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Role of Dietary Calcium and Dairy in Modulating Oxidative Stress, Inflammatory Stress and Lifespan

Antje Eerenstein Bruckbauer
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To the Graduate Council:

I am submitting herewith a dissertation written by Antje Eerenstein Bruckbauer entitled "Role of Dietary Calcium and Dairy in Modulating Oxidative Stress, Inflammatory Stress and Lifespan." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Nutritional Sciences.

Michael B. Zemel,, Major Professor

We have read this dissertation and recommend its acceptance:

Jung Han Kim, Guoxun Chen, Michael McEntee

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Role of Dietary Calcium and Dairy in Modulating Oxidative Stress, Inflammatory Stress and Lifespan

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Antje Eerenstein Bruckbauer

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I dedicate this work to my parents who taught me to set high goals,

and to my family and friends

who supported and motivated me over the past years to achieve those.

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Abstract

Oxidative stress and inflammatory stress have been implicated as a cause of tissue damage in multiple organ systems, leading to the development of chronic diseases such as obesity, diabetes, hypertony and atherosclerosis. They are also recognized as major factors contributing to the physiological process of aging. Previous studies have demonstrated that dietary calcium regulates reactive oxygen species production (ROS) production in adipocytes in vitro and in vivo, and inhibits adipocyte-derived inflammatory cytokine expression by suppression of calcitriol. In addition, high calcium diets modulate energy metabolism and partitioning between adipose tissue and muscle resulting in a decrease in fat storage and an increase in fat oxidation in muscle. Providing calcium in form of dairy appears to cause greater effects than supplemental calcium in both mice and humans, most likely mediated by additional components in dairy products such as branched-chain amino acids (BCAA) and angiotensin converting enzyme inhibitor (ACEi) peptides. In consideration of the multiple effects of dietary calcium and other components of dairy on adipocyte and muscle metabolism, a high density oligonucleotide microarray approach was used to identify common and differential pathways related to energy metabolism, inflammation and oxidative stress in adipose and muscle tissue in response to milk and milk components. In addition, considering the protective role of dietary calcium against oxidative and inflammatory stress, which otherwise accelerate the process of aging, a lifespan study in a mouse model of diet- induced obesity was conducted to evaluate the effects of dietary calcium from both non-dairy and dairy sources on mouse lifespan and on lifespan-related biomarkers. The results of this research confirm that calcium and BCAA contribute to the alteration of energy partitioning between adipose tissue and muscle and provide

new evidence for calcium independent effects of BCAA and ACEi in energy metabolism and inflammation. Further, present data demonstrate that milk diet attenuates adiposity, protects against muscle loss and reduces oxidative and inflammatory stress. Although these did not alter maximum lifespan, they significantly suppressed early mortality.

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Part One

Introduction

Elderly individuals (defined as the number of people over age 65) have been increasing worldwide, both as an absolute number and as a proportion of the total, for the past century. In the United States the number increased from 3.1 million in 1900 to 35.0 million in 2000, and according to the U.S. census Bureau projections this trend will continue as the first Baby Boomers turning 65 in 2011. It is projected that the older population will increase up to 72 million in 2030 and represent up to 20 percent of the total U.S. population. This increase is also accompanied by changes in the age composition of the population. The median population age, increased from 22.9 years in 1900 to 35.3 years in 2000, and projected 39 years in 2030 (1). However, the changes in age structure of the population have tremendous impact on families and society as it changes the number of working-age people and creates challenges to provide long-term care for the elderly. Therefore, aging research became an important and expanding area of science, with the goal not only to increase life expectancy but also to understand the biological process of aging in order to optimize 'successful aging', defined as avoidance of disease and disability, maintenance of mental and physical function, and continued engagement with life (2).

Oxidative stress and inflammatory stress have been implicated as major factors causing the physiological process of aging as well as playing a role in the pathogenesis of numerous chronic diseases which contribute to morbidity, disability and mortality of the older population (3, 4). Accordingly, diverse lifespan models either reducing or increasing their production have been developed to investigate the underlying molecular mechanisms (5-8).

Over the past decade, the view of the adipose tissue has changed from a passive storage site for energy to an active endocrine organ that secretes multiple hormones and

cytokines regulating energy, glucose and lipid metabolism (9-11). Adipose tissue is an important contributor to reactive oxygen species (ROS) and inflammatory cytokine production (12). Consequently, obesity, and particularly visceral obesity, is associated with a chronic low-grade inflammation status, a principal causative factor to the development of metabolic diseases (13-16).

Dietary calcium and other dairy components have been demonstrated to significantly alter adipose metabolism and overall energy partitioning between muscle and adipose tissue (17). Multiple studies, both *in vitro* and *in vivo*, have confirmed that dietary calcium reduces adiposity, promotes lean tissue mass and decreases oxidative and inflammatory stress, thereby reducing obesity risk and improving metabolic status (18-23). In addition, dairy products have been shown to exert greater effects than supplemental calcium due to additional components in dairy such as branched-chain amino acids (BCAA) and angiotensin-converting enzyme inhibitory (ACEi) peptides (24, 25).

Therefore, considering the role of dietary calcium in modulating oxidative and inflammatory stress and considering the role of additional dairy components on these effects, the purpose of this research was as follows: a) to elucidate the common and differential pathways related to energy metabolism, inflammation and oxidative stress in adipose tissue and muscle in response to milk and milk components by using a high-density oligonucleotide microarray approach; and b) to evaluate the effects of dietary calcium and dairy on lifespan and on lifespan related biomarkers in an animal model of diet-induced obesity.

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Part Two

Literature Review

2.1 Aging and lifespan

2.1.1 Overview

Aging is a diverse and complex process associated with variable phenotypes and different rates of progression across species as well as within the same species. Accordingly, to find a universal definition and a distinct cause is nearly impossible, and today's view that aging is a multifactorial process has replaced earlier attempts to find a single explanation. In general, aging causes a loss of efficiency of physiological functions and of the capacity to maintain internal homeostasis. As a consequence the organism is less resistant to stress and more susceptible to diseases, infections and cancer leading to death. Factors that influence the individual's lifespan can be genetically or environmentally (1).

The term 'senescence' is often interchangeably used with aging. Senescence refers to the biological process resulting in death of the individual but is also used as 'cellular senescence' to describe the aging process at the cellular level (2).

Longevity is often used as synonym for life expectancy and includes two different concepts; the maximal and mean lifespan (3).

2.1.2 Theories of aging

In order to understand the phenomenon of aging many theories have evolved to give explanations for origin, occurrence, progression and consequences. With advances in technology and new experimental methods, new ideas developed either replacing or improving older theories or giving rise to new ideas. Today, there are more than 300 theories counted and their classification can vary widely. While some authors group them on the

basis of history, others divide them into stochastic vs. programmed and evolutionary theories (4). Further they can be grouped depending on the level of biological organization, which is as evolutionary, molecular, cellular and systemic (1).

Meanwhile well accepted, theories should explain the four specific criteria of aging suggested from Strehler in 1959 (5):

- Universality: the phenomenon is part of a general aging process in all individuals of a species
- Intrinsicality: changes are due to the inherent instability of the system
- Progressiveness: the onset of the changes is gradual and accumulative
- Deleteriousness: the change should result in a decreased survival capacity of the old organism

However, many of the older theories do not meet those criteria**.**

2.1.2.1 Evolutionary

Evolutionary theories focus on the force of natural selection which declines with age (6). According to Darwin's theory that natural selection favors processes which increase reproductive fitness, aging with increased rate of mortality and reduced capacity of reproduction seems to be in contradiction to this theory (7).

Weissmann in 1891, was one of the first biologists shaping the evolutionary era of aging. He believed that organisms must show a decline ('wear and tear') analogous to that of mechanical devices but also argued that there was a specific death mechanism designed by natural selection to eliminate the old (8). However, he never indicated any precise mechanisms and his ideas were criticized as illogical and inconsistent. Also the hypothesis that aging and programmed death occurs to limit population size and to facilitate the

turnover of generations was confuted by the fact that natural death contributes only negligibly to the mortality in a natural environment where extrinsic hazards such as famine and predation predominates (9).

Further solutions to this enigma were suggested by Haldane in 1941, who proposed that natural selection would have little force at late ages based on his observations of Huntington Disease (10). Since this detrimental disease, caused by a single dominant gene mutation, affects only individuals at later age (30-40 years old), carriers are able to reproduce before death occurs and therefore are able to escape natural selection. Inspired by this observation, Medawar expanded this idea to the 'Mutation accumulation theory', proposing that deleterious mutation with a late onset may accumulate in the population and, since unopposed by natural selection, may cause pathology and senescence (1).

Williams focused specifically on the idea of trade-offs between reproductive fitness and later survival which was later named as the 'antagonistic pleiotropy' by Rose in 1982 (10). This idea suggests that genes that are beneficial during youth are actively favored by natural selection, although they may have deleterious effect with age.

Related to the pleitropic gene hypothesis is also the 'disposable soma theory', originally proposed by Kirkwood 1977 (11). This theory predicts that the accumulation of unrepaired somatic damage through life will cause senile degeneration and ultimately deaths. Since somatic maintenance and reproduction are both energy-consuming processes there must be a balance between both. Therefore, the optimal level of investment will depend on the species' ecological niche. Species with high external mortality may benefit by investing in rapid reproduction while species with low accidental mortality may profit by investment in somatic repair and a longer life (8).

While evolutionary theories in general focus on the aging process of the whole individual organism, the molecular and cellular theories take also different tissue specific aging pattern into account (4). However, although the age-related decline of physiological functions varies within an individual, many changes may not be important for longevity.

2.1.2.2 Molecular

Molecular theories provide mechanistic explanations for the pathological changes of aging in tissues and cells by focusing on the damage occurring to macromolecules. The main categories of molecular damage within a cell occurs to DNA, RNA , or proteins (12). However, most of the theories have focused only on a single category of molecular damage. For example, the 'Somatic mutation theory' of Szilard in 1959 assumes that the elementary step for the process of aging is an 'aging hit' which destroys a chromosome of the somatic cell with the consequence that all genes of that chromosome become inactive (13). These 'aging hits' are random events that accumulate with a constant rate throughout life. This will result in a decreased number of functional somatic cells at a given age and the fraction of surviving functional cells decreases with age at an accelerating rate. In addition, the rate of hits is characteristic of the species and therefore responsible for the similar lifespan of individuals within a species.

The 'Gene regulation theory', proposed by Kanungo in 1970 (14), is based on the concept of cellular differentiation due to regulation of gene activity. Kanungo postulated that each organism undergoes phases of differentiation, growth, maturity and senescence, which have a unique characteristic, duration and speed for each species. This linear order of phases occurs due to sequential activation and repression of genes. Addition or deletion of one or more genes in the sequence may also accelerate or decelerate the sequence in the new

species and thereby decrease or increase its life span. Thus this model offers an explanation for the two important characteristic features of aging: the gradual decline in activity after maturity and the specific life span of a species.

The ‘Dysdifferentiation hypothesis’, based on work from Cutler (15), focuses on impaired regulation of gene expression due to random molecular damage. This hypothesis suggests that aging results from a natural instability of proper gene regulation with cells drifting away from their proper state of differentiation towards dysdifferentiation. Thus, longevity must result from mechanisms acting to stabilize the proper differentiated state of cells. Cutler proposed that a common set of ‘longevity determinant genes’ may exist in mammalian species which control the expression and timing of the defense and protective system acting against the unfavorable side reactions of normal and essential biological processes. These regulatory processes, and not specific kinds of genes, would govern the characteristic aging rate of species.

Other theories emphasize on the changes occurring in proteins. For instance, the ‘error catastrophe theory’, introduced 1963 by Orgel (16), suggested that errors leading to reduced specificity of protein-synthesizing enzymes increase rapidly the error frequency of proteins. These processes accumulate over the lifespan of the organism and may reach a value at which necessary processes for the existence of cells becomes critical and therefore may result in death. Accordingly, the ability of a cell to produce functional proteins depends not only on the correct genetic background but also on the competence of the protein-synthetic apparatus.

Strehler (5) developed his ‘Codon restriction theory’ which is based on the principle that the kinds of proteins synthesized by a cell are controlled by the set of code words a cell

can decode. The types of tRNA and aminoacyl-tRNA synthetases determine the kinds of syntheses a cell will carry out and are different during cell growth, differentiation and aging. They also vary in different tissues and therefore are responsible for the characteristic protein profile of a tissue.

In constrast, the ‘membrane hypothesis’ of aging, introduced by Zs.-Nagy in the late 1970s (17), explains aging as a consequence of the formation of damaging waste products such as lipofuscin accumulating in the plasma membrane which are due to an imbalance between the rates of protein synthesis and elimination. These alterations are due in part to free radical attacks and residual heat formation, which induce molecular damage. Despite a continuous replacement of the damaged components, a certain amount of residual damage accumulates during life, which consequently leads to an increase in dry mass due to a passive loss of permeability of the cell membrane for potassium and water. This dehydration process causes a decrease in enzyme activities and a decline in physiological functions. Since all types of cells have basically identical structure, these processes can be observed in every cell, however, in different magnitude.

2.1.2.3 Cellular

Cellular theories concentrate on the characteristic changes of aging in cells and often overlap with molecular theories, since these changes are often also caused by molecular damage. The most important theories in this category are the ‘Free radical theory’ from Harman, the ‘Cellular senescence theory’ from Hayflick, and the ‘Telomere shortening theory’.

The ‘Free radical theory of aging’ from Harman in 1956 postulates that the common aging process is the initiation of free radical reactions (FRR) in cells and tissues which lead

to the progressive deterioration of biological systems over time due to their innate ability to produce random changes (18). Therefore, measures to decrease the chain lengths of FRR or their rates of initiation can, in turn, decrease the rate of aging and of disease pathogenesis. Since most FRR take place in the mitochondria and life span is determined by the free radical damage to mitochondria, this theory was extended to the 'mitochondrial aging theory' in 1972 (19).

The 'cellular senescence theory' or also called the 'Hayflick Limit theory', named after its discoverer Dr. L. Hayflick in 1965, proposes that senescence is caused by a limit in replicative capacity of normal cells (1). In his experiments, Hayflick demonstrated that normal diploid cells could undergo only a certain number of cell divisions (50-100 times) under *in vitro* conditions. Conversely, cells which don't have a finite lifetime share properties of cancer cells (20).

One of the explanations for this 'replicative senescence' was given by the idea of 'telomere shortening' in 1971 (21). Telomeres, which are repeated terminal DNA sequences of eukaryotic chromosomes, are synthesized by the polymerase 'telomerase', a ribonucleoprotein reverse transcriptase. The function of the telomeres is to stabilize the ends of chromosomes (22). Subsequent research indicated that these telomeres shorten every time a cell divides because of a loss of telomerase activity, eventually leading to the cessation of cellular proliferation (23) . In contrast, immortal cells such as cancer cells and embryonic stem cells maintain telomerase activity and, consequently, a stable telomere length (24).

2.1.2.4 Systemic

Systemic theories are system-based views of aging with the concept that the homeostatic network which integrates functions of different cells and tissues and gives the

organism the ability to communicate and to adapt to the environment, declines with age (25). This approach combines evolutionary aspects with molecular and cellular models of aging rather than providing an alternative (26); however, it considers the alterations of single cells as secondary effects and as irrelevant for the whole organism. Key players in this coordinating network are the neuroendocrine and the immune system (25).

The ‘neuroendocrine theory’ proposes that aging is due to changes in neural and endocrine function. It considers the hypothalamo-pituitary-adrenal axis as the master regulator which regulates to a greater or lesser extend all body functions. Progressive deterioration of this system leads to changes of hormones and neurons leading to secondary alterations in other tissues and organs. Overall, the organism’s ability to survive to stress decreases and it becomes more susceptible to diseases and death (1, 25).

The ‘immunologic theory’ emphasizes on the decline of the immune function resulting in decreased resistance to infectious diseases and cancer, and increased incidence of autoimmune responses (1). This model is based on the assumption that the immune system is the leading apparatus to maintain body integrity because it must control and eliminate foreign particles invading the host while at the same time protect it from self-destruction. A progressive deterioration of the immune system with age such as seen in the thymus in humans, which undergoes a continuous decline starting from puberty, leads to a global reduction in the capacity to deal with a variety of stressors. This immunosenescence will lead to an increase in the pro-inflammatory status which is one of the characteristics seen with aging (27).

Since the immune system is also regulated by signals from the neuro-endocrine system and, on the other hand, the neuro-endocrine system is modulated by signals derived

from the immune system such as lymphokines and cytokines, there are new attempts to integrate changes to both networks into one model (25).

Another systemic theory, however, in contrast to the above theories not focusing on a network system, is the ‘rate of living theory’ which originally was developed by Pearl in 1928 (28). In essence, this hypothesis assumes that any organism has a fixed amount of life or energy expenditure. Animals that choose to utilize this amount fast will live shorter while individuals, who use this amount slowly, will live longer. In many aspects this theory is similar to the evolutionary ‘wear and tear’ idea assuming that a living organism deteriorates with higher usage similar to malfunctioning of mechanical objects (28).

2.1.3 Focus on ROS

Reactive oxygen species (ROS) are one of the most important free radicals in biological systems. Superoxide Anion ($O_2^{\bullet -}$) is considered the primary ROS and can further interact with other molecules to generate secondary ROS such as the hydroxyl radical ($^{\bullet}OH$) and peroxyl radicals ($ROO^{\bullet}$) (29). ROS are products of normal cellular metabolism and exist at low but measurable concentrations in living systems. The balance between ROS production and their elimination by the anti-oxidant defense system determines the effects in cells which may be beneficial or harmful (30).

