cachexia after anti-Gr-1 treatment was reproduced by a collaborator in an independent
Therefore, mature neutrophils demonstrated not to be essential in CAC development. Importantly, anti-Gr-1 treatment did not reduce the stimulus in bone marrow for releasing neutrophils and other cells. Thus, bone marrow might still overproduce cells in a very energetic demanding process. The receptor for G-CSF is expressed in granulocytic precursors, also in mature neutrophils (Panopoulos and Watowich 2008), and Csf3r (-/-) mice are neutropenic (Trikha and Carson 2014). These knockout mice were then subjected to the CHX-mediated CAC xenograft mouse model. CHX tumor-bearing Csf3r (-/-) mice showed less granulocytes even in cachexia condition. However, this did not prevent loss of weight, suggesting once more that the levels of granulocytes (and neutrophils, as the majority population) and thus, the increase in GLR, are likely consequences of CAC and not a cause. Nonetheless, one still open question is the immunometabolism profile of neutrophils. It might be that the released neutrophils from hematopoietic organs are immature (e.g. Ly6Gint vs. the mature Ly6Ghi) and/or not completely functional, and therefore these cells might cause a negative impact on CAC development. In addition, other granulocytic populations such as myeloid derived suppressor cells (MDSC) would also be affected by anti-Gr-1 treatment and the lack of Csf3r as part of the myeloid lineage, to further complicate the interpretation of the results. More detailed analysis of sorted neutrophils (Ly6C+; Ly6Ghi) will help to obtain more robust conclusions from the final role of these cells in the development of CAC, as well as to design future experiments.

**S100a9 and Lcn-2**

As part of the potential modifications of neutrophil phenotype in CAC, two neutrophil-related molecules were analyzed in this work. S100a9 demonstrated not to be essential in CAC development, although it represents around 45% of neutrophil cytosolic proteins, together with S100a8 in normal conditions (Schiopu and Cotoi 2013). S100a9 is a chemoattractant for neutrophils and it can be expressed in endothelial cells, thus S100a9 is important in neutrophil migration (Vandal et al. 2003; McNeill et al. 2007). No differences in tumor size were detected between cachectic S100a9 (-/-) and cachectic wild-type mice, suggesting that S100a9 is not essential for tumor growth. Interestingly, S100a9 (-/-) mice with CAC showed an increase in total circulating WBC, in which granulocytes and also lymphocytes tended to be upregulated as well. However, these high levels of leukocytes do not modify CAC, supporting the conclusion that the number of these immune populations are not essential in CAC development. Interestingly enough, S100a9 might be involved in cardiac inflammation and remodeling (Wu et al. 2014) and it is associated with
doxorubicin-induced cardiotoxicity in diabetic mice (Pei et al. 2016), therefore it could have an effect in the heart phenotype of CAC observed in K5-SOS Tg GEMM and CHX transplantation model. Additional experiments would be needed to determine the role of S100a9 in cardiac wasting in CAC.

Similarly to the previous experiment, Lcn-2 (-/-) tumor-bearing mice did not show amelioration of CAC compared to wild-type tumor-bearing mice. As in the case of S100a9, Lcn-2 is able to modulate neutrophil chemotaxis and cytokine secretion, being also important for neutrophil extravasation (Schroll et al. 2012), but any of these mechanisms did not affect the cachectic phenotype or tumor size. This suggests that Lcn-2 is not involved in CAC development. Notably, Lcn-2 (-/-) mice are described to show inflammatory and metabolic phenotype, including increased circulating insulin and cholesterol levels, bigger size of adipocytes, abnormal thermogenesis or impaired glucose tolerance. Although no differences in the analyzed parameters were detected, I did not include additional measurements regarding the mentioned features, that could mask some differences regarding the cachectic phenotype.

Nevertheless, in this work I demonstrate that Lcn-2 is highly upregulated in sera from cachectic mice, thus I propose Lcn-2 as a potential biomarker of CAC. Importantly, there is a striking increase of Lcn-2 expression in hepatocytes not related to liver cancer in all the three analyzed models. I hypothesize that liver detects systemic signals, eg circulating IL-6, which are able to induce changes in the expression pattern of hepatocytes, as suggested by Flint T. et al. (Flint et al. 2016). An alternative explanation could be the anemic status or an early stage of liver injury (Asimakopoulou, Weiskirchen and Weiskirchen 2016). Molecular signature analysis would be needed for the identification of mechanism involved in this dramatic upregulation of Lcn-2 in hepatocytes. By all means, this is the first time that Lcn-2 overexpression in liver is reported in CAC and further investigations in human and in animal models are deeply recommended.

Lymphocytes

Interestingly, antiGr-1 treatment induced a reduction in lymphocyte levels in K5-SOS Tg mice compared to controls, and a significant reduction of CD8 T cells in particular; this might be explained by the role of Ly6C in controlling CD8 T cells as also lymphocytes can express that antigen (Hänninen et al. 2011; Hickey 2018). As lymphopenia was a consistent feature in all the studied mouse models of CAC, lymphocytes might be protective in CAC.
Unexpectedly, having no mature lymphocytes did not aggravate the wasting syndrome, as shown in this work by inducing CAC in \textit{Rag1} (-/-). However, this experiment does not answer the question whether lymphocytes protects against CAC, as it was suggested by Joana Davis lab (Wang \textit{et al.} 2008). Experimentally, it would be necessary to induce CAC in a model of lymphocytosis or, ideally, to recover the normal levels of lymphocyte levels. According to Tisdale, non-Hodgking lymphomas usually do not lead to CAC in human patients (Tisdale 2009); still, a correlation between CAC and clinical outcomes was found in aggressive B-cell non-Hodgkin lymphoma (Karmali \textit{et al.} 2017). This might indicate the importance of studying lymphocyte subsets separately; thus specific techniques should be selected on order to obtain robust conclusions. In the context of lymphocytes, Flint \textit{et al} showed that the metabolic impairment in CAC could lead to the inefficiency of immunotherapy and more studies are needed to elucidate the interaction between metabolic impairment in CAC and lymphocytes (Flint \textit{et al.} 2016).

The experiments shown in this thesis demonstrated that there are mechanisms leading to CAC in which mature neutrophils or lymphocytes are not involved. My results indicate that CAC development is independent of the number of neutrophils and lymphocytes, whereas very likely this tumor-associated wasting syndrome induces the alterations in those immune cell populations. Thus, the hypothesis claiming that proportion of immune cells (increase in neutrophils and decrease in lymphocytes) is a cause of CAC, is necessarily refused by the data. This does not necessarily imply that other important phenotypic aspects of these and other immune cell populations are irrelevant for cancer and CAC, or for the patient prognosis. Besides, it is important to consider, that these approaches are not specific, and conditions cannot be controlled.

The data on this thesis suggest that the complexity of the CAC syndrome might require more precise and flexible tools to properly address the exact role of granulocytes and lymphocytes in the initiation or progression of this deadly systemic condition. \γδ\>-T cells are a subtype of T cells important for regulating adipocyte physiology: mice lacking \γδ\>-T cells are not able to normally regulate core body temperature at thermoneutrality and after cold challenge (Kohlgruber \textit{et al.} 2018). Consequently, \γδ\>-T cells might be involved in the abnormal metabolism in the adipose tissue in CAC, including lipolysis or browning.

Regarding other immune cells, macrophages are reported as important agents in adipose tissue homeostasis as well, and even were postulated as one of the causes of adipose tissue loss through lipolysis and browning through the secretion of catecholamine’s by the alternative activated macrophages (Qiu \textit{et al.} 2014), although
this theory has been now refused (Fischer et al. 2017). If monocytes or macrophages play a role in CAC development remains still unknown.

Is CAC orchestrated by neuro-endocrine system?

CAC is typically considered a problem of energy imbalance: according to the literature, adipose tissue is lost before loss of skeletal muscle (Petruzelli et al. 2014), suggesting that lipolysis is an early event for obtaining energy. Besides, during lipolysis triglycerol and FFA are also released, and those would be material for anabolism for the tumor.

Appetite / Anorexia

The implication of the brain is evident and was previously discussed (Grossberg, Scarlett and Marks 2011). Cachectic patients frequently suffer from anorexia. The triggers for anorexia can be derived from the tumor itself, directly causing dysphagia due to its location or by secreting anorexigenic factors, or derived from antitumoral therapies (Ezeoke and Morley 2015).