2.1.3.1 ROS production

ROS can be generated either as a byproduct of other biological reactions or can be generated as a primary function of an enzyme system. Locations where ROS is produced as a byproduct are the mitochondria, peroxisomes, and cytochrome P-450; the major ROS producing enzyme are NADPH oxidases (31).

Approximately 2% of the total oxygen consumption in isolated mitochondria are converted to ROS under physiological conditions (32). This is caused by 'electron-leakage' preferentially in complex I and III of the electron transport chain, which generates superoxide instead of contributing the electrons to the reduction of oxygen to water (29). Most of the produced superoxide is converted to the non-radical hydrogen peroxide (H_2O_2) by Superoxide dismutase. However, extra-mitochondrial escaped superoxide can participate in further reactions (e.g. Fenton reaction, Haber-Weiss reaction) to produce the highly reactive hydroxyl radical.

The first identified system producing ROS as a primary function was the phagocytic enzyme NADPH oxidase (31). The phagocytic form as well as the other nonphagocytic isoforms belongs to a family of transmembrane heme-containing proteins which function to transport electrons across the membrane from a cytosolic donor such as NADPH to an electron acceptor such as oxygen. In this process superoxide can be generated which has physiological relevance as a first line defense against environmental pathogens as well as in cellular signaling and regulation of gene expression (33). There are seven family members of NOX oxidases with a wide tissue distribution. NOX2 is the most abundant isoform in phagocytes and NOX4 is most widely expressed (34). Other enzymes producing ROS include 5-lipooxygenase and cyclooxygenase; however, the evidence for a physiological role in ROS generation is still scarce (30).

2.1.3.2 Antioxidant defense system

To maintain a low concentration of ROS in the cell, different defense mechanisms have evolved; these include preventive mechanisms, repair mechanisms, physical defenses and antioxidant defenses (29). The antioxidant system includes the enzymatic defense by the

enzymes Superoxide Dismutase (SOD), Catalase, and Glutathione Peroxidase (GPx), and non-enzymatic defense involving antioxidants such as ascorbic acid (Vit C), α-tocopherol (Vit E), carotenoids, flavonoids and glutathione (GSH) (29).

2.1.3.3 'Redox signaling', 'Redox homeostasis' and 'Oxidative stress'

Under normal physiological conditions the concentration of ROS in a cell is low. This 'steady state' concentration is determined by the balance between the rate of ROS production and the rate of ROS removal (29). During 'redox signaling' this balance is shifted temporarily towards more oxidative conditions, which will induce redox-sensitive signal cascades. Direct feedback regulatory processes will inhibit further ROS production and stimulate the antioxidant defense mechanisms to reset the original state. This reestablishment of the original redox state after a temporary exposure to increased ROS concentration is called 'redox homeostasis' (30). However, under certain conditions the oxidative event may be more strongly or persistently, and the defense mechanisms are not sufficient to reach baseline levels. If this pro-oxidative shift is severe enough, it may result in oxidative stress for the cell and may lead to the development of pathological conditions (30).

2.1.3.4 Redox signaling under physiological conditions

The increased ROS production as an 'oxidative burst' by the phagocytic NADPH oxidase is one of the best studied redox-responsive signaling pathways. In an inflammatory environment, activated macrophages and neutrophils can produce large quantities of superoxide and hydrogen peroxide, and the latter can reach concentrations of 10-100 μM (30). These antimicrobial and tumoricidal ROS act as a first line defense against the

invading pathogens. In addition, the increased levels of ROS enhance the activation of T-lymphocytes further stimulating the immune response (35).

Another role of redox signaling is the regulation of cell adhesion of leukocytes to endothelial cells and cell adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), CD11b/CD18, and L-selectin, which play an important role in chronic inflammation, cell growth and differentiation, and wound repair. It has been shown that ROS induces the adherence of leukocytes to endothelial cells and induces the production of adhesion molecules via activation of transcription factors such as AP-1, nuclear factor (NF)-kB and hypoxida inducible factor (HIF) -1 (36).

In addition, there are multiple positive bidirectional interactions between signal transduction pathways and ROS production. For example, multiple growth factors, hormones and cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin- 3 (IL-3), tumor necrosis factor-α (TNF-α), angiotensin II (ANGII), platelet derived growth factor (PGDF), nerve growth factor (NGF), transforming growth factor-β1 (TGF-β1), granulocyte-macrophage colony-stimulating factor (GM-CSF), and fibroblast growth factor (FGF-2) activate NADPH oxidase. The generated ROS, in turn, will induce positive feedback effects on the signal transduction from the corresponding membrane receptor or act as second messenger to activate tyrosine kinases, protein tyrosine phosphatases or serine/threonine kinases involved in other signal transduction pathways such as mitogen-activated protein kinase (MAPK) and PI3K/AKT pathway (29, 30) .

Furthermore, an increase in intracellular ROS can activate intracellular Ca^{2+} stores as well as stimulate the influx of extracellular calcium, resulting in increased intracellular Ca^{2+} concentration (37, 38). However, $[Ca]_i^{2+}$ as an ubiquitious intracellular second messenger is

tightly regulated and involved in regulation of many different cellular functions such as contraction, metabolism, gene expression and cell survival/death. In addition, there is a bidirectional connection between $[Ca]_i^{2+}$ and ROS with $[Ca^{2+}]_i$ also stimulating ROS production in mitochondria and by NADPH oxidase (38). Therefore, the cross-talk of both important signaling systems can be stimulatory or inhibitory, depending on cell type and extent of exposure.

2.1.3.5 Redox signaling under pathological conditions

The response to ROS at the cellular level depends on the duration and intensity of the oxidative insult. While low concentrations stimulate proliferative and survival pathways, higher concentrations contribute to cell arrest and apoptosis (39). The harmful effects of prolonged and intense ROS production (oxidative stress) are caused by different mechanisms. First, the increase in ROS can damage other molecules such as DNA, proteins and lipids (40-42). This can result in DNA mutation and breakage, loss of protein and enzyme function, and rupture of membranes. On the other hand, chronic stimulation of above mentioned signaling pathways contributes to chronic inflammation, proliferation and cell death. Accordingly, oxidative stress has been implicated in a number of chronic diseases including diabetes, cardiovascular diseases and cancer (43-46).

2.1.3.6 ROS and Aging

Harman (1956) was the first proposing the hypothesis that the accumulation of free radicals in cells and tissues is responsible for the progressive deterioration and the process of aging of biological systems (47). Later this idea was incorporated in other theories of aging such as ‘protein error theory’ and the ‘membrane hypothesis’. Chen et al. also demonstrated that ‘replicative senescence’ could be induced by H_2O_2 (48).

A growing body of evidence suggests that aging involves free radical induced changes of molecules and of regulatory processes. For example, it was shown that shorter-lived species produce relatively higher amounts of ROS than longer-lived species (49). Other studies revealed that aging is associated with an accumulation of oxidized biomolecules such as 8-oxo-2'-deoxy Guanosine (8-oxo-dG) in DNA and oxidized proteins in tissues (50-54). Further support is given by the fact that oxidative damage can result in age-characteristic phenotypes (55); for instance, oxidative damage to brain cortex correlated with impairment in memory and cognitive function (52). However, most evidence comes from transgenic studies that show that decreasing ROS by over-expression of antioxidant enzymes such as SOD and catalase can increase resistance to oxidative stress and prolong lifespan (56-58). Also pharmacological treatment with antioxidants resulted in longevity extension in some studies (59-61).

2.1.4 Mitochondrial aging

More than 90% of the oxygen used by aerobic cells is consumed by mitochondria in the process of oxidative phosphorylation. As a byproduct, 1-2 % of the consumed oxygen is converted to superoxide in the respiratory chain which will mainly be dismutated to H_2O_2 by the mitochondrial SOD (32). This continuously spontaneous production can contribute immensely to the total ROS amount in cells, since hundreds to thousands of mitochondria are within each cell, depending on cell type.

The 'rate of living theory', proposed by Pearl, was the first theory to consider an inverse relationship between the rate of oxygen consumption and maximum life span of species (28). However, there were some exceptions to this theory, as seen in some species of

birds, which show a high oxygen consumption and long lifespan. Later in 1972, Harman focused his work on mitochondria and described them as ‘biological clock’ since as a major source of endogenous free radical production they would determine the lifespan (18, 19). Miquel further developed the idea of ‘mitochondrial aging’ which was based on studies of insects and mammals (62). While insects showed consistently many age-related changes in mitochondria of flight muscles in association with increased H_2O_2 production, such as reduction of total mitochondrial volume, decrease in total amount of mtDNA, structural changes as a loss of cristae as well as a decline in activity of key enzymes involved in respiration and energy production, results of studies in mammals were conflicting depending of the methodology and experimental design used. Similar age-related changes were found in fixed postmitotic cells and cells with a low replication rate such as skeletal and heart muscle, while cells with a high replication rate, such as gastro-intestinal cells, appeared to be free of age-related changes. Thus, Miquel concluded that due to the increased free radical production with age damage mainly to the inner mitochondrial membrane and DNA accumulates because of lipid oxidation and cross-linking which makes the cell unable to divide; however, the degree of damage is more evident in postmitotic cells and cells with a low replication rate.

A large body of evidence supports this idea. Mitochondrial DNA (mtDNA) is more susceptible to oxidative damage and mutation than nuclear DNA because of the close proximity of ROS production and the characteristics of mtDNA. The lack of protective histones and an effective repair system makes them more vulnerable to oxidative damage. In addition, mtDNA has no introns. Consequently random mutations will usually strike a coding sequence. Furthermore, mtDNA is inherited maternally and do not recombine so that

normal and mutant DNA can coexist within the same cell. This heteroplasmy allows otherwise lethal mutation to persist and to accumulate (63). As a result the transcription of mitochondrial genes may be impaired, resulting in mitochondrial dysfunction which can lead to a further increase of ROS production due to damage to the electron transport chain (45). In addition, oxidative damage of mitochondrial proteins and membrane lipids further contributes to morphological and functional changes (64). Accordingly, mitochondrial dysfunction has been linked to the pathogenesis of a wide range of pathologies as well as to aging (45, 63, 65).

Numerous studies conducted in multiple species support the relationship between mitochondrial dysfunction and aging, although some have yielded inconsistent results. Some investigators focused on the damage to the mtDNA; for example, Melov et al. found a substantial increase in the number and variety of mtDNA rearrangement in muscle from older humans compared to muscle from younger individuals (66). Others, such as Short et al. (63), investigated structural and functional changes in human skeletal muscle and found that there was a continuous decline in mitochondrial protein and DNA content which was associated with a decrease in mitochondrial ATP production rate (67). Barja et al. also examined the relationship of mtDNA damage of heart and brain to the lifespan in mammals and compared it to the damage found in nuclear DNA. He identified an inverse relationship between oxidative damage to mtDNA and maximum lifespan, while no such correlation was found for nuclear DNA (68). In another study the causative link between mtDNA mutations and aging was examined in knock-in mice expressing a proofreading-deficient DNA polymerase (69). These knock-in mice developed a phenotype with 3 to 5 fold increase in mtDNA mutations and deletions which was associated with reduced lifespan and premature

onset of aging-related phenotypes such as weight loss, alopecia and osteoporosis. However, in a follow-up study the authors showed that these age-related phenotypes in the mtDNA mutator mice were not related to increased ROS production or a decrease in antioxidant enzyme activities since the increase in oxidative damage was only minor despite profound respiratory chain deficiency (70). Therefore, the authors proposed that the respiratory chain dysfunction per se, and not oxidative stress, was the primary inducer of the premature aging. Further conflicting data came from a study, in which life-long reduction of mitochondrial SOD activity resulted in increased DNA damage but did not accelerate aging (71). On the other hand, other studies demonstrated that decreasing mitochondrial ROS production by over-expressing mitochondria targeting enzymes or by administration of antioxidants could prolong lifespan and prevent age-related phenotypes (58, 72, 73). Despite some conflicting data, the overall consensus seems to support the concept that mitochondrial oxidative stress and dysfunction are important contributors to the process of aging, although detailed mechanisms still needs to be fully elucidated.

2.1.5 Lifespan

The term ‘lifespan’ can be applied to an individual or to a population of a species. The individual lifespan specifies the period between birth and death for that individual (74). In contrast, the population lifespan can be characterized by the maximum lifespan, which is the highest verified age at death in a specified time period or ever recorded, or it can be measured by the mean life expectancy, which is the statistically calculated estimate of number of years of an individual to live at a given time. Other measures include the median lifespan and range, all of them are always dependent on the number of individuals sampled

(75). While the maximum lifespan seems to be relatively constant and mainly genetically determined, the mean life-expectancy depends more on environmental conditions and external mortality (9).

The lifespan between species varies widely from days in flies up to 200 years in whales, but there is also substantial variation within the same species. Evolutionary biology suggests that this variation is caused by adaptation processes resulting from selection due to demographic circumstances, duration of infantile period and the species' ecological niche (76). Despite some exceptions, there is generally a positive correlation between body size, relative brain size, possession of armor and lifespan (75). However, growth seems to negatively impact the life span within mammalian species (77).

In humans, there was a major increase in mean life expectancy over the past century; this was associated with decreased early mortality due to improved nutrition, housing, sanitation and health care. In contrast, there is no evidence for an increase in maximum lifespan over that time period, with the highest recorded age of 122 years (74). There are some indications for sex differences, with females living longer than males, possibly due to factors such as men having riskier behavior, larger body size and different hormone levels (78). In general, longevity shows a clear heritable component but also non-genetic factors and gene-environment interactions are very important in determining the lifespan (74).

2.1.5.1 Models of lifespan

As the mean life expectancy dramatically increased over the last century due to socio-economic factors it became also more evident that both lifespan and aging is under moderate genetic control (79). Therefore, aging research over the last several decades has focused on understanding the aging process at the molecular level with the inevitably

consequence of an intensive search for models of lifespan (80). Models of lifespan can be a helpful tool to study pathways influencing metabolism and mechanisms of maintenance and repair, and may also contribute to finding molecular candidates to extend health and longevity. Since aging is a diverse and complex process associated with various phenotypes, dissecting it into components and studying each component separately in experimentally suitable models may be necessary; however, the results need to be interpreted in light of the broad context of the process which is a complex network of inter-related pathways often controlled by diverse mechanisms (80). Validity of findings are further complicated as they critically depend on laboratory conditions and settings which often are very dissimilar to the normal environment (79). Therefore, depending on the complexity and research questions suitable models can reach from single cells to whole organisms.

2.1.5.1.1 Cellular models

According to Hayflick who discovered first that normal human fibroblasts have a limited number of cell divisions and then enter into a state of cell senescence, the accumulation of senescent cells may contribute to the process of aging in the whole organism (81). In support of this hypothesis, single gene alterations such as loss of telomerase or over-expression of p53 which alter the amount of cell divisions, are also associated with overall changes in longevity of the whole organisms (82). In addition, many mouse models of accelerated aging are caused by gene alterations affecting DNA repair, cell cycle and apoptosis (82).

Technical advances in cell culturing and subcultivation in the 1960s and the development of reliable biomarker for cell senescence such as the senescence associated β-

galactosidase (SA-β-gal) assay, flow cytometry and green fluorescent protein (GFP)-expressing reporter constructs for gene expression, revolutionized biological aging research (83). In cell culture the number of possible cell divisions until reaching a state of replicative senescence seems to be cell type and species specific (84). Although most cell types undergo cell senescence, there are some exceptions, such as transformed cells and post-mitotic cells as neurons. Studying the pathways which enable immortal cells to escape senescence has significantly contributed to the identification of signaling cascades important for human aging and age-associated diseases; these include the telomerase pathway, the IGF-Akt pathway and the mitochondrial/oxidative stress pathway (84). Therefore, cells can be an easy and good model for finding key mechanisms and studying genetic mutations.

2.1.5.1.2 Invertebrates

Invertebrates such as *S. cerevisiae*, *C. elegans* and *D. melanogaster* have been extensively used in aging research. Advantages of these models are in general their short lifespan, easy maintenance and low costs, excellent background genetics, and the ready availability of large numbers of genetically homogenous individuals (85, 86). Despite their advantages, they also have some limitations that will be discussed below.

Saccharomyces cerevisiae, or baker's yeast, is a unicellular organisms belonging to the ascomycete family. Aging in *S. cerevisiae* can refer to the 'clonal', also called 'replicative' lifespan, or to the 'chronological' age. The 'replicative' lifespan is defined by the finite number a mother cell can bud into smaller daughter cells, whereas the 'chronological' lifespan means the gradual loss of viability when grown to saturation. The main difference between both is the reliance of mitochondrial respiration; under clonal

conditions the energy derives from anaerobic glycolysis and mitochondrial respiration is actively repressed, therefore it is an unsuitable model for studying energy metabolism (87).

Caenorhabditis elegans is a pseudo-coelomate nematode and very attractive as model organism because of its large number of offspring, short generation time (around 20 days) and the ability to be stored frozen. However, an important difference in comparison to mammals is the dependence of lifespan on the temperature. Lowering the ambient temperature by $6^{o}C$ can double the lifespan of either species. Aging studies are usually centered on the 'dauer stage', an alternative, dormant and stress resistant life stage induced by food shortage and characterized by a very long lifespan (87, 88).

The fruit fly *Drosophila melanogaster* has been a favored model for aging. Advantages to the others described above are the short time until reproduction/adulthood and being essentially a postmitotic organism, therefore ideal to study accumulation of aging changes. In addition, it represents a relatively complex organism because of its ability to fly which requires engagement in various sensory and motor-driven behaviors that can be followed with age in addition to its lifespan (85).

In contrast to cell studies, studies in invertebrates make it possible to examine changes in a whole organism but still having the advantage of low cost and easy maintenance. They can be easily genetically modified and used for targeted single gene mutations. They also can be used for profile gene expression analysis to find age-related gene expression differences and similarities between short-lived and long-lived animals (89). Studies in invertebrates have contributed immensely to the identification of specific genes and signaling pathways important for the aging process.