In this work, the signals detected in the hypothalamus from cachectic mire did not induced anorexia. Instead, levels from orexigenic peptides were increased, and a significant reduced expression of anorexigenic neuropeptides was detected in the hypothalamus from cachectic K5-SOS Tg mice. Leptin levels were significantly reduced in CAC, thus the hypothalamus response agree with leptin signaling. Indeed, cachectic K5-SOS Tg mice increase their food intake (Petruzelli et al. 2014), although hyperphagia does not lead to an increase body weight or an amelioration of the cachectic phenotype. At pre-cachexia, relative expression of these neuropeptides was similar in K5-SOS Tg, indicating that hunger pathway is activated even at early time-points in that GEMM. Likely, this work demonstrated an increase in AgRP and NPY levels and decrease in POMC in CHX cachectic mice compared to controls, as also occurs in other models like Lewis Lung Cancer (LCC) and C26 (Dwarkasing et al. 2014, 2015). According to the authors, food feeding does not necessarily correlate to the levels of these peptides from the melanocortin system, as C26 was hyperphagic but not LLC, and they proposed serotonin signaling as a potential mediator for anorexia in CAC conditions. Additionally, pro-inflammatory cytokines such as TNF-α, IL-6 or IL-1β are able to pass through the BBB, induce hypothalamic inflammation and lead to anorexia (Grossberg, Scarlett and Marks 2011). Importantly for this thesis, a recent paper published in Nature demonstrated that Lcn-2 is also able to cross the BBB and
suppress appetite through the activation of MC4R in hypothalamus (Mosialou et al. 2017). Thus, future experiments need to be performed to determine if Lcn-2 might play a similar role in CAC.

**Adrenal hormones**

Adrenal hormones are key regulators of systemic events (stress, blood pressure). Glucocorticoids are important mediators involved in the stress pathway and are essential in metabolism of glucose, proteins and adipose tissue. Significant high levels of corticosterone were detected in CAC in K5-SOS Tg and CHX cachectic mice, similarly to published data in sera from C26 cachectic mice (Rivadeneira et al. 1999). However, here we show that expression levels of CRH did not significantly change; this suggests that the trigger for corticosterone release might be the activation of sympathetic nervous system although a deeper analysis of HPA, as well as the levels of ACTH would be needed for a definitive conclusion. Importantly, the blockade of glucocorticoid receptor did not modify CAC in C26 (Rivadeneira et al. 1999), and in the Yoshida AH-130 rat model (Llovera et al. 1996), indicating that the effects derived from glucocorticoids are not essential in CAC development.

Aldosterone was reported as a potential new target for CAC (Springer et al. 2014), and the role of the RAAS, which leads to aldosterone secretion, was known to be important in CHF and cardiac cachexia (Ferrara et al. 2002). RAAS was activated K5-SOS Tg and CHX mouse models, which show statistical differences compared to controls in the majority of the analyzed peptides; similar data were published in humans (Penafuerte et al. 2016), in particular regarding Ang II levels. According to the literature, Ang II is able to directly promote muscle wasting (Cabello-Verrugio, Córdova and Salas 2012) and also via TNF-α through the TNFR-1 (Shen et al. 2017). Ang II also increases the generation of Reactive Oxidative Species (ROS) through the activation of caspases -3 and -8 in skeletal muscle (Cabello-Verrugio, Córdova and Salas 2012), and even induce anorexia (Du Bois et al. 2015; Penafuerte et al. 2016). Remarkably, RAAS is also activated in obesity; Ang II secretion from abdominal subcutaneous adipose tissue was demonstrated in obese individuals (Schütten et al. 2017). In vitro studies demonstrate that acute exposure to Ang II reduces lipolysis in human adipocytes (Goossens et al. 2007), and that lipogenesis and glucose oxidation can be modified by different blockers of RAAS blockers (Caminhoto et al. 2016).

Aldosterone values were higher in K5-SOS Tg and CHX transplantation mouse model at pre-cachectic time-point and tended to be also similarly increased in the last time-point, consistently with other models of CAC (Suzuki et al. 2013). Indeed, aldosterone
was postulated as an important factor in adipose dysfunction and muscle atrophy (Delafontaine and Yoshida 2016), hence future work is needed to better understand the potential role of aldosterone in CAC.

This work also reveals higher levels of other intermediate peptides from RAAS, in particular Ang (1-7), which is part of the alternative RAAS pathway. Although there it was proposed as a potential therapy in experimental models in cachexia (Ebner and von Haehling 2017), this is a novel input to the field as no data from the alternative pathway and Ang (1-7) has been previously reported in CAC. Ang (1-7) and Ang (1-5) are significantly upregulated in CAC at the end stage in CHX and in K5-SOS Tg mice. The alternative pathway was reported to have the opposite functions than the classical pathway, which main peptide is Ang II. I hypothesize that the system is switched to the alternative RAAS in order to counteract the negative effects of the classical pathway, mainly driven by Ang II.

Importantly, some publications relate the immune cells with RAAS (Crowley and Rudemiller 2017), and also that inhibition of Ang II provide anti-inflammatory effects (Suzuki et al. 2003; Dandona et al. 2007). Additionally, there are local RAAS components in tissues, such as ocular, renal and adipose tissue (Holappa 2015). What the contribution of those local mini-systems in the total of the organism is still to be determined, although some works indicate a local paracrine/autocrine signaling of Ang II, also based in the short lifespan of the peptide (Cabello-Verrugio, Córdova and Salas 2012).

Although additional research is needed, further confirmation of the contribution of RAAS to cachexia development would be of great impact since there are lots of therapeutic agents that interfere with this system as a first line treatments for hypertension and diverse cardiovascular diseases. The inhibition of RAAS may improve the clinical outcome in cachectic patients and consequently the quality of life and the response to the current cancer therapies.

The heart: another muscle in CAC

CAC is associated with cardiac wasting and heart failure rodent models such as the Yoshida AH-130 hepatoma rat model (Springer et al. 2014) and the C26 mouse model (Tian et al. 2010). Hearts from cachectic animals are published to be smaller, with reduced left ventricle areas and lighter, and are functionally impaired, in terms of a reduced heart ratio and shortening fraction. Not in compliance with those results, cachectic mice from my research showed an increase in diastolic and systolic areas, thus an increase in the left ventricle volumes in the GEMM and in at least one of the
transplantation models. K5-SOS Tg cachetic mice showed another particularity, which is an increase in heart weight. Besides, the tumor in those mice can reach 1/3 of the total body weight of the mouse, so I hypothesize that the heart needs to compensate tail enlargement. Due to the location and the volume of the tumor (in the tail), blood pressure could not be measured. It might be that the blood pressure is too high in these mice, that they have an additional heart valve disease.

The link between RAAS and heart data might be physiological, because of RAAS in blood vessels which modify the resistance to heart pumping, and hypervolemia due to aldosterone. More probably, RAAS is a consequence of heart impairment, which might be directly address by the same stimulus than skeletal muscle wasting. Research at earlier time points are needed to be further investigated to find what is the first event that causally leads to CAC.

CAC in human patients

Human samples are extremely precious samples in CAC as scarce number of clinical trials are ongoing. One of the challenges of human trials is regarding the variability in the patients, in terms not only of the cachetic status but also the tumor type and subtype as well as the treatments (cancer treatments and additional drugs for co-morbidities). In this work I show the analysis from a small cohort of human cancer patients. Pancreas and Lung cancer are highly associated to CAC; additionally, lungs are known to be important for systemic production of ACE, as kidney is the main producer of circulating renin. Thus, we selected these tumor types to analyze RAAS in CAC and to understand the differences associated to the location of the primary tumor.

The variability was remarkable. However, even considering the heterogeneity of the groups, we were able to identify the increase in the RAAS as the high levels of circulating IL-6 in the sera, suggesting that there are common factors not affected by other factors eg gender, age, treatment etc.

Future directions

CAC is quite a new research niche where many things still need to be done. It is a challenge due to the complexity of disease and all the limitations in research with animal models and in humans. But then again, it is urgent to advance in treatments, even if they are palliative, as no effective alternatives are available in clinics.