2.1.5.1.3 Vertebrates

Findings in different invertebrate models support the possibility that these pathways are highly conserved. Accordingly, vertebrate models are needed to test the key findings of invertebrate models for relevance in mammals and eventually humans. Mice are mainly used as vertebrate lifespan models because they still have a relatively short lifespan (2 to 3 years), their basic biology and genomes are known, and they are relatively easy experimentally manipulated.

Since there was a great body of evidence in *C. elegans* and *D. melanogaster* that the insulin/IGF-1 pathways plays an important role in the aging process, there were several genetic mouse models created with mutations in growth hormone (GH), IGF-1 or insulin signaling. Examples are the following (90):

1) Dwarf mice: these mice have a mutation in the pituitary-specific transcription factor 1 (Pit1) gene causing hereditary dwarfism. Depending on their mutation and background, there are the 'Snell', 'Jackson', 'Ames', and 'Ames and Snell' dwarf mice. Dwarf mice do not produce growth hormone (GH), thyroid stimulating hormone (TSH) and prolactin (PRL); therefore they show severe growth retardation, infertility, and reduced metabolism and metabolic rate. They also have alterations in insulin/IGF-1 signaling with severely reduced circulating plasma insulin, IGF-1 and glucose levels. Some of these dwarf mice also have an increased resistance to oxidative stress. Although reports on lifespan are controversial, most investigators found an increase of up to 68% in these mice.

2) Growth hormone receptor/binding protein (GHR/BP) knockout mice: these mice show severe growth retardation starting at 4 weeks of age and delayed sexual maturation in

females. They have very high serum GH levels while IGF-1 levels are decreased by 90%. Lifespan extension of up to 55% was reported compared to wild-type mice.

3) Insulin-like growth factor type I receptor (IGF-1R) knockout mice: while homozygous knockout are not viable and die at birth, heterozygous ($IGF1r^{+/-}$) animals look normal. However, IGF-1R and IGF-1 signaling was reduced up to 50% in some tissues. Most markers such as body weight, glucose levels and metabolic rate were normal or only slightly different when compared to wild-type mice; however, they showed an increased resistance to oxidative stress and an increase in mean lifespan up to 33%.

4) Fat-specific insulin receptor knockout (FIRKO) mice: in these mice the insulin receptor (IR) gene is mutated in adipose tissue specifically leading to 90% reduction of insulin-stimulated glucose uptake. These animals show a 15-25% reduction in total body weight accompanied by a 50-70% reduction in fat mass despite higher food consumption. In addition, FIRKO mice had lower fasted insulin plasma levels while maintaining normal blood glucose level; however, they developed impaired glucose tolerance due to insulin resistance with increasing age. Despite that, the median lifespan was increased 18% compared to littermate controls.

Although these mouse models seem to support the link between GH/IGF-1 and insulin axis and longevity, interpretation needs to be done with caution. Some of these models represent rather multiple hormone deficiencies than single gene mutations; therefore the precise role of GH and IGF-1 in regulation of lifespan remains equivocal. In addition, these hormones have critical actions in development and early adulthood such as increasing body weight, bolstering bone and muscle mass, enhancing immune function and increasing

general reproductive fitness (91). Therefore, a balance between the beneficial and deleterious consequences of these hormones needs to be found.

Another often-used mouse model with extended lifespan is the $p66^{shc}$ knockout mouse. The $p66^{shc}$ gene encodes for a Src-homology2 (SHH2) domain protein with two major functions: cytoplasmic signal transduction of mitogenic signals activated from receptors to Ras, and the p53 stress activated induction of apoptosis and production of ROS. Knockout mice show increased resistance to oxidative stress, lower plasma levels of oxidative stress markers (isoprostane, LDL-oxidation) and mean survival was increased 40% compared to wild-type mice (91)

Additional insight into the aging process may also be gained from models with shortened lifespan. One of the oldest biogerontological resources is the senescence accelerated mouse (SAM), which was developed by selective breeding in Jackson laboratory in 1968 based on early onset of age-associated pathologies such as loss of activity, hair loss, skin coarseness, osteoporosis and short lifespan. There exist 14 different senescence-prone inbred strains (SAMP) and 4 senescence–resistant inbred strains (SAMR) with normal aging which are used as controls. Additionally neurological age-related changes, including deficits in learning and memory and hearing, as well as brain atrophy make them also a useful model for studies of age-related neurodegenerative diseases (92).

Another model of shortened lifespan is the Telomerase knockout mouse. Since the replicative senescence of human cells is caused, at least in part, by telomere shortening, it was assumed that telomerase deficient animals would exhibit a shorter lifespan (93). However, murine somatic cells have long telomeres and maintain telomerase activity, in contrast to human somatic cells; therefore, telomerase-based replicative senescence is

unlikely to occur during the normal aging process in mice. Consequently, a mouse model was created by homologous recombination and successive intergenerational crosses that was telomerase deficient and had undergone sufficient telomere shortening. These late generation ($mTerc^{-/-}$) animals demonstrated a shortened lifespan and premature aging phenotypes such as alopecia, hair graying and reduced resistance to stress (93).

The Klotho mouse is an unintentionally created mouse model with shortened lifespan. It was generated by incidental insertional mutation of a transgene in an unknown gene that was later named klotho gene. The disruption of that gene contributes to an accelerated aging process that is very similar to the normal aging in mammals. Animals show calcification of small arteries, decreased bone mineral density, skin atrophy, and cognitive impairment. Death usually occurs at 8 to 9 weeks of age. It is suggested that the klotho gene inhibits the insulin/IGF-1 signaling pathway, since overexpression of klotho is associated with moderate insensitivity to IGF-1 and insulin, and prolonged lifespan (94).

Since cancer suppression is an integral component of longevity, it was also expected that increased activity of p53, a tumor suppressor gene, would cause an extension in lifespan. However, hypermorphic p53 mutant mice with increased p53 activity exhibited enhanced cancer resistance but reduced longevity and accelerated onset of aging-associated phenotypes. In contrast, inactivation of p53 in mice resulted in drastically increased tumor susceptibility but those animals in which tumor development could be prevented showed increased longevity (95).

To determine the role of ROS production in mammalian longevity, many manipulations of the antioxidant defense system have been studied. In general, potential approaches can be divided into two categories: endogenous studies involving gene

modifications or exogenous studies with exogenously administered toxicants or antioxidants (96). Endogenous modifications involve complete knockouts to tissue specific disruption as well as total and tissue targeted over-expression of antioxidant enzymes and proteins. For example Sod2 (mitochondrial isoform) nullizygous mice die within the first week of life, associated with severe pathologies such as dilated cardiomyopathy, hepatic lipid accumulation, metablic defects and DNA oxidative damage. These symptoms were partly rescued by administration of a catalytic antioxidant MnTBAP (97). In contrast, inactivation of the other two isoforms of SOD (cytosolic and extracellular) resulted in a negligible phenotypes and essentially normal lifespans (96). On the other hand, transgenic mice with over-expression of catalase targeted to mitochondria, peroxisomes or nucleus were more resistant to oxidative stress, had reduced mitochondrial deletions, delayed cardiac pathology and cataract development, and had an increase in median and maximum lifespans (58). Furthermore, over-expression of thioredoxin in mice resulted in median and maximum lifespan extension compared to wild-type mice (98). However, some conflicting results came from a newer study using transgenic mice over-expressing SOD and catalase either alone or in combination, as none of these genotypes exhibit any effects on mean, median or maximum survival (99).

To obtain results, which are physiologically more similar to humans, some studies are performed in non-human primates. For instance, rhesus monkeys were used to investigate the effects of adult-onset caloric restriction (30%) on disease pattern and longevity (100). After 12 years of CR, the animals showed an expected reduction in body weight, reduced adiposity, increased insulin sensitivity and reduced levels of oxidative damage. Furthermore, middle-aged monkeys had reduced risk for cardiovascular disease and

diabetes mellitus and an increased median survival (101). However, these animals have long lifespans compared to rodent and other models; consequently, lifespan studies in this model are quite resource intensive and, consequently, infrequently performed.

2.1.5.1.4 Relevance for humans

Although many of the lifespan regulating pathways identified in invertebrate model organisms are highly conserved throughout evolution, they often have become more complex and diverse in higher developed species (79). For example, the mammalian genome often contains several homologues of a single invertebrate gene such as the forkhead transcription factor (102). On the other hand, some genes have no homologues in invertebrates (103). In addition, signaling pathways such as the insulin/IGF-1 have become more complicated with different receptors and different functions for insulin and IGF (104). Therefore, gene alterations prolonging the lifespan in invertebrates may lead to paradoxically results in mammals, e.g. reduced p53 activity lead to lifespan extension in flies (105) but is associated with increased tumor formation in mammals (106). In addition, environmental conditions influence the expression patterns of genes and can result in gene variations. Since laboratory conditions often poorly mimic the evolutionary niche, results may affect genes, which have no relevance in the natural environment. Furthermore, gene-environment interactions such as epigenetic modifications contribute to phenotypic variations and can cause lifespan differences within species (79). Therefore, extrapolating results from lifespan models to the human population should be done with caution. To overcome these challenges, human diseases of premature aging are sometimes used to examine the underlying mechanisms and to interpolate it to the normal aging process. One

model of human aging is the Werner Syndrome, a rare autosomal recessive disorder. Patients are normal until the second decade of life, and then they develop symptoms of premature aging (alopecia, accelerated atherosclerosis, osteoporosis, cataracts, type II diabetes, cancer) and usually die within the fourth decade (107). The gene mutation affects a gene encoding for a DNA helicase that is important for maintenance of genome stability, DNA replication and repair. However, the specific functional role of this protein and its interaction with other proteins in human cells needs still to be fully elucidated.

Other human approaches include twin studies and studies in centenarians to look for specific gene polymorphisms or longevity genes. Although all these models bring some insight of understanding molecular genetic determinants and environmentally relevant modulators, the multitude of mechanisms of the aging process remains a compelling area of research.

2.1.5.2 Caloric restriction

Caloric restriction (CR) refers to a diet which is usually 20 to 40 % reduced in calories compared to an ad libitum diet but contains all essential nutrients (108). While there were some conflicting data if food or dietary restriction of various nutrients, e.g. proteins or specific amino acids, without changing calorie intake can also prolong lifespan, it is now well accepted that CR is the only dietary intervention able to extend lifespan and to delay the onset of age-associated phenotypes (100, 109).

The idea of performing experiments on life extension by caloric restrictions is based on the ‘rate of living’ hypothesis, assuming that there is an inverse correlation between energy expenditure and longevity (4). In the 1930s, Mc Cay et al. demonstrated first credible evidence that the long-term reduction of caloric intake without malnutrition prolonged the

lifespan in rats (110). Since then multiple studies have been carried out in a wide variety of organisms ranging from yeast to mammals giving further support that CR extends mean and maximum lifespan (100, 111-113).

The physiological effects of CR depend on the duration and extent of restriction, usually beginning with an acute phase followed by an adaptive phase of several weeks to reach a stable, altered metabolic state. This state is characterized by lower body temperature, lower blood glucose and insulin levels, reduced body fat and weight, and increased resistance to external stressors such as heat and oxidative stress (114, 115).

Although the physiological changes of CR are well characterized, the underlying molecular mechanisms to link CR to the extension of lifespan remain elusive. The first mechanistic explanations were based on early findings that the metabolic rate was reduced. This reduction in O_2 consumption would consequently reduce ROS production during respiration and therefore, fitting the free radical theory of Harman, would result in less oxidative damage (116). However, this theory was refuted by the observation that the metabolic rate was unchanged when normalized to body weight (117), and there was even some evidence that respiration is increased by CR (118, 119). Many other hypotheses have been proposed to explain the effects of CR on longevity, including alteration of glucose-insulin system and growth-hormon/IGF axis (109). However, newer evidence points to the nutrient-sensitive proteins such as sirtuins and forkhead transcription factors which are key regulators of energy metabolism (100, 109).

Although the longevity effect of CR is still controversial in primates and humans, the health benefits and the delay of age-associated diseases are well recognized. However, lifelong caloric restriction is not very attractive to most humans and therefore in most cases

unfeasible. Thus, a lot of research focuses on the development of drugs, which mimics the effects of CR. One promising substance is resveratrol, a natural occurring polyphenolic compound, mainly found in the skin of red grapes (120). In addition to its beneficial cardiovascular effects, anti-cancer and anti-inflammatory effects, it also extended the lifespan in some species including *C. elegans*, *D. melanogaster*, and mice, which seemed to depend on the presence of the sirtuins Sir2 and Sirt1, respectively (121-124). However, in another study the lifespan extending results could not be repeated (125). Overall, the changes produced by resveratrol are similar to those produced by CR; these include increased insulin sensitivity, increased AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α activity, and increased mitochondrial function (121). Although very promising, the application in humans is still limited because of extremely low bioavailability, toxic effects at higher doses and expensiveness of this compound (120, 122).

2.1.5.3 Role of Sirtuins

The silent information regulator (Sir) proteins, also called sirtuins, belong to a conserved family of deacetylases and mono-ADP-ribosyltransferases that either deacetylase histones and proteins or transfer ADP-ribose to an acceptor amino acid using NAD^+ as a co-substrate (126). Because of the NAD^+-dependence they are linked to the metabolic activity of cells regulating numerous metabolic pathways including adipogenesis, gluconeogenesis, insulin and glucose homeostasis (127). Accordingly, they have been implicated in the prevention of mitochondrial dysfunction, metabolic disorders and aging (128, 129).

Sir2 in yeast, the first of the sirtuins identified, is involved in the regulation of transcriptional silencing and DNA damage responses (126) and has emerged as the major

genetic determinant of replicative lifespan in yeast with and without CR (115). For example, integration of an extra copy of Sir2 increased the lifespan in *S. cerevisiae* up to 30% whereas deletion of Sir2 shortened it (130). Likewise, studies in different species such as *C. elegans* and *D. melanogaster* with extra copies of Sir2 orthologs revealed similar results (131, 132). In addition, CR did not extend the lifespan when Sir2 was deleted suggesting that CR activates this pathway (132, 133); however, this finding was challenged by other reports showing that CR prolonged lifespan in yeast independently from Sir2 (134).

There are seven sirtuins (Sirt1 to Sirt7) in mammals which are ubiquitously expressed in different tissues (135). They can be classified into four classes according to their conserved NAD-dependent core domain (136). In addition, they differ in their sub-cellular localization; Sirt1, 6 and 7 predominate in the nucleus while Sirt2 resides mainly in the cytoplasm, and Sirt 3, 4 and 5 are predominantly found in the mitochondria (126). Moreover, they vary in terms of activity with Sirt1 and Sirt5 having robust and weak deactylase activity, respectively, Sirt4 and Sirt6 being mono-ADP-ribolsyl transferases, and Sirt2 and 3 having both. The activity of Sirt7 is not yet fully understood (126).

The Sir2 ortholog in mammals is Sirt1 and is most extensively studied. Sirt1 interacts with a large number of substrates including p53, nuclear factor (NF)-κB, forkhead proteins, peroxisome proliferator-activated receptor-γ (PPAR-γ) and peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α, thereby influencing adipogenesis, differentiation of muscle cells, glucose and fat metabolism, resistance to oxidative stress, apoptosis and cell survival. These activities also connect them to the observed induction of Sirt1 by CR (115, 127).

2.1.5.3.1 Stress resistance, cell survival and apoptosis

The role of Sirt1 in cell survival and apoptosis is versatile and depends on tissue localization and other stress factors. For instance, suppression of apoptosis in response to DNA damage is promoted by deacetylation of lysine residues on p53 resulting in their inhibition of transcriptional activity (137). Although this may not advantageous for all cells and may promote cancer, it also stimulates survival of post-mitotic cells such as neurons and may be beneficial in neurodegenerative diseases (127). On the other hand, p53 inhibition as well as FOXO activation evoke cell-cycle dependend autophagocytosis, a catabolic process which can degrade cellular proteins, membranes and organelles (138). It has been observed that autophagic degradation declines during aging and efficient maintenance of autophagy may have a significant role in regulation of longevity (138). Furthermore, FOXO activation by Sirt2 under severe stress can promote cell death and reduce ROS production by activating expression of manganese superoxide dismutase (139). In addition, Sirt1 can induce apoptosis by inactivation of NF-κB, which plays not only an important role in regulating inflammation but also in regulating cell survival. It has been demonstrated that deacetylation of NF-κB in HEK 293 cells by SIRT1 sensitizes the cells to apoptosis in response to TNFα in a p53-independent manner (140).

2.1.5.3.2 Regulation of insulin and glucose homeostasis

Sirt1 regulates insulin and glucose homeostasis in different ways. In pancreatic β-cells it was shown that Sirt1 over-expression in BESTO mice positively promoted glucose-stimulated insulin secretion and improves glucose tolerance (141); however, these effects were lost with advanced age but could be restored by administration of nicotinamide

mononucleotide, an important metabolite for the maintenance of normal NAD-biosynthesis (142). These effects were also associated with a down-regulation of mitochondrial UCP-2 gene expression suggesting that Sirt1 promotes more efficient energy generation in pancreatic β-cells. Interestingly, Bordone et al. demonstrated that overnight food deprivation restored UCP2 levels via reduction in NAD resulting in down-regulation of Sirt1 (143).