As important as finding therapies, identification of new biomarkers would be desirable to predict which cancer patients will develop the disease and which of them will not.
Basic Science is an essential tool for investigating those treatments, as well as *in vitro* experiments but mainly to understand the pathophysiology of the disease; animal models allow us to characterize the stages of the disease and even considering the limitations that would be extremely important for our comprehension of CAC.
Conclusions
1. Splenomegaly is a common feature in CAC as well as anemia. In circulation, percentage of neutrophils as well as total number of granulocytes are increased in CAC while lymphocytes are decreased in number. At early time points (pre-cachexia) a trend to a higher number of granulocytes and enlargement of spleens are observed.

2. GLR negatively correlates to carcass weight in mouse models of CAC. Tumor-bearing mice injected with shIL-6 knockdown C26 cells develop milder CAC and milder inflammation, with a reduction in the GLR ratio compared to cachectic mice injected with IL-6 proficient C26 cells.

3. Systemic circulating levels of Lcn-2 and G-CSF are increased in CAC.

4. No changes in body weight loss are observed in mice lacking several neutrophil-associated peptides including S100a9 (-/-), Csf3 R (-/-) and Lcn-2 (-/-) tumor-bearing mice compared to wild-type controls. Neutrophilia and granulocytosis are not necessary for CAC as anti-Gr1 treatment does not ameliorate loss of body weight, muscle and fat mass. However, the treatment induces a significant reduction in tumor size.

5. Lack of lymphocytes do not impair or ameliorate CAC severity as Rag-1 (-/-) tumor-bearing mice show similar parameters than controls.

6. Hypothalamus is altered in CAC. Orexigenic pathway is upregulated while anorexigenic peptides like POMC are decreased. Thyroid axis is not upregulated in CAC. Besides, CRH does not show an increase expression in hypothalamus although corticosterone synthesis and release are upregulated.

7. RAAS is active in CAC. There are increasing levels of Ang I and Ang II in sera from 2 mouse of CAC, as well as an increase in the peptide Ang (1-7), which suggests an activation of alternative RAAS as well. Same trend is observed in samples from pancreatic and lung cancer patients but not in kidney cancer patients.

8. *In vivo* experiments show increase left ventricle area and volume in CAC, indicating potential cardiac adaptation to changes in the circulation and demands of the organism.
Conclusiones
1. La esplenomegalia es una característica frecuente en CAC, así como la anemia. El porcentaje de neutrófilos circulantes, así como el número total de granulocitos se incrementa en CAC. En pre-caquexia ya se observa una tendencia hacia el aumento de granulocitos, así como un mayor volumen del bazo.

2. El GLR se correlaciona negativamente con el peso corporal en modelos de ratón de CAC. Ratones inyectados con células C26 silenciadas para IL-6 desarrollan menos CAC y menor grado de inflamación, con una reducción del GLR comparado con los ratones caquécticos C26.

3. Los valores de Lcn-2 y G-CSF se encuentran incrementados en suero en CAC.

4. No se observan cambios en el fenotipo de CAC cuando se induce el proceso en animales noqueados para péptidos asociados con neutrófilos, incluyendo S100a9 (-/-), Csf3 R (-/-) y Lcn-2 (-/-), comparados con los controles. La neutrofilia no es necesaria en CAC dado que el tratamiento con antiGr1 no mejora la pérdida de peso corporal, músculo o grasa. Sin embargo, el tratamiento induce una reducción significativa del tumor.

5. La ausencia de linfocitos no es beneficia ni empeora la gravedad de la CAC, ya que los ratones caquécticos Rag-1 (-/-) presentan valores similares a los controles.

6. El hipotálamo está alterado en CAC. La vía orexigénica está incrementada mientras que péptidos anoxigénicos como POMC están disminuidos. El eje tiroideo no está aumentado en CAC. Por otro lado, CRH no muestra una mayor expresión en el hipotálamo, a pesar de que se detecta un aumento de la síntesis y secreción de corticosterona.

7. El RAAS está activo en CAC: los niveles de Ang I y Ang II se incrementan en el suero de dos modelos murinos, así como los niveles de Ang (1-7), lo cual sugiere la activación del eje alternativo del RAAS. En muestras de pacientes con tumores pancreáticos y de pulmón se observa una tendencia similar, no así en pacientes con cáncer renal.

8. Experimentos in vivo muestran un incremento de áreas y volumen del ventrículo izquierdo en CAC, indicando una posible adaptación cardíaca a cambios en la circulación y necesidades del organismo.
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