In liver, Sirt1 stimulates gluconeogenesis via activation of FOXO and PGC-1α (127, 144). Sirtuin activation by resveratrol or by oxidative stress promoted FOXO1 mediated transcription of gluconeogenic genes and glucose release from cultured hepatoma cells (145). Sirt1 also deacetylates and activates the transcriptional coactivator PGC-1α, a master regulator of energy homeostasis and mitochondrial biogenesis (146). While levels of PGC-1α mRNA are very low under ad libitum conditions in liver, fasting and diabetic conditions dramatically increased PGC-1α gene expression resulting in increased expression of gluconeogenic genes and decreased expression of glycolytic genes (146). In addition, PGC-1α contributes to improved insulin sensitivity in muscle by increasing simultaneously mitochondrial respiration and glucose uptake by insulin-sensitive glucose transporter Glut-4 (146).

Sirt1 also improves insulin signaling in adipocytes. Studies in 3T3-L1 adipocytes have demonstrated that Sirt1 knockdown inhibited insulin signaling events and glucose uptake while treatment of these cells with an Sirt1 activator reversed the effects (147). In addition, activation of FOXO by Sirt1 increases adiponectin levels which further improves insulin sensitivity and regulates glucose and lipid metabolism (126).

2.1.5.3.3 Regulation of adipogenesis and fat metabolism

The action of Sirt1 on adipogenesis and fat metabolism are mainly mediated by repressing PPAR-γ via direct interaction and by activating the PPAR-γ negative cofactors N-CoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoid and thyroid receptors) (115). The nuclear receptor PPAR- γ is a ligand-activated transcription factor and the master regulator of adipogenesis. It is induced during the differentiation of preadipocytes in both white and brown adipose tissue but also regulates fat storage into adipocytes (148). Repression of PPAR-γ by Sirt1 over-expression or activation resulted in inhibition of adipogenesis, enhanced lipolysis and release of free fatty acids leading overall to decreased adiposity (149).

2.1.5.3.4 Sirtuins, caloric restriction and longevity

It has been demonstrated that aging is associated with changes in glucose and fat metabolism, such as increased adiposity and decreased insulin sensitivity. As described above, CR and activation of Sirt1 can improve glucose metabolism, lower insulin levels, increase insulin sensitivity and pancreatic β-cell function, and can reduce overall adiposity. In addition, both have also neuroprotective effects and thus delay the onset of age-related neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and stroke (108). Although their effects on maximal lifespan in humans is controversial, they do appear to have beneficial effects on overall health and may thereby contribute to a 'healthy aging process'.

2.1.5.4 Role of FOXO

The winged helix/forkhead class of transcription factors are important regulators of stress resistance but are also, since regulated by insulin signaling pathway, an alternate nutrient sensing pathway in cells linking caloric restriction, insulin signaling pathway and longevity (150). Their function in the cell is versatile and reaches from regulation of cell cycle arrest, DNA repair and apoptosis to cell differentiation, antioxidant defense, glucose metabolism and energy homeostasis (102). There are over 100 members identified from yeast to human, which are subdivided according to their phylogenetic basis. All have the common characteristic 100-amino acid winged helix DNA-binding domain, giving them their name (151).

In mammals, four FOXO genes are found (FOXO 1, 3a, 4 and 6); these are vertebrate orthologs of DAF16 in C. elegans, the first identified forkhead protein. They are tightly regulated by post-translational modification in response to environmental stimuli that directs them either to the cytosol for proteasomal degradation or to the nucleus for specific transcriptional activity (152, 153). One of the main regulatory mechanisms is the sequential phosphorylation by Akt /PKB on serine and threonine residues (Ser 256 and 319, Thr 24) which prevents their nuclear translocation, interferes with binding of the co-activators p300/CBP and targets them for degradation thereby inhibiting their transcriptional activity (153). Conversely, phosphorylation on threonine residues 447 and 451 by the stress-activated JNK (c-Jun N-terminal kinase) results in nuclear accumulation and enhanced transcriptional activity to induce expression of antioxidants such as MnSOD (154).

Another modification is acetylation/deacetylation. In response to stress signals, FOXO proteins transported to the nucleus associate with other proteins that have histone

acetylase activity such as p300/CBP and PCAF (p300/CBP associated factor) to form the initial transcriptional activation complex. However, paradoxically acetylation reduces their transcriptional activity but recruits Sirt1 for deacetylation and consequently fully activation. FOXO-Sirt1 interaction results in preferential activation of genes involved in cell-cycle arrest/cell death and resistance to oxidative stress, and in reduction of pro-aptotic targets (152).

The third layer of posttranslational modification involves ubiquitination. Akt-dependent phosphorylation of serine 256 creates a binding site for the cytoplasmic ubiquitin ligase Skp2. Ubiquitination targets them for proteasomal degradation which results in reduction of total cellular levels of FOXO and therefore reduced activity (152).

This intricate interaction of posttranslational modifications in response to external signals balances the functions of the FOXO proteins carefully and determines the fate of the cell either to proliferation and survival, or to cell arrest and death, or to survival, stress defense and longevity (153).

2.1.5.4.1 Insulin/IGF-1/FOXO axis and longevity

As discussed earlier the FOXO transcription factors are nutrient sensing signaling regulators linking caloric restriction, insulin signaling pathway and longevity. Studies demonstrate that FOXOs are positively regulated by oxidative and nutrient stress stimuli and negatively regulated by growth factors and insulin (154-156). In addition to insulin, nutrient status is also signalized by the energy sensor AMP-activated protein kinase (AMPK) which directly phophorylates FOXO on sites distinct from the Akt phosphorylation sites, resulting in its activation (157). FOXO target genes include the anti-oxidant enzyme MnSOD, the

glycolytic gene Glucose-6-phosphatase, and the gluconeogenic genes phosphoenolpyruvate carboxykinase 1 (Pck1) and PGC-1α (158). In addition, it regulates the hypothalamus by inducing the expression of agouti-related protein (Agrp) and neuropeptide Y (Npy) to increase food intake. In energy-utilizing tissues such as skeletal muscle it induces autophagy and muscle atrophy in response to nutrient deprivation. Thus, FOXO activation acts in concert to increase systemic glucose levels, to increase oxidative stress resistance, and to set the whole organism in a self-preservation state to survive harsh environmental conditions in anticipation of improved nutrient availability (158). These alterations of cellular metabolism may contribute to an extended lifespan.

The first evidence for a role of FOXO in regulating longevity was demonstrated by the identification of DAF-16 in *C. elegans* (159-161). DAF-16 is a downstream target of DAF-2 pathway, the ortholog to the mammalian insulin/IGF-1 signaling cascade, and the effects of DAF-2 mutants on longevity, metabolism and development are suppressed by DAF-16 mutations. (161). Since the insulin/IGF-1 pathway is a highly conserved signaling mechanism, it was assumed that alteration in this signaling pathway may also affect disease development and longevity in higher eukaryotes including mammals. However, data in mammals are conflicting since the mammalian insulin/IGF pathway is much more complicated than in nematodes and flies with different functions for IGF and insulin, and early disruption is associated with increased risk of age-related diseases and increased mortality due to decreased insulin-sensitivity (104). Nonetheless, some evidence is provided by studies in mice that both inactivation of IGF-1 receptor and insulin receptor substrate (IRS) 1 extended the lifespan (162, 163). In addition, CR has been demonstrated to interfere with the insulin/IGF-1/FOXO pathway as an underlying mechanism to prolong lifespan

(150, 164), and sirtuins are also activators of FOXO, as described above. In humans, evidence for the involvement of the insulin/IGF-1 pathway in controlling longevity is still scarce. However, it has been reported that centenarians showed increased insulin sensitivity but lower IGF-1 levels compared with aged subjects (165, 166). Moreover, polymorphic variants of the IGF-1 receptor and phosphatidylinositol 3 kinase genes have been identified. These variants were associated with lower plasma IGF-1 levels and more often found in long-lived individuals (167).

2.1.6 Changes in body composition in aging

Changes in body composition with aging are mainly caused by a decline in bone and skeletal muscle mass, and by changes in fat mass resulting in altered tissue functions and fat to muscle ratio. These changes typically begin in the fourth decade of life and contribute to impaired physical functioning, metabolic diseases, disability and frailty of the elderly (168).

2.1.6.1 Loss of bone densitiy and osteoporosis

Osteoporosis is characterized by a low bone mass and microarchitectural deterioration of bone tissue, resulting in increased bone fragility and susceptibility to fracture (169). Diagnosis is based on a WHO set of criteria comparing the bone mineral content (BMC) or bone mineral density (BMD) of an individual to the young healthy adult mean of the population (T-SCORE): values within 1 SD are regarded as normal, values between -1 and -2.5 SD are defined as osteopenia, and values more than -2.5 SD below the mean are labeled as osteoporosis. However, in clinical practice the T-score may not always be relevant and the Z-score may be more important. The Z-score compares a person’s bone mass with the mean bone density of adults of the same age and gender and also is used to

determine between primary and secondary osteoporosis (170). Osteoporosis can also be diagnosed based on the presence of a fragility fracture regardless of the T-score or Z-score (171). Primary fracture sites are locations with larger proportions of trabecular bone such as hip, vertebrae and forearm. The incidence of fractures is higher in women and increases exponentially with age (172).

Osteoporosis can occur primary due to age-related bone loss, or secondary as a result of other diseases or conditions e.g. hyperparathyroidism, medication or poor nutrition (172). In general, it is a multifactorial process caused by genetic, nutritional and environmental factors, and occurs in both sexes. Risk factors include female sex, increased age, low weight, and estrogen deficiency contributing to a negative balance in bone remodeling (173).

Bone remodeling is a lifelong process and is thought to support calcium homeostasis as well as to maintain the load-bearing capacity and to prevent accumulation of old bone. From infancy to adulthood BMD increases progressively to reach the 'peak bone mass' around 25 years, then remains stable until the age of 45 to 55 and starts declining thereafter in both men and women. In women, the decline occurs abruptly due to estrogen withdrawal after the onset of menopause and causes a loss of 25 to 30 % of the skeletal mass over a period of 5 to 10 years while in men the loss occurs more gradually at a rate of up to 1 % per year (172, 174). Regulation of bone remodeling involves several endocrine hormones, including estrogen, Vitamin D, parathyroid hormone (PTH), and insulin growth factor (IGF)-1, as well as locally produced factors such as interleukin (IL) 6, receptor activator of nuclear factor kappa B (RANK), its ligand RANKL, and macrophage colony-stimulating factor (M-CSF) (172).

Estrogen has a very important role in bone homeostasis because it is responsible for the increase of BMD during puberty and also controls maintenance of bone mass later in life in both men and women. The suggested underlying mechanism involves suppression of bone resorbing cytokines such as IL6, IL1, tumor necrosis factor (TNF)-α and M-CSF, which otherwise increase osteoclast formation and differentiation. In addition, estrogen upregulates transforming growth factor (TGF)-β, an inhibitor of bone resorption, and may positively influence intestinal calcium absorption. Accordingly, estrogen deficiency increases bone resorption and contributes to a negative calcium balance due to impaired calcium absorption (172). Estrogen loss is not only one of the main contributors to the risk of osteoporosis in postmenopausal women, but also in older men since testosterone and free estradiol levels significantly decline with age while sex hormone binding globulin (SHBG) increases, further reducing bioavailability of sex steroids (174).

2.1.6.2 Loss of muscle mass and sarcopenia

Age-related progressive muscle loss is called sarcopenia and is characterized by quantitative and qualitative deterioration of muscle cells, resulting in gradual slowing of movement and decline in strength (175). Sarcopenia affects both genders similarly. Multiple contributing factors have been identified such as neuronal and hormonal changes, inadequate nutrition, low-grade inflammation and physical inactivity (176). Histologically, there is a decrease in fiber size and number, an associated loss of motor units as well as an infiltration of fat and other non-contractile material with preferential impact on type II (fast twitch) muscle fibers (175).

The age-related decline in hormones such as sex hormones (testosterone, estrogen) and other anabolic hormones such as growth hormone (GH) and IGF-1 contribute not only

to the development of osteoporosis but also to the progressive loss of muscle mass. Androgens have been shown to increase satellite cell numbers in animals and humans (177, 178), and testosterone levels have been positively correlated to muscle strength in older men whereas no association of sex hormones with muscle strength was found in women (179). Growth hormones promote directly muscle growth and regulates mitochondrial function (180). In addition, it stimulates IGF-1 secretion in liver and muscle further promoting muscle growth but also motor neuron survival (175).

Many studies have linked an increase in proinflammatory cytokines to sarcopenia, and elevations in IL6 and CRP are associated with greater risk of losing muscle mass and strength (181), while low levels of IL6 can act as a muscle growth factor (182). Furthermore, there is evidence that elevated levels of TNF-α contribute to muscle loss by activation of apoptosis (183).

Age-related changes in muscle are also associated with a decline in mitochondrial function. Conley et al. measured key mitochondrial fluxes, ATP synthesis and O_2 uptake *in vivo* to determine mitochondrial coupling efficiency. They found that there was a significant difference of mitochondrial dysfunction between muscles within the same individual, with more damage in muscles high in type II muscle fiber content (184). Oxidative stress may play a role for mitochondrial dysfunction since level of oxidative DNA lesions were increased in older people which was associated with a reduction of protein content and key oxidative enzyme activity as well as a decline in mitochondrial capacity for ATP production (67).

Muscle loss in elderly may be further facilitated by inadequate nutritional status and decreased physical activity. Anorexia and malnutrition secondary to decreased appetite, poor

dentition, disease co-morbidities and/or isolation are well known problems in older people (185). As a consequence, protein intake may become insufficient for optimal muscle protein metabolism, although it remains unclear to what extent this contributes to sarcopenia (186). Moreover, physical activity declines with age, further promoting the decline in muscle mass and strength, and it has been demonstrated that exercise training (especially resistance training) can maintain and reverse muscle strength (187).

2.1.6.3 Loss of fat mass and central adiposity

Changes in fat mass and redistribution occur throughout the lifespan. The peak in fat mass is reached in middle or early old age, followed by a substantial decline in total body fat in advanced old age, while the percent body fat remains basically constant due to concomitant loss of body weight and fat free mass (188). In addition, fat depots are redistributed from subcutaneous fat depots to visceral locations as well as to other non-fat tissues such as bone marrow, muscle and liver. These changes may contribute to systemic lipotoxicity and increased prevalence of metabolic syndrome in older populations (189).

Aging has a substantial effect on fat cell dynamics. Fat depot size increases with middle age due to an increase in adipocyte number and size. However, there is a decrease in fat depot size in old age; this is mainly related to a decrease in fat cell size whereas accompanied by an increase in both preadipocyte and adipocyte number. However, preadipocytes of old individuals have a reduced capacity to differentiate into fat cells and to accumulate lipid compared to those from younger individuals. This is associated with a decline in several differentiation dependent genes (188). For example, the expression of C/EBPα, one of the key regulators of adipogenesis and fat cell function, declines substantially with aging (190). Since C/EBPα augments PPAR-γ expression, a decline in

C/EBPα and PPAR-γ may contribute to the decrease in fat cell size and altered fat cell functioning resulting in decreased lipid accumulation in fat cells (188). In addition, aging is associated with increased levels of pro-inflammatory cytokines such as TNF-α, released from preadipocytes (191, 192). TNF-α inhibits C/EBPα and PPAR-γ expression and activity, thereby further enhancing the above mentioned effects and contributing to reduced insulin responsiveness in fat cells (193).

Ectopic fat accumulation is increased with aging. There are several mechanisms suggested to explain this phenomenon (188). First, diminished adipose tissue function increases the likelihood that lipids are stored in non-fat tissues. For example, lipodystrophic syndromes are associated with a lack of fat tissue and accumulation of ectopic fat depots (194). Another cause may be the dysdifferentiation of non-adipose mesenchymal cells such as muscle satellite cells, osteoblasts and macrophages, into adipocyte-like cells. These cells can acquire features of adipocytes, including the ability to express aP2 and PPAR-γ2 and can accumulate lipids (195). However, lipid accumulation in non-adipose tissues has deleterious effects and can result in metabolic dysfunction and cellular damage due to increased lipid peroxidation (188).

Loss of fat mass with age is found primarily in subcutaneous fat depots, with substantial retention of fat in visceral depots (196). Since visceral adiposity is associated with a higher production of pro-inflammatory cytokines, this increase in the ratio of visceral to subcutaneous fat storage tends to decrease insulin sensitivity, and increase the risk of atherosclerosis and diabetes (197) and may thereby contribute to the development of age-related metabolic disorders.

2.1.6.4 Consequences

Epidemiological studies in humans have demonstrated positive correlation of changes in body composition with age to risk of metabolic disease, physical disability and frailty in the elderly population. For example, a 5.5 longitudinal study in healthy older people (mean baseline age 71 years found that both genders showed a significant decline of appendicular and leg free fat mass (FFM) while waist circumference and total fat mass (FM) increased despite overall weight stability over the study period. This was associated with reduced ability to perform activities of daily living (198). In another prospective study performed in men aged 60 to 79, muscle mass was inversely associated with mortality while high waist circumference was positively correlated to mortality (199).

Some studies have demonstrated an age-related decline in resting energy expenditure due to the loss of fat-free mass and alterations in metabolically active components (200, 201), and a decrease of total energy expenditure due to decreased physical activity (202). This may lead to a positive energy balance contributing to the development of obesity and central adiposity, often observed in middle and early old age.

The increase in central obesity with age causes a higher production of pro-inflammatory cytokines that contributes to a low-grade inflammatory state. As previously discussed, inflammatory cytokines are implicated in the development of both, osteoporosis and sarcopenia, and thus may further enhance the age-related changes in these tissues. Furthermore, the pro-inflammatory cytokines are involved in the development of insulin insensitivity (184), which is then exacerbated by the decreased metabolic functioning and wasting of muscle tissue (175).

2.1.7 Inflammatory stress

Aging is associated with an imbalance between the adaptive and innate immunity leading to a pro-inflammatory phenotype; this phenomenon has been termed 'inflamm-aging' (27). While the adaptive immune system declines with age (immunosenescence), innate immunity seems to be activated. Epidemiological studies indicate that serum levels of inflammatory markers are 2-4 fold elevated in aged people compared to their younger counterparts (203). The most important cytokines in this process appear to be TNF-α, IL-6, IL-1β, TGF-β and IL-10, and studies show that high levels of these markers are correlated with greater morbidity, disability and mortality (204). Accordingly, inflammatory stress has been implicated as one of the most important factors in the development of chronic diseases of aging (204).

Various stimuli and factors are involved in the cytokine production, including immunological stimuli, the renin-angiotensin system (RAS), ROS, adipose tissue metabolism and PPARs (204). Most of these pathways converge on modulating the transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), which is key player in activating the expression of pro-inflammatory genes such as those for TNF-α, IL-6 and IL-1β. Genetic polymorphisms may also influence the individual level of produced cytokines and therefore the intensity of the inflammatory response (204).

The transcription factor NF-κB has a crucial role in regulating the inflammatory response in multiple cell types. In the resting state, NF-κB is associated with inhibitory proteins IκBs and thereby sequestered in the cytoplasm. However, IκBs are rapidly phosphorylated and degraded by the proteasomes in response to various stimuli, resulting in

free NF-κB that translocates to the nucleus to regulate gene expression of its target genes (205). In addition to the IκB-kinase (IKK)-dependent regulation, NF-KB can be posttranslationally modified independently of IκB by ubiquitination, acetylation and isomerization of specific amino acid residues (206); e.g. deacetylation by Sirt1 leads to its inactivation (140).

There is a bidirectional interaction between oxidative stress and inflammation. H_2O_2 produced from the mitochondria leads to NF-κB activation in the cytosol and consecutively to the production of pro-inflammatory cytokines. Although the response to oxidative stimuli is short-lived under normal conditions, it can lead to chronic inflammatory responses when the oxidative stimuli are not well controlled (207). On the other hand, induction of TNF-α can increase cellular ROS production, mainly by activation of NADPH oxidase. Because of a positive feedback loop, this results in further activation of NF-κB and production of TNF-α (208, 209). The interaction between oxidative stress and inflammation appears to play an important role in vascular inflammation and the development of atherosclerosis even in the absence of other traditional risk factors. For instance, it was demonstrated that TNF-α in isolated carotid arteries from young rats can promote an atherosclerotic phenotype by increasing superoxide production and NF-κB activation, and treatment with an anti-TNF-α agent exerted vasculoprotective effects in aged rats (210).

The local renin-angiotensin system (RAS) plays also a role in NF-κB stimulated inflammation, particularly in endothelial cells (209). Most of these effects are mediated by angiotensin II (Ang II). The classical mechanism involves the phosphorylation of IκB; however, binding to its AT1 receptor can also activate NADPH oxidase resulting in

increased ROS production and NF-κB activation (211). In addition, there is evidence for a Ras/MEK/ERK1/2 signaling pathway dependent activation of NF-κB (212)

Adipose tissue is a key source of pro-inflammatory cytokines, including IL-6, TNF-α, C-reactive protein, plasminogen activator inhibitor-1 (PAI-I) and angiotensin (213). Therefore, the increase of visceral adipose tissue with age is a major contributor to the pro-inflammatory state of older people. In support of this concept, obese people show increased levels of pro-inflammatory markers and a greater incidence of inflammation-based diseases than lean people (214).

Newer evidence from genetic analysis of candidate genes in centenarians and patients with age-related diseases indicates that some genes related to inflammation are implicated in human longevity. There are positive associations between polymorphic markers of IL-6, IL-10 and TGF-β1 and longevity. Moreover, additive effects of gene variants of cytokines with other genes involved in metabolic pathways such as PPARγ were reported to influence the lifespan (215, 216). Thus, genetic polymorphism may influence the individual level of circulating cytokines and consequently determine overall survival.

2.1.8 Insulin sensitivity

Aging is associated with a high prevalence of type 2 diabetes and impaired glucose tolerance, which contributes to a substantial risk for cardiovascular disease, and consecutively to early morbidity and mortality. Although the interaction of many factors associated with aging such as the development of central adiposity, the decrease in physical activity, the decrease in muscle mass, medications and coexisting illness contribute to the

age-related insulin resistance, the influence of the aging process *per se* on this impairment is still not fully understood (217).

Although obesity is clearly a major contributor to the development of insulin-resistance, there is also a slight age-related increase in fasting glucose levels and a reduction in insulin sensitivity in healthy older and lean people (218). The impairment of insulin action can appear at all levels of the insulin signaling pathway and affects glucose metabolism as well as lipid synthesis, protein synthesis and other insulin signaling pathways. While the insulin signaling system is highly conserved and practically expressed in all cell types, the major insulin responsive tissues are muscle, liver and adipose tissue (217).

A great body of evidence suggests that the increased production of adipocytokines is the underlying link between central adiposity and insulin resistance (193). The most important cytokine in this process is TNF-α. Studies have shown that TNF-α can inhibit the tyrosine kinase activity of the insulin receptor (IR) and induces serine phosphorylation of IRS-1 in adipocytes and hepatoma cells resulting in reduced autophosphorylation and activity of the IR (219, 220). In addition, TNF-α decreased Glut-4 mediated glucose uptake and stimulates the mobilization of stored trigylcerides in human adipocytes in cell culture (221). However, the increased release of free fatty acids can result in insulin resistance of muscle and liver (222, 223). In addition, TNF-α, released into the systemic circulation by adipose tissue can have direct effects on other tissues, such as muscle and liver. For example, chronic TNF-α exposure impaired both insulin-stimulated glucose uptake and Glut4 translocation in primary myotubes (224), and it inhibited insulin signaling and insulin-induced glucose production in liver (225, 226). Furthermore, TNF-α exerts indirect effects

on insulin signaling by suppressing the transcription of adiponectin in adipocytes. Adiponectin has a wide range of actions, including increasing whole body insulin sensitivity by reducing endogenous hepatic glucose production and promoting glucose uptake and oxidation in muscle. However, in contrast to other adipokines, circulating plasma levels are reduced in individuals with visceral obesity and insulin resistance (227). Since TNF-α levels rise with age even in non-obese people, it may also play a role in the development of insulin resistance in healthy older people (228).

A cross-talk between Insulin/IGF- and ROS-redox pathways may suggest a link between oxidative stress and insulin resistance (229). It has been shown that activation of the insulin receptor activates NADPH-oxidase to generate H_2O_2. This endogenous burst of low-level H_2O_2 reversibly inhibits the protein tyrosine phophatase (PTB) activity that negatively regulates the autophosphorylation of the insulin receptor (IR) (230). Thus, the IR mediated ROS production implicates a positive autoregulatory mechanism in early insulin signal transduction. In addition, it has an essential role in the downstream insulin cascade by enhancing phosphoinositide 3 kinase (PI3K) and Akt activity (231). On the other hand, long-term exposure and/or higher concentration of H_2O_2 exert inhibitory effects on insulin action by multiple molecular mechanisms. These include direct oxidant-induced protein modifications of insulin signaling molecules, changes in gene regulation as well as impaired signal transduction due to enhanced serine phosphorylation of IR and IRS by activated Serine/Threonine-kinases such as ERK, JNK and IKK (232). Therefore, the increase of endogenous oxidative stress with age leading to impaired insulin signal transmission may be part of the mechanisms of the age-related insulin resistance.

The renin-angiotensin-system (RAS) is mainly responsible for adverse structural changes of arteries leading to endothelial dysfunction and cardiovascular disease seen in patients with hypertension and diabetes (233). However, multiple studies suggest also an involvement of the RAS in the pathogenesis of insulin resistance and diabetes. This hypothesis is based on the observation that inhibition of RAS, either by angiotensin-converting enzyme (ACE) inhibitors or by angiotensin receptor blockade (ARB) reduced the incidence of type II diabetes in patients with and without hypertension in these clinical trials (234). Proposed underlying mechanisms are mainly mediated by angiotensin II (ANG II) stimulation of the AT 1 receptor. This can lead to increased ROS production by activation of NADPH oxidase and increased expression of cytokines such as TNF-α, IL-6 and NF-κB leading to impairment of insulin signaling and glucose uptake in muscle and other tissues. Moreover, ANG II increases the serine phosphorylation of IR β-subunit, IRS-1, and the p85 regulatory subunit of PI-3-kinase via activation of the serine/threonine kinases JNK and ERK 1/2 (234). Additionally, it was proposed that RAS blockade increases the number of small insulin-sensitive adipocytes and inhibits lipid storage in muscle and other tissues, thus improving insulin sensitivity (235). Since adipocytes contain all components of RAS, and RAS is up-regulated in obese patients, the RAS induced insulin resistance may be a further link between obesity and insulin resistance (236).

Insulin levels are generally decreased in older people due to insulin secretory defects, suggesting pancreatic β-cell dysfunction. Thus, the compensatory hyperinsulinemia, needed to maintain normal glucose metabolism during a state of decreased insulin sensitivity can not occur. Consequently, the age-related insulin secretory dysfunction contributes to the glucose intolerance and the prevalence of type 2 diabetes in the elderly (237).

2.2 Adipose tissue

2.2.1 Background

Adipose tissue has been intensively studied over the last decades as it transformed from an energy storage depot to an active endocrine organ participating in energy homeostasis, glucose metabolism and immune function. It differs from many other tissues as it is distributed around the body and forms clusters around other organs such as heart and kidneys (238). Dispersed fat cells can also occur within other tissues such as bone and muscle and are more often found in elderly and under lipodystrophic conditions (188, 194).

Adipose tissue in mammals can be distinguished into white adipose tissue (WAT) and brown adipose tissue (BAT) with almost opposed physiological roles. In contrast to WAT, BAT serves primarily in thermogenesis by dissipating energy in form of heat established by increased mitochondrial biogenesis and increased expression of the uncoupling protein (UCP)-1 (239). Despite their different functions, they are most likely derived from common precursor cells and maintain the ability to transform into each other depending from the metabolic needs and stimuli (240).

In humans, the WAT can be distinguished into subcutaneous and visceral WAT, which have different gene expression profiles of several genes, specific functional characteristics and gender specific distribution. The visceral fat is found in the intraperitoneal and retroperitoneal compartments. Intraperitoneal fat can be further divided into omental, mesenteric and in rodents perigonadal/epididymal fat. The retroperitoneal fat is located along the ventral surface of the kidney and dorsal from of the intestines, and represents only a minor part in humans. The subcutaneous can be subdivided into superficial

and deep layers and is found to a greater extent around the hip, gluteal and femoral region (241). Premenopausal females appear to accumulate a greater proportion of their body fat in the gluteal and femoral region (gynoid type), whereas men tend to store their excess fat in the abdominal cavity (android type) (242). Visceral fat is unique compared to other adipose regions because of its close proximity and direct connection to the liver by the portal vein. In addition, visceral adipocytes are smaller than subcutaneous cells, accumulate fewer lipids, show a greater response to β-adrenergic agonists and glucocorticoids, have a higher lipolytic rate, and are metabolically more active by secreting higher amounts of adipocytokines (243). All these different characteristics contribute to the greater impact of visceral adiposity to the development of insulin resistance and metabolic diseases.

Adipose tissue growth can appear by an increase in both adipocyte size (hypertrophy) and number (hyperplasia), and is controlled by interactions of multiple factors including hormones, transcription factors, autonomic nervous system and diet (244, 245). While studies suggest that hyperplasia is highest during the first years of life and at puberty, hypertrophy seems to be more responsible for fat mass expansion in adulthood. However, the capacity to increase the adipocyte number remains throughout life, although it is hypothesized that a critical fat cell size is necessary before hyperplasia takes place and that the mature adipocyte stimulates the proliferation of preadipocytes in an autocrine/paracrine manner (246-248).

The cell dividing capacity of mature adipocytes is very limited, thus hyperplasia involves the differentiation of precursor cells to new adipocytes. Differentiation to adipocytes can be induced from embryonic stem cells (249), and it has been reported that mulitpotent adipose derived stem cells (MADS) have been isolated from human adipose

tissue harvested by liposuction, which can differentiate into mesenchymal cells giving rise to adipogenic, osteogenic, chondrogenic and myogenic precursor cells (250). However, molecular and cellular differentiation events have been best studied in *in vitro* culture systems using undifferentiated fibroblast-like preadipocytes (251). To undergo differentiation, committed preadipocytes have to withdraw from the cell cycle (growth arrest) and receive a combination of mitogenic and adipogenic signals, which may vary depending on the specific cell culture model used. In general, the adipogenic induction cocktail includes supraphysiological concentrations of insulin, dexamethason (DEX), and isobuthylmethylxanthine (IBMX), indicating the involvement of insulin/IGF-1, glucocorticoid and c-AMP signaling pathway as required signals for differentiation. Adipogenesis is accompanied by characteristic morphological and gene transcription changes. Morphological hallmark is the alteration of the cell shape from a fibroblastic to a spherical shape. Transcriptionally gene expression changes can be divided into early, intermediate and late mRNA/protein markers. Key regulators include the transcription factor families CCAT/enhancer binding protein factor (C/EBP α, β and δ) and peroxisome proliferator activated receptor (PPAR)-γ as well as the adipocyte determination and differentiation factor (ADD)-1 and the sterol regulatory-element binding protein (SREBP)-1c. Activation of the transcriptional cascade leads finally to increased activity and mRNA levels for enzymes involved in lipid metabolism, insulin sensitivity and glucose uptake, and adipocyte-specific production of hormones and cytokines (251, 252).

2.2.2 Cell composition

In addition to adipocytes, adipose tissue consists of a variety of other cell types. These include stromal/vascular cells, immune cells and nerve fibres of the autonomic nervous tissue (213, 245).

Studies have demonstrated that there is a dynamic interplay between adipogenesis and angiogenesis (253-255). Adipocyte differentiation took place in adipogenic/angiogenic cell clusters and depended significantly on the secretion of the vascular endothelial growth factor (VEGF) of the non-fat cells in these clusters. On the other hand, VEGF antibodies could inhibit angiogenesis and decrease the formation of adipogenic/angiogenic cell clusters (255). Similarly in the other study, preadipocytes induced angiogenesis in a *vivo*-model while PPAR-γ inhibition reduced preadipocyte-induced angiogenesis and VEGF-receptor antibodies inhibited angiogenesis and subsequent further predadipocyte differentiation suggesting a paracrine interaction between adipocytes and stromal/vascular cells (253).

The stromal/vascular fraction of adipose tissue constitutes also an important residence for macrophages that could originate from either preadipocytes or blood monocytes. Cousin et al. provided evidence that preadipocytes could function as macrophage like cells and that this ability was lost with differentiation into adipocytes (256). In addition, studies indicate that the macrophage population in adipose tissue resulted from recruitment of blood monocytes. Adipocyte-derived proteins such as monocyte chemoattractant protein (MCP)-1, IL-8 and leptin in mature adipocyte-conditioned medium were capable to activate endothelial cells to secrete soluble adhesion molecules such as intercellular adhesion molecule (ICAM)-1 and platelet endothelial cell adhesion molecule (PECAM)-1 that stimulated the adhesion and diapedesis of blood monocytes (257). In

support of this concept, obesity is positively correlated with higher levels of adhesion molecules such as ICAM-1, vascular cellular adhesion molecule (VCAM)-1, E- and P-selectin (258, 259) and fat tissue from adipose individuals shows a greater infiltration of macrophages (257). The latter was also confirmed in a gene expression profile study in mice demonstrating that adiposity up-regulated a large proportion of transcripts encoding for proteins characteristic for macrophages (260). In addition, visceral fat was associated with increased expression of adhesion molecules mRNA compared to subcutaneous fat, supporting the link between visceral adiposity, inflammation and increased risk of cardiovascular disease (258).

It has been reported that the adipose tissue is regulated by the autonomous nervous system directly and indirectly as a result of its effect on insulin secretion (261). While sympathetic activation in white adipose tissue led to an increase in lipolysis and release of free fatty acids, and inhibited adipocyte proliferation *in vivo* (262, 263), it increased thermogenesis and proliferation of brown adipose tissue (239). Furthermore, adipose site-related differences were suggested with a weaker catecholamine-induced lipolytic response in subcutaneous fat than in visceral fat, which was more evident in women than in men. This effect could be explained by the different distribution of α_2-adreno and $\beta_{1,2}$-adrenoreceptors, which is not only sex-specific but also depend on the extent of fat mass and fat cell hypertrophy (261). In contrast, the evidence for parasympathetic innervation is scarce and controversial. However, Kreier et al. reported an overall anabolic role for the vagal input to white adipose tissue (264).

2.2.3 Endocrine function

The view of the adipose tissue as a passive energy-storing tissue has changed with the discovery of leptin. Since then, many new hormones and factors secreted from adipocytes or by other cell types within the adipose tissue such as macrophages have been identified, and its active endocrine role is meanwhile well recognized (213). In addition to regulating energy and nutrient homeostasis locally, adipose derived hormones such as leptin and adiponectin (discussed below) are involved in modulating energy balance, lipid and glucose metabolism in other tissues, and cytokines such as TNF-α and Il-6 (discussed below) play a central role in the pathogenesis of inflammation and metabolic diseases (265). Furthermore, adipose tissue expresses enzymes influencing glucocorticoid conversion such as 11 β-hydroxysteroid dehydrogenase (11 β HSD) that converts inactive cortisone to the active form cortisol, and sex hormone production promoting the formation of estrogen from androgenic precursors (266). Particularly increased 11 β-HSD activities may contribute to visceral adiposity and metabolic syndrome (267, 268). In addition, adipose tissue expresses all enzymes of the renin-angiotensin-system (RAS) that contributes locally and systemically to inflammation and oxidative stress production (269, 270).

2.2.3.1 Leptin

The hormone leptin, a 167 amino acids long protein product of the obesity (*ob*) gene, is derived almost exclusively from the adipose tissue. Its main function is to regulate energy homeostasis by repressing food intake and promoting energy expenditure; however, it also modulates neuroendocrine and immune function (271).

The main action of leptin is mediated by interaction with leptin receptors in the CNS. Highest expression of these receptors is found on neurons in the hypothalamus. Binding to

its receptor causes rapid activation of Janus activating kinase (JAK) 2 leading to tyrosine phophorylation of the receptor and consecutively to binding of src homology 2 (SH2)-domain containing proteins such as STAT3 and SH-2-domain phosphotyrosine phosphatase (SHP-2). Downstream targets of this signaling cascade include activation of the extracellular signal-regulated kinase (ERK1/2), c-FOS and PI3K (272).

Serum leptin levels are proportional to the amount of adipose tissue and abdominal obesity is generally correlated with higher circulating leptin concentrations. However, despite higher levels, obese individuals seem to develop a state of leptin-resistance in the CNS either caused by decreased uptake by the brain blood barrier or by overexpression of negative regulators of the leptin signaling cascade such as suppressor of cytokine signaling (SOCS) 3 protein and protein phosphatase 1B (PTP-1B) (227, 272).

In addition to its central action, leptin receptors are present in peripheral tissues such as adipose tissue, liver, skeletal muscle and pancreatic β-cells. Leptin signaling in these tissues regulate glucose homeostasis, improve insulin sensitivity and inhibit accumulation of triglycerides in liver and muscle (265). Moreover, anti-immunosuppressive effects by interacting with T lymphocytes were reported, which may activate the innate immune system. While this activation may have beneficial effects under acute conditions and starvation, chronically activation may be deleterious and may contribute to the pro-inflammatory state associated with obesity (213, 272).

2.2.3.2 Adiponectin

Adiponectin, also known as adipocyte complement-related protein 30 (Arcp30), adipose most abundant gene transcript 1 (apM1) and adipoQ, is a 30 kDA protein and mainly synthesized by adipocytes (273). It circulates in high concentration in the plasma

with circulating levels in females two to three times higher than in males. In contrast to most other adipocytokines, adiponectin levels decrease with increasing fat mass, particularly visceral obesity, and are negatively correlated with insulin resistance and type II diabetes (273, 274).

The adiponectin monomer consists of three domains, which are a variable amino-terminal signal peptide, a collagen-triple helix formation, and a globular head at the carboxyl-terminus. In serum adiponectin exist in form of homotrimers, which can further combine to multimeric complexes. These complexes can be classified into two major groups: low molecular weight (LMW) oligomers which include trimers and hexamers, and high molecular weight (HMW) multimers consisting of four to six trimers or more (12-, 18-mers and larger). Furthermore, adiponectin can undergo several kinds of post-translational modification including hydroxylation and disulphide bond formation, which play a role for oligmerization (273).

There are two adiponectin receptors identified. AdipoR1 is ubiquitously expressed but most abundant in skeletal muscle, AdipoR2 is most abundantly expressed in liver. In addition to lower circulating adiponectin levels during hyperinsulinemia, the expression of both receptors is also decreased by insulin (265, 273).

Adiponectin is a multifunctional protein with potent insulin-sensitizing effects. It increases fatty acid oxidation and glucose uptake in skeletal muscle, and it reduces glucose production in liver; most of these effects are mediated by activation of AMPK (275). It also suppresses TNF-α expression thereby exerting anti-atherosclerotic, anti-atherogenic and anti-inflammatory effects which are beneficial in protection against vascular disesase (276).

2.2.3.3 Tumor necrosis factor (TNF) -α

One of the most potent pro-inflammatory cytokines is TNF-α that is mainly produced by activated macrophages within the adipose tissue rather than by adipocytes (213). It is a 26 kDa plasma membrane protein which is cleaved in the extracellular domain by matrix metalloproteinases to release a 17 kDa soluble form. Responses to TNF-α are mediated by binding to one of two distinct cell surface receptors, TNFR1 and TNFR2, which differ in their cellular and tissue distribtion and show both distinct and overlapping activities (277). In addition, soluble forms of TNF-α receptors are known and it is suggested that these compete for binding with the cell surface receptors to inhibit TNF-α activity (271). Binding of TNF-α to its cell-surface receptors results in subsequent signaling events, which include activation of NF-κB, JNK and p38 MAPK. Overall, these signaling cascades may lead to wide range of cellular responses such as death, survival, proliferation, differentiation and migration (277).

Similarly to the other cytokines, higher levels of TNF-α are found in visceral adipose fat depots than in subcutaneous fat (278). Circulating TNF-α concentrations rises with increasing obesity and are positively correlated with insulin resistance (213). In addition, TNF-α plays a pivotal role in the pathogenesis of vascular inflammation and endothelial dysfunction (279), providing an important link between visceral adiposity, insulin resistance and vascular diseases.

2.2.3.4 Interleukin 6 (IL-6)

IL-6 is an important regulator of inflammation and immune response. It is produced by a number of cells such as endothelial cells, fibroblasts, osteoblasts, myocytes and

macrophages; however, the adipose tissue is recognized as one of the major production sites and contributes 10 to 35 % of circulating plasma levels in resting, healthy humans. Within the adipose tissue, the adipocytes release only a fraction of the total IL-6; non-fat cells in the adipose matrix such as macrophages and stromavascular cells produce the remaining amount. Hypertrophic enlargement of adipocytes is correlated with increased IL-6 production, and visceral fat contribution is greater than subcutaneous fat production. Accordingly, circulating levels are slightly but significantly elevated with visceral adiposity and contribute to a chronic low-grade inflammation state in obese people (213, 280).

The IL-6 receptor consists of two forms: a membrane bound form and a soluble form (IL-6Rs) generated by alternative splicing or proteolytic cleavage of the membrane-bound receptor. In addition, IL-6 in form of IL-6/IL-6Rs, can bind to gp130, a common signal transducer unit for IL-6 type cytokines including IL-11, leukemia inhibitory factor and oncostatin M. After binding to the receptors, the IL-6/receptor complex leads to activation of the JAK/STAT pathway and MAPK signaling cascade (271, 280).

IL-6 has auto-, para-, and endocrine regulatory function. While in muscle insulin sensitizing effects with increased insulin-stimulated glucose uptake are reported, it suppresses insulin-signal transduction in liver and adipocytes resulting in reduced glucose uptake and increased release of free fatty acid from the adipose tissue (280). Similarly, double-sided actions are found in terms of inflammation. Some evidence points to anti-inflammatory functions because it inhibits TNF-α production and stimulates the production of anti-inflammatory IL-1 receptor antagonist and IL-10. On the other hand, it promotes the release of C-reactive protein and other acute phase proteins from the liver, promotes the migration of polymorphnuclear leukocytes and increased expression of adhesion molecules

in endothelial cells, and leads to activation of local RAS thereby favoring chronic inflammation and vascular damage (213, 281)

2.2.4 Energy metabolism

The adipose tissue plays a major role in total body lipid and energy homeostasis. First, it is the most important site of energy storage and manages the release or uptake of free fatty acids and triglycerides according to the body's supply and demand. Secondly, it acts as an endocrine organ in a hormonal and neuronal network by integrating signals from other part of the body and by secreting adipocytokines that regulate food and energy balance locally and peripherally.

Lipid storage in adipocytes is regulated by fatty acid uptake, lipogenesis and lipolysis, and reflects the balance or imbalance between energy intake and expenditure. Triglyceride synthesis can start as de novo lipogenesis from acetyl-CoA that involves the key enzymes acyl-CoA carboxilase (ACC) and fatty acid synthase (FAS), or from the uptake of circulating free fatty acids (FFA). In humans, de novo lipogenesis seems to be of minor importance in adipocytes and plays mainly a role under high caloric, low-fat high-carbohydrate-diets (282). Under normal condition, most triglycerides derive from the uptake of FFA, which are released from circulating lipoprotein-triglycerides by lipoprotein lipase. In the adipocytes, the FFA will be esterified to glycerol-3-phophate and stored in the lipid droplets (276).

Under fasting or enhanced energy demand adipose tissue fat stores are used to supply the body's energy requirements. The generation of FFA (lipolysis) involves the hydrolysis of the esterbonds from the glycerol backbone, which is mediated by the three lipases:

adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoglyceride lipase (MGL) (283). Although HSL shows broad substrate specificity, it preferentially hydrolyzes diglycerides, whereas ATGL prefers triglycerides over diglycerides.

Adipose tissue is also involved in regulation of glucose homeostasis. It participates in postprandial insulin-mediated glucose uptake and disposal, and both obesity and lipodystrophy are associated with hyperglycemia and insulin-resistance. In addition, the secretion of several hormones such as leptin, adiponectin, resistin, and visfatin can modulate insulin sensitivity and glucose metabolism (284). Furthermore, circulating non-esterified fatty acids, primarily released from the adipose tissue during fasting, may reduce glucose uptake in muscle and adipose tissue, promote hepatic glucose production, and stimulate pancreatic insulin secretion (222, 285). These effects are most likely caused to spare glucose utilization for the CNS. However, prolonged exposure as seen under insulin-resistant and diabetic conditions may lead to β-cell apoptosis and lipotoxicity of other tissues such as muscle and liver, thus producing a vicious cycle by further increasing insulin resistance (286, 287)

Glucose and lipid metabolism is controlled by hormonal and neuronal stimuli, which also depend on other conditions such as age, gender, physical activity and nutritional factors. The most potent stimulators of lipolysis are catecholamines acting on β1 and β2 adrenergic receptors in human adipose tissue. Binding to the receptor stimulates G_s proteins to activate adenylate cyclase, which results in increase in c-AMP and subsequently to activation of c-AMP-dependent protein kinase-A (PKA). Other hormones that act via G_s protein-couple receptors and stimulate PKA are glucagon, parathyroid hormone, thyrotropin, α-melanocyte-stimulating hormone, and adrenocorticotropin (283). PKA phophorylates a

number of target proteins including lipolytic enzymes such as HSL and lipid droplet associated proteins. On the other hand, there are several anti-lipolytic factors acting through inhibitory G_i protein-coupled receptors. Examples are α2-adrenergic receptor catechomines, prostaglandins, adenosine and nicotinic acid. However, the most potent inhibitors of lipolysis are insulin and IGF, which interact with the insulin signaling cascade and leads to activation of several protein kinases such as 3-phosphoinositide-dependent protein kinases (PDK1 and 2) and protein kinase C (PKC). Inhibition of lipolysis includes the phosphorylation of phophodiesterase 3B resulting in degradation of c-AMP and decreased PKA activity.

Insulin, released in the fed state, is also one of most important hormones influencing lipogenesis. The stimulating effect is caused by several mechanisms, these include increased uptake of glucose in the adipose cell, activation of lipogenic and glycolytic enzymes via covalent modification, and activation of the transcription factors sterol regulatory element binding protein (SREBP)-1 and PPAR-γ (288).

Lipogenesis is also stimulated in an endocrine/autocrine manner by the acylation stimulating protein (ASP), a small peptide produced by the adipose tissue. In *vitro* and in *vivo* studies indicate that ASP stimulates triglyceride clearance from the plasma and accumaltion in the adipocytes while simultaneously decreasing lipolysis. However, the specific underlying molecular mechanisms are still not known (275).

2.2.5 Source of ROS

Adipose tissue is a significant source of ROS production, and obesity can lead to an increase in systemic oxidative stress. Supporting evidence is provided by studies, which

show that BMI and fat accumulation correlates with systemic oxidative stress markers in rodents and humans (289, 290). Furthermore, abdominal fat and weight loss after bariatric surgery was correlated with decreases of plasma derivatives of reactive oxidative metabolites (291).

The major source of ROS production in adipocytes is NADPH oxidase, and two isoforms, NOX2 and NOX4, have been identified (31). Furukawa et al. showed that adipose tissue NOX4 is up-regulated in obese mice (290). They also indicated that NOX4 mediated ROS production seemed to be stimulated by fatty acids, since ROS production was increased concomitantly with the accumulation of fat into the differentiating 3T3-L1 adipocytes, and was also elevated in differentiated adipocytes after incubation with free fatty acids in the medium. Inhibitors of NADPH oxidase suppressed both effects. In addition, adipose tissue plays a pivotal role in the production of TNF-α and other cytokines, and TNF-α appears to be an important activator of ROS production. Most of this effect is mediated by inducing NADPH oxidase gene expression and activity as demonstrated in multiple tissues such as cerebral vascular endothelial cells, colon epithelial cells and vascular smooth muscle cells (292-294). However, since ROS stimulates the NF-κB-dependent activation of pro-inflammatory cytokine expression including TNF-α (295), this bidirectional interaction may act as a vicious cycle under conditions of either enhanced ROS or cytokine production as seen in obesity.

Mitochondrial ROS production is dependent on the mitochondrial potential, which is determined by the backflow of H^+ across the mitochondrial membrane. The uncoupling proteins (UCP) are a family of proteins in the inner mitochondrial membrane, which uncouple the proton flux from ATP-synthesis, thereby reducing the mitochondrial potential

and ROS formation (296). UCP 2 and 3 are ubiquitously expressed and played an important role in modulating mitochondrial ROS production in several tissues and cells including macrophages and endothelial cells (297). For example, over-expression of recombinant UCP-2 in primary cultured human vascular smooth muscle cells reversed high glucose and Angiotensin II stimulated ROS production (298), whereas defective UCP2 expression in endothelial cells increased intracellular and extracellular oxidative stress markers (299). Since UCP2 is also highly expressed in adipose tissue and tissues rich in macrophages (300), it is very likely to play a major role in adipocyte ROS production. In addition, UCP2 expression appears to function as a free fatty acid transporter that support the fatty acid (FA) cycling to the mitochondria for oxidation (296), Accordingly, it has been postulated that free fatty acids up-regulate UCP expression by mechanisms that may involve the PPAR-γ transcription factors (301, 302). They also seem to be up-regulated in obese animals and humans (303, 304), suggesting a protective and compensatory role of UCP2; although, some evidence indicates decreased levels in obese subjects, which is compatible with reduced energy expenditure in obesity (305).

2.3 Dietary calcium and dairy modulation of adipose tissue function

Studies over the last decade indicate that dietary calcium has not only health promoting effects on bone and mineral metabolism but also on adipose tissue function by modulating adiposity, fat metabolism and endocrine activity. Dairy products are the major contributors to dietary calcium and are often used to study the effects of calcium; however,

effects are often augmented when dairy products are compared to supplemental calcium, which is due to additional components in dairy as discussed in the next chapter.

2.3.1 Energy storage and adiposity

Epidemiological and experimental evidence pointed to an inverse relationship between calcium intake and body weight and adiposity in multiple studies (306). Although these effects are usually seen in all population groups, some studies show gender and ethnic differences such as a cross-sectional survey in 3246 Israelis aged 25 to 60 years, which showed a significant inverse association between daily calcium intake and waist circumference for women but not for men (307), and a cross-sectional study constructed among US adults from the National Health and Nutrition Examination Survey 1999-2004 data, in which the effects of a variety of dairy products and their related nutrients on obesity, central obesity and metabolic syndrome were assessed. Ethnic differences in metabolic outcomes were found, with higher BMI for Non-Hispanic Blacks and Mexican Americans, which could be at least in part explained by variations in dairy-related nutrients (308). Lower weight, BMI and body fat mass and higher lean body mass were also found for the highest calcium intake group in a study conducted in 96 normal weight and obese adolescents providing further support for the importance of an adequate calcium intake during childhood and early adulthood (309).

Calcium and dairy appear to have augmenting effects on weight and fat loss under caloric restriction and decreases adiposity under eucaloric conditions in individuals with low calcium intake. For example, Zemel et al. conducted a randomized, placebo-controlled weight loss trial over 24 weeks in 32 obese adults with habitual low calcium intake, in which

patients were maintained on a standard hypocaloric diet either supplemented with placebo, 800 mg of calcium or 1200 to 1300 mg of calcium in form of dairy. The high calcium and dairy group increased their weight loss by 26% and 70%, respectively, compared to the placebo group; calcium and dairy also significantly augmented fat loss from the trunk region (310). This effect seems to be diminished in individuals whose calcium intake is adequate or only moderately low (311). Although no weight loss effects of high calcium treatment is seen under eucaloric conditions, it still significantly modulates body composition by reducing fat mass and increasing lean tissue mass (312).

Mechanistic explanations are suggested by multiple animal and cell studies. Low calcium intake causes an increase in parathyroid hormone secretion and 1, 25-dihydroxy-vitamin D (calcitriol) production. Calcitriol stimulates rapid calcium influx into the cells by acting on the membrane-associated rapid response to steroid receptor (MARRS), which results, paradoxically, in an increased intracellular calcium concentration ($[Ca^{2+}]_i$) (313, 314). The calcitriol-stimulated increase in $[Ca^{2+}]_i$ promotes energy storage in adipocytes by activating key enzymes of lipogenesis such as fatty acid synthase (FAS) and inhibiting lipolytic genes (315, 316). Accordingly, dietary calcium suppresses circulating calcitriol that leads to a decrease of $[Ca^{2+}]_i$ and to a reversal of calcitriol action. In addition, calcitriol acts via the classical nuclear vitamin D receptors to inhibit UCP2 expression in adipocytes, leading to inhibition of apoptotic pathways at physiological calcitriol concentration; consequently, suppression of calcitriol with high calcium diets results in up-regulation of UCP2 and apoptosis in adipose tissue further enhancing the anti-obesity effect of high calcium diets (317, 318). Additionally, UCP2 is involved in fatty acid transport to the mitochondria, and its up-regulation increases fatty acid oxidation; that together with

increased uncoupling leads to increased energy expenditure (319, 320). These concepts are confirmed by experimental studies in mice. After induction of obesity by feeding a low calcium high sucrose diet in aP2-agouti transgenic mice, a weight loss phase was implemented with either a low-calcium hypo-caloric diet or different high calcium hypo-caloric diets. The high calcium groups showed significantly greater weight and fat pad loss and an increase in core temperature, which was associated with a decrease in FAS activity and increase in lipolysis as well as an up-regulation of UCP2 expression in WAT (321). In another study, the calcium effects on refeeding were examined after a weight loss period. The high calcium groups demonstrated less weight gain, stimulated adipocyte lipolysis, inhibited lipogenesis and adipogenesis, and were associated with higher UCP2 expression in WAT and UCP3 expression in muscle indicating a repartioning of energy from adipose tissue to muscle (320).

Studies have shown that the anti-obesity effect induced by high calcium diets resulted in greater reduction of visceral fat depots (322). This effects is most likely attributable to a decreased activity of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), which converts inactive cortisone to active cortisol in adipose tissue (319). In fact, calcitriol has been shown to stimulate cortisol production in human adipocytes (323), and selective over-expression of 11β-HSD in white adipose tissue in mice resulted in central obesity (268). In addition, results of a microarray study demonstrated suppression of 11β-HSD1 expressions in adipose tissue by dietary calcium in diet-induced obese mice (324). Thus, it seems very plausible that the reduced cortisol production due to suppression of calcitriol caused the preferential loss of visceral adipose tissue.

2.3.2 Adipose tissue – muscle crosstalk

Adipose tissue funtions as a large energy reserve that provides fuel for metabolic processes in other tissues in form of fatty acids during fasting or during periods of high activity. This energy is primarily used in muscle. In addition, adipose tissue secretes factors that regulate energy balance and may alter substrate utilization in muscle (252). For example, adiponectin secreted from the adipocytes is a potent activator of AMPK in muscle, which results in increased fatty oxidation and glucose uptake, thus improving hypergycemia and insulin sensitivity. Leptin also stimulates repartitioning of fatty acids towards oxidation via activation of AMPK and enhances the insulin response in muscle (325). In addition, pro- and anti-inflammatory cytokines play a role in the interaction between adipose tissue and muscle. Culture of human skeletal muscle cells with conditioned media from mature human adipocytes derived from subjects with a wide range of BMI demonstrated an inverse correlation between insulin-stimulated Akt/PKB and BMI and fat cell size (326). The selected adipokine concentration in the conditioned media also revealed an inhibitory effect TNF-α on Akt/PKB phosphorylation. Likewise, chronic exposure to TNF-α induced insulin resistance in rodents (220, 226), and TNF-α expression was significantly increased in skeletal muscle of insulin resistant humans (327). Furthermore, TNF-α appeared to impair mitochondrial biogenesis *in vitro* in white and brown adipocytes, and muscle satellite cells as well as *in vivo* in fat and muscle tissue of genetically and environmentally obese mice (328), which may contribute to the altered lipid metabolism observed in insulin resistant individuals. Conversely, the cytokine IL-6 seems to have insulin-sensitizing effects and catabolic effects on lipid metabolism; IL-6 infusion in diabetic patients decreased plasma insulin concentration and increased fatty acid utilization in muscle (329). Further, Il-15, a

cytokine highly expressed in skeletal muscle, appeared to have anabolic action on muscle fiber growth in cell culture, and administration to adult rats decreased white adipose tissue mass and circulating triacylglycerols levels, indicating significant effects on both adipose and muscle tissue (330).

Dietary calcium, particularly in form of dairy products, has been demonstrated to reduce adiposity and to promote lean tissue mass in multiple studies (310, 321, 331), indicating a role of calcium and calcitriol on energy partitioning between adipose tissue and skeletal muscle. Thus, in a follow-up cell study the effects of calcitriol and calcium antagonism in regulation of adipose-muscle cross-talk were investigated in an adipocyte-skeletal myocyte coculture system (332). Calcitriol significantly decreased fatty acid oxidation in muscle cells and concurrently increased FAS gene expression in adipocytes, while the calcium channel blocker nifedipine partially inhibited these effects. Additionally, it was found that calcitriol inhibited adiponectin production in adipocytes, which was reversed by addition of nifedipin. Calcitriol also regulated the interaction of macrophages and adipocytes, which resulted in enhanced pro-inflammatory cytokine production, as demonstrated in an adipocyte-macrophage coculture system (333), which may further contribute to alteration in energy metabolism as discussed earlier.

2.3.3 ROS

As described before, adipose tissue is major site of ROS production and oxidative stress is increased in obesity and associated disorders (290). It has been demonstrated that calcitriol stimulates ROS production in adipose tissue via both genomic and non-genomic actions, as follows (319). The calcitriol induced increase in $[Ca^{2+}]_i$ resulted in an increased

expression of NADPH oxidase, the major contributor to cytosolic ROS production, in human and murine adipocytes (334), while suppression of calcitriol by dietary calcium inhibited adipocyte NADPH oxidase expression and ROS production in aP2-transgenic mice (335). Further, calcitriol suppressed adipocyte UCP2 expression via interaction with nVDR, resulting in increased mitochondrial potential and, consequently, enhanced mitochondrial ROS production (334). This effect was augmented by addition of a mitochondrial uncoupler inhibitor and reversed by over-expression of UCP2. Moreover, the calcitriol induced an increase in adipocyte cortisol production via activation of 11β-HSD1, thereby promoting visceral adiposity, as described above, and visceral fat appeared to be associated with a higher ROS production than subcutaneous fat (336). Accordingly, suppression of calcitriol by dietary calcium resulted in a greater reduction of ROS in visceral fat than in subcutaneous fat. Overall, all these effects support a role of dietary calcium in modulating oxidative stress, and is supported by both *in vitro* and *in vivo* studies (335-337).

2.3.4 Inflammation

Dietary calcium has been shown to attenuate obesity-associated inflammatory stress in mouse models of obesity by inhibiting the expression of pro-inflammatory factors, such as TNF-α and IL-6, in visceral fat, and stimulating the expression of the anti-inflammatory factors such as IL-15 and adiponectin. Conversely, calcitriol stimulated the expression of TNF-α, IL-6 and Il-8 in differentiated adipocytes (338). Further it was demonstrated that calcitriol induced the production of an array of other inflammatory cytokines in adipocytes and/or adipose tissue derived macrophages, which include macrophage surface specific protein CD14, macrophage inhibitory factor (MIF), macrophage colony-stimulating factor

(M-CSF), macrophage inflammatory protein (MIP) and monocyte chemoattractant protein-1 (MCP-1). In addition, it regulated the cross-talk between adipocytes and macrophages, resulting in an augmentation of inflammatory cytokine production (333). Both the calcium-channel antagonist nifedipine and the mitochondrial uncoupler dinitrophenol were able to block these effects, suggesting that the calcitriol induced inflammatory cytokine production involves both calcium-dependent and mitochondrial uncoupling-dependent mechanisms.

2.4 Role of additional bioactive components in dairy in modulating adipose tissue function

Beneficial effects of milk on health are known for centuries; however, more recently physiologically active compounds in dairy products have gained major interest. Milk consists of various components and cow milk contains approximately 5% lactose, 3.2% protein, 4% lipids and 0.7% mineral salts (339). The major proteins of cow milk are a micellar casein fraction (about 80%) and a soluble whey protein fraction (about 20%) that includes β-lactose, α-Lactalbumin, and immunoglobulins. Both, caseins and whey proteins, may act as precursor for many peptides produced by enzymatic proteolyses with bioactive functions such as opiate agonist/antagonist and ACE inhibitory activities (339). In addition, milk proteins have a high nutritional quality score and contain a high proportion of the branched chain amino acids (BCAA) leucine, isoleucine and valine (340).

2.4.1 BCAA and Leucine

Dairy proteins, and particularly whey protein, provide a rich source of essential amino acids and BCAAs. Although the recommended daily allowance (RDA) guidelines for protein intake is based on maintaining short-term nitrogen balance under conditions of controlled energy intake, which is 0.8 g protein/kg body weight/day (341), higher intake levels of BCAAs are required for fulfilling other metabolic functions than serving as substrate for protein synthesis (342). Recommended intake levels also depend on the kind of method used, thus measurements based on nitrogen balance revealed lower levels than newer methodologies measuring direct amino acid oxidation and direct amino acid balance (343). Therefore, some investigators recommend protein and amino acid intake levels of up to three times the RDAs, reaching over 8g/day for leucine (342).

In contrast to the other amino acids, BCAAs are not degraded in the liver; therefore plasma and peripheral tissue levels are proportional to dietary intake (344). Their primary function is to serve as substrates for protein synthesis; additional roles are to function as precursors for alanine and glutamine synthesis and to participate in signaling pathways (342). Leucine, in particular, has a unique role in regulating muscle protein synthesis. Administration of leucine, alone or in combination with the other amino acids, stimulates muscle protein synthesis after exercise and inhibits muscle degradation during catabolic periods such as food deprivation and prolonged endurance training (345-347). In addition, leucine supplementation stimulated protein synthesis rate to the same amount in older men compared to younger men after fulfilling activities of daily living suggesting that it may be beneficial in preventing the age-related muscle loss in the elderly (348). Supplementation with whey (rich in leucine) led to greater fat loss and less reduction of lean tissue during a

weight loss period in obese people when compared to the control diet with equal energy value (349). Similarly, dairy administration exerted greater effects on weight loss, fat loss and prevention of fat regain after refeeding in obese mice and humans compared to supplemental high calcium diets (310, 320). Although these effects may be attributable to several dairy components, recent data demonstrate that leucine alters energy partitioning between adipose tissue and muscle by stimulating skeletal muscle fatty acid oxidation and suppressing lipid accumulation in adipocytes (332).

The underlying molecular mechanism for leucine's unique role in regulating protein synthesis is mainly mediated by stimulation of the mTOR complex 1 (TORC1) in skeletal muscle, which results in activation of eukaryotic initiation factor-4E (eIF4E) and S6 ribosomal protein. Leucine also activates the eukaryotic initiation factor-4G (eIF4G) through a mTOR-independent mechanism. Although these effects are largely insulin-independent, baseline fasting levels of insulin seem to be necessary for optimal effects (350).

2.4.2 Angiotensin-converting enzyme inhibitor (ACEi) peptides

Bioactive peptides with ACE inhibitory activity in the casein and whey fraction of dairy have been demonstrated to play a role in modulating adipose tissue function. ACE is one of the enzymes necessary for the successive enzymatic cleavage of angiotensinogen to the major active form angiotensin (ANG) II, and it is well documented that in addition to the classical endocrine pathway of ANG II synthesis, multiple tissues, including adipose tissue, contain all the enzymes to produce locally ANG II (270, 351).

The action of ANG II is initiated through interaction with the angiotensin receptor 1 or 2 (AT1 and AT2), which mediate partly counteracting actions. AT1 mediated effects are

mainly responsible for vascular, renal and adrenal actions of ANG II resulting in arterial vasoconstriction, renal sodium absorption and sympathetic nervous system stimulation. AT2 expression is much lower in these tissues and results in vasodilation by stimulation of endothelial nitric oxide synthase (eNOS) and bradykinin release, and in antiproliferation (234).

Adipose-derived angiotensinogen (AGT) may not only exert its effects locally at the adipose tissue but also contribute to systemic circulating levels, as numerous studies have demonstrated that circulating RAS components are increased in obesity and reduced with weight loss in both animals and humans (269, 352-354). In addition, the amount of visceral fat correlates with higher levels of RAS components than subcutaneous fat (355).

A role of RAS in adiposity is suggested by studies that demonstrated that ANG II regulates both adipogenesis and lipogenesis. For example, ANG II increased triglyceride content and activities of two key lipogenic enzymes (fatty acid synthase (FAS) and glycerol-3-phosphate dehydrogenase (GPDH)) in 3T3-L1 and human adipocytes (356). Regulation of adipogenesis is more complex and seems to be different for mesenchymal stem sells and preadipocytes. While stimulation of AT1 receptor on mesenchymal stem cells promoted differentiation to adipocytes, AT2 receptor activation inhibited adipogenesis (357). In primary cultured human preadipocytes, stimulation of AT1 receptor, which is the major expressed receptor in preadipocytes, inhibited differentiation resulting in poorly differentiated adipocytes. Accordingly, blockade of RAS, either by ACEi or angiotensin receptor blockers, promotes recruitment of preadipocytes and an increase in small insulin-sensitive adipocytes (235, 358). Furthermore, co-culture with mature adipocytes inhibited adipogenic differentiation, and this effect was abolished by AT1 receptor blockade (359)

The beneficial effects of RAS blockade also results in improvement of metabolic abnormalities. Since poorly differentiated insulin resistant adipocytes promote lipid deposition in other tissues such as muscle and bone with consecutively cell damage, blockade of RAS prevented ectopic fat deposition (360). In addition, blockade of RAS by ARB reduced the production of ROS, TNF-α and other cytokines in adipose tissue of obese mice which otherwise may exaggerate the inflammatory response (361). Moreover, adipose-derived RAS components contribute to systemic levels causing vasoconstriction and impairing the insulin signaling cascade in muscle and liver promoting overall hypertension, and insulin resistance (362). Accordingly, blockade of RAS either, by ACEi and/or ARB, resulted in significant improvement of insulin resistance and metabolic disorders in both humans and rodents (360, 363-365).

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Part Three

The Effects of Dairy Components on Energy Partitioning and Metabolic Risk in Mice: A Microarray Study[1]

[1] This manuscript has been published in similar form with co-author Julia Gouffon, Bhanu Rekapalli and Michael B. Zemel in: Journal of Nutrigenetics and Nutrigenomics 2009;2:64-77

3.1 Abstract

Background/Aim: High calcium diets modulate energy metabolism and suppress inflammatory stress. These effects are primarily mediated by calcium suppression of calcitriol. We have now investigated the contribution of additional components in dairy (branched-chain amino acids [BCAA] and Angiotensin-Converting-Enzyme inhibitors [ACEi]) on adipocyte and muscle metabolism in an animal model of diet-induced obesity.

Methods: aP2 agouti mice were fed four different 70% restricted diet groups (basal (0.4% Ca), milk (1.2 % Ca), calcium depleted milk (0.4 % Ca), or basal (0.4 % Ca) with supplemented BCAA/ACEi for 6 weeks. A high density oligonucleotide microarray approach was used to compare the effects on energy metabolism.

Results: Lipogenic genes in adipose tissue were downregulated in the milk group while in muscle protein synthetic pathways were stimulated by the Ca depleted and low Ca/BCAA/ACEi diet groups. Pathways involved in inflammation were altered in adipose tissue and muscle by all three diet treatment groups.

Conclusions: The results support our previous findings that calcium and BCAA contribute to the alteration of energy partitioning between adipose tissue and muscle. They provide further evidence for a calcium independent effect of BCAA and ACEi in energy metabolism and inflammation.

3.2 Introduction

Dietary calcium appears to play a significant role in modulating energy metabolism and obesity risk (1-3) Although these effects are mediated, in part, by calcium suppression of circulating calcitriol, providing calcium in form of dairy products causes a greater effect than supplemental calcium in both mice and humans (4-6). Additional components in dairy products that appear to contribute to this effect include branched-chain amino acids (BCAA) and angiotensin converting enzyme inhibitor (ACEi) peptides (7,8).

Dairy proteins contain a high proportion of the BCAA leucine, isoleucine and valine. Of these, leucine plays an important role in metabolic signaling pathways regulating muscle protein synthesis (9-11). Accordingly, leucine-stimulated protein synthesis may create a metabolic demand for increased fatty acid oxidation to support the energetic requirements of increased protein synthesis. In support of this concept, we recently demonstrated coordinated cross-talk between adipose tissue and skeletal muscle in response to leucine (8), with leucine suppressing lipid accumulation in adipocytes while stimulating skeletal muscle fatty acid oxidation.

ACEi lowers blood pressure by inhibiting the conversion of angiotensin I to angiotensin II (12). However, the local renin-angiotensin system (RAS) also plays an important role in adipogenesis and in the development of inflammation associated with obesity. In humans, angiotensin II inhibits preadipocyte differentiation, resulting in poorly differentiated adipocytes which may exhibit exaggerated inflammatory and metabolic abnormalities (13,14). Accordingly, blockade of RAS, either by ACEi or angiotensin receptor blockers, promotes recruitment of preadipocytes and an increase in small insulin-

sensitive adipocytes (15,16). Consequently, the ACEi peptides found in dairy foods may contribute favorably to improvements in obesity and metabolic syndrome.

Increased production of reactive oxygen species (ROS) has been implicated as a major factor in the development of chronic diseases associated with obesity (17-19). One of the underlying mechanisms is the dysregulation of adipocytokines producing a low grade systemic inflammation (20). Recent data demonstrate that calcitriol plays a role in the regulation of adipocyte ROS and adipocytokine production, suggesting that suppression of calcitriol by dietary calcium may suppress the oxidative and inflammatory stress associated with obesity (21,22).

In consideration of the multiple effects of dietary calcium and other components of dairy on adipocyte and muscle metabolism, we used a high density oligonucleotide microarray approach to identify common and differential pathways related to energy metabolism, inflammation and oxidative stress in adipose and muscle tissue in response to milk and milk components in an animal model of diet-induced obesity.

3.3 Material and Methods

3.3.1 Animals and Diets:

aP2-agouti mice have been used in our previous studies of the effects of calcium and milk on diet-induced obesity. These mice express normal agouti protein under the control of the aP2 promoter in adipose tissue (23). They are not obese on a standard AIN-93 G diet but develop mild to moderate obesity when fed high sucrose/high fat diets. Accordingly, we induced obesity in male 7-week old aP2 agouti mice by feeding a sub-optimal Ca (0.4%),

high fat, high sucrose diet (Table 3-1) for 6 weeks (Phase I). After this time they were randomized into an ad libitum control group and four energy-restricted diet groups which received 70% energy of the ad libitum group (10 animals/diet group). The four 70% restricted diets contained either soy protein with low Ca (basal restricted (BR), 0.4% Ca), non-fat dry milk (NFDM, 1.2% Ca), Ca-depleted NFDM (0.4% Ca), or soy protein with low Ca (0.4% Ca) and supplemental BCAA/ACEi (Table 3-1). Calcium carbonate ($CaCO_3$) was added to each diet to achieve the individual final level of calcium as indicated. The animals were kept on these diet groups for 6 weeks (phase II). At the end of this phase they were terminated by exsanguination under pentobarbital anaesthesia, and fat pads and muscle tissue were collected for analysis. This study was approved from an ethical standpoint by the University of Tennessee Institutional Animal Care and Use Committee.

3.3.2 Total RNA extraction:

The Ambion ToTALLY RNA isolation kit (Ambion, Inc., Austin, Texas) was used to extract total RNA from visceral abdominal adipose tissue and skeletal muscle (gastrocnemius) from 4 representative animals from each diet group according to the manufactor's instruction. The concentration and purity of the isolated RNA were measured by using the ND-1000 Spectrophotometer (NanoDrop Technologies Inc., DE).

3.3.3 Quantitative Real-time PCR

Fatty acid synthase (FAS) and 18S expression was measured by real time reverse transcriptase- polymerase chain reaction (RT-PCR) using the 7300 Real Time PCR system with TaqMan universal PCR Master Mix, no AmpErase UNG (2X), and reverse transcriptase and RNAse inhibitor from the TaqMan 1000 Reaction Core Reagent Kit

Table 3-1: Mouse diet composition

	Diet			
	Low Ca (Basal) (0.4% Ca)	*Milk (NFDM) (1.2% Ca)*	*Ca-depleted Milk (0.4% Ca)*	*LowCa/ BCAA/ACEi (0.4% Ca)*
Ingredient (g/kg)				
Soy protein	161	0	0	164
Milk, non-fat dry	0	400	0	0
Milk, decalcified, dry	0	0	400	0
DL-Methionine	3	0	0	3
Sucrose	637.9	429.7	421	628
Cellulose	50	50	50	50
Soybean oil	100	41.8	41.8	44
Lard	10	65	65	66
Mineral Mix S10022B	7	7	7	7
Calcium carbonate	9.2	17.4	5.5	9.2
Potassium phosphate, monobasic	8	8	24	12.7
Potassium Citrate, 1 H_2O	1.6	1.6	0	0
ACE inhibitor peptide	0	0	0	5.1
Vitamin Mix V10037	10	10	10	10
Choline Bitartrate	2.5	2.5	2.5	2.5
t-butyl hydroqinone	0.014	0.014	0.014	0.014
Macronutrients (g/kg diet)				
Protein	143.9	144.8	152.5	152.5
Carbohydrate	647.9	647.7	639.0	639.0
Fat	110.0	110.0	110.0	110.0
Fiber	50	50	50	50

Table 3-1, Continued.

	Diet			
	Low Ca (Basal) (0.4% Ca)	*Milk (NFDM) (1.2% Ca)*	*Ca-depleted Milk (0.4% Ca)*	*LowCa/ BCAA/ACEi (0.4% Ca)*
Macronutrients (kcal/kg diet)				
Protein	576	579	610	610
Carbohydrate	2592	2591	2556	2556
Fat	990	990	990	990
Total	4157	4160	4156	4156
Macronutrients (kcal%)				
Protein	14	14	15	15
Carbohydrate	62	62	62	62
Fat	24	24	24	24
Total	100	100	100	100
Calcium, gm	4.0	12.0	4.0	3.99
Phosphorus, gm	3.11	5.7	5.5	5.5
Potassium, gm	3.74	10.81	7.62	4.51
BCAA (g/kg diet)				
Leucine	11.4	13.8	13.8	13.4
Isoleucine	6.92	8.5	8.5	8.35
Valine	7.08	9.4	9.4	9.22
BCAA (%/kg diet)				
Leucine	1.14	1.33	1.33	1.34
Isoleucine	0.69	0.82	0.82	0.83
Valine	0.71	0.91	0.91	0.92

(all from Applied Biosystems, Foster City, CA), according to manufacturers instructions. Standard curves were made for each gene of interest by pooling 1µl of each sample (concentration 10 ng/µl), then using ten-fold dilutions for each progressive standard point ranging from 100 ng to 0.5 pg total RNA. RT-PCR reaction mixture contained TaqMan universal PCR Master Mix, no AmpErase UNG (2X), murine leukemia virus reverse transcriptase (50 U/µl), RNAse inhibitor, and 20X Assays-on-Demand Gene Expression Assay Mix for FAS and 18S (all from Applied Biosystems, Foster City, CA). Polymerase chain reaction was performed according to instructions of the 7300 Real Time PCR system (Applied Biosystems) using 50 to 100 ng total RNA per sample plated in Thermo-Fast 96Detection plates (ABgene, Epsom, Surrey, UK). FAS mRNA levels were normalized to 18s levels prior to statistical analysis.

3.3.4 Microarray:

The Affymetrix Gene Chip Mouse Genome 430 Plus Array, release 2.0 (Santa Clara, Calif., USA) was used and the Affymetrix protocol for One-cycle cDNA synthesis and labeling was followed. Total RNA (3 µg from abdominal adipose tissue and 1 µg from gastrocnemius muscle, 4 animals/diet group) was converted into single stranded cDNA. One cycle cDNA synthesis was followed by cleanup using Affymetrix GeneChip Sample Cleanup Modules. Biotin-labeled cRNA was prepared using Affy 3'IVT Kit (Santa Clara, Calif., USA). After cleanup of the in vitro transcription products using Affymetrix GeneChip Sample Cleanup Modules for IVT reactions, the purified cRNA was fragmented. The fragmented cRNA (5 µg from adipose tissue, 1 µg from gastrocnemius) was mixed with a hybridization cocktail per Affymetrix directions in the Technical Manual. The solution

was hybridized to a GeneChip at 45^{o}C for 16 hours. After hybridization, the cocktail was removed from the GeneChip and stored for potential future analysis. Using an Affymetrix Fluidics 450 wash station, the GeneChip was washed and stained with streptavidin-phycoreythrin (Molecular Probes), followed by a wash with biotinylated antibody (Vector Labs) and IgG (Sigma) and then another staining with strepatavidin-phycoerythrin. The GeneChips were immediately scanned with a GeneChip 3000 scanner. The individual GeneChip scans were quality checked for the presence of image abnormalities, outliers, control genes and background signal values. The GeneChips were processed at the University of Tennessee Knoxville Affymetrix Core Facility.

3.3.5 Analysis of Array Data:

The data obtained from the GeneChip scanner were further analyzed using Affymetrix ® Microarray Suite 5.0 Algorithm. Hybridization of B2 oligo served as positive control by the software to place a grid over the scanned image. Noise, which is the pixel-to-pixel variations of probe cells on the array and the average background (varies between 20 and 100) were subtracted from the signal intensity value. The control genes were analyzed (poly A controls and hybridization controls) for appropriate signal intensity. The software uses a detection algorithm to call the expression of each gene as present (P), marginal (M) or absent (A). This is based on the target-specific intensity of the probe pair relative to the overall hybridization intensity (i.e., perfect match-mismatch/ perfect match + mismatch). The genes that are considered as present were selected and their signal intensities were converted into a signal log base 2 value (log2). Using Partek Genomics Suite (Partek GS) software (St. Louis, Missouri) for Affymetrix microarray data, differentially expressed genes

for each treatment groups compared to the control group were obtained with a step-up FDR-corrected statistical significant p-value of ≤0.05 (24). The extracted genes from each diet group were compared in the Venn diagram to identify genes in common among the treatment groups (25). The web-based tool David bioinformatics (http://david.abcc.ncifcrf.gov/) was used to classify genes according to their biological pathways in which the gene expression changes occurred (26).

All microarray data were submitted in compliance with Minimal Information about Microarray Experiments (MIAME) (27). All data can be accessed at the UTMD data repository under category 'Mouse'.

3.4 Results

To identify gene alterations caused by different milk components, we used three different diet treatment groups and one control group (low Ca basal group, BR) varying in the amount of calcium and/or the amount of BCAA/ACEi (Table 3-1). At the end of the study the animals showed significant differences for weight loss, fat mass and muscle mass in all three treatment groups compared to the control group, with greatest changes found in the milk group (Table 3-2). For the microarray data analysis, we identified the genes which were statistical significantly (FDR corrected p≤ 0.05) altered in each treatment group compared to the low calcium basal control group and then compared these lists to determine similarities and differences among the treatment groups (Figure 3-1). Since the level of BCAA/ACEi is similar in all three treatment groups, the effects of these components can be identified by comparing common gene alterations among all three treatment groups to the control group. Specific calcium-mediated effects can be found in the unique gene alterations

Table 3-2: Effects of the diet groups on weight change, fat pad mass and muscle mass.

	Diet				
	Low Ca (BR)	NFDM	Ca-depleted	LowCa/ BCAA/ACEi	
Weight loss (g)	-8.25 ± 1.06 [a]	-14 ± 1.06 [b]	-10.97 ± 0.85 [c]	-10.52 ± 1.32 [c]	non-matching superscripts denote significant different, $p<0.01$
Fat pad mass (g)	3.98 ± 0.21 [a]	1.73 ± 0.18 [b]	2.52 ± 0.28 [c]	2.75 ± 0.22 [c]	non-matching superscripts denote significant different, $p<0.001$
soleus mass (g)	0.017 ± 0.0005 [a]	0.028 ± 0.0009 [b]	0.022 ± 0.0.001 [c]	0.019 ± 0.0001 [d]	non-matching superscripts denote significant different, $p<0.05$

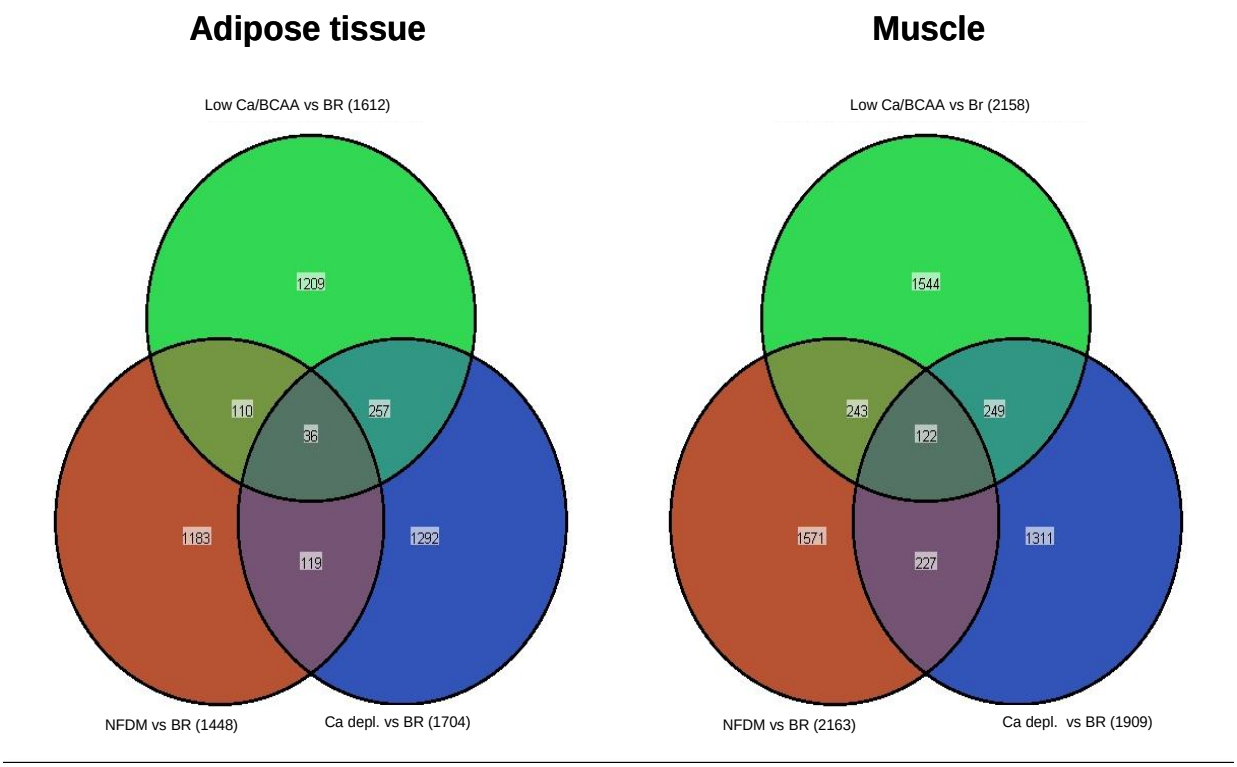

Figure 3-1:

Venn Diagram presenting the number of genes significantly altered by milk (NFDM), Ca-depleted milk and LowCa/BCAA/ACEi diets in adipose tissue and muscle relative to a 70% low calcium basal restricted diet (BR) (step-up FDR corrected $p \leq 0.05$).

in the milk diet group, while changes in common between milk and the other treatment groups are calcium independent.

Figure 3-1 summarizes the genes differentially regulated by the milk (NFDM), calcium-depleted milk (Ca-depleted) and LowCa/BCAA/ACEi treatments in adipose tissue and skeletal muscle. There were 1448, 1704 and 1612 genes in adipose tissue altered in the NFDM, Ca-depleted milk and LowCa/BCAA/ACEi diet groups, respectively, compared to the 70% low calcium basal restricted group (BR). Thirty-six of these were in common among all three diet groups (Table 3-3), including an upregulation of transformation related protein 53.

In muscle, 2163, 2158 and 1909 genes were altered in NFDM, Ca-depleted and LowCa/BCAA/ACEi diet groups, respectively. 122 of these genes were in common between all three groups (Table 3-4), including a downregulation of platelet derived growth factor D.

In adipose tissue, 1183 genes were uniquely altered in the NFDM group. The pathway analysis revealed a significant involvement of insulin signaling, PPAR signaling and pyruvate metabolism (Table 3-5). Most lipogenic genes in these pathways (e.g. acetyl-coenzyme a carboxilase beta (ACCβ), stearoyl-coenzyme a desaturase1 (SCD1), acyl-coenzyme a synthetase (ACS) were downregulated (Table 3-6). Consistent with this, RT-PCR analysis of Fatty acid synthase (FAS) in adipose tissue showed a significant down regulation in the NFDM diet group and to a lesser degree in the Ca-depleted and LowCa/BCAA/ACEi group (Fig.3-2).

In muscle, 1571 genes were found uniquely altered by the NFDM group. The pathway analysis (Table 3-7) identified changes in the adipocytokine signaling pathway; there was a significant upregulation of adiponectin receptor 1 and AMP-activated protein kinase (AMPK) (Table 3-8).

Table 3-3: List of genes commonly altered among all three diet groups compared to the low calcium basal restricted diet group (BR) in adipose tissue (step-up FDR corrected $p \leq 0.05$).

Column ID	Name	Fold change		
		BR/Ca-depl.	BR/ NFDM	BR/LowCa/ BCAA/ACEi
1416977_AT	|| 1416977_AT || SIGNAL TRANSDUCING ADAPTOR MOLECULE (SH3 DOMAIN AND ITAM MOTIF) 2 || 172086	-1.34046	-1.61335	-1.43847
1419467_AT	|| 1419467_AT || RIKEN CDNA 1200003C23 GENE || 203227	1.97756	1.61547	1.28154
1419531_AT	|| 1419531_AT || RIKEN CDNA 1700102P08 GENE || 184390	-12.3923	-7.75211	-14.6248
1422224_AT	|| 1422224_AT || T-COMPLEX PROTEIN 10B || 167969	-9.64146	-6.7559	-14.2911
1422417_AT	|| 1422417_AT || EPIDYMAL SPERM GENE || 197408	-5.40085	-4.54111	-4.94442
1425948_A_AT	|| 1425948_A_AT || SOLUTE CARRIER FAMILY 25, MEMBER 30 || 191699	-4.2969	-2.93277	-2.61668
1427739_A_AT	|| 1427739_A_AT || TRANSFORMATION RELATED PROTEIN 53 || 201660	-1.85564	-1.53007	-1.82481
1428398_AT	|| 1428398_AT || UDP-GAL:BETAGLCNAC BETA 1,3-GALACTOSYLTRANSFERASE, POLYPEPTIDE 5 || 190551	-6.95665	-4.39804	-4.51513
1429351_AT	|| 1429351_AT || RIKEN CDNA 4930429H24 GENE || 167678	1.3697	1.36996	1.21694
1430676_AT	|| 1430676_AT || PROCOLLAGEN, TYPE XIX, ALPHA 1 || 185892	-1.48811	-1.44295	-1.65256
1431720_AT	|| 1431720_AT || RIKEN CDNA 2810404F17 GENE || 197914	-4.00553	-4.08327	-3.8094
1431870_AT	|| 1431870_AT || RIKEN CDNA 4930463O16 GENE || 189877	-10.4209	-10.0796	-7.13554
1436250_AT	|| 1436250_AT || RIKEN CDNA 5430405G05 GENE || 199709	-4.77521	-7.74733	-4.67203
1436928_S_AT	|| 1436928_S_AT || ADENYLATE CYCLASE 3 || 163001	1.59706	2.62345	1.29188
1437748_AT	|| 1437748_AT || FUCOSYLTRANSFERASE 11 || 196868	-1.51246	-1.368	-1.38191
1439303_AT	|| 1439303_AT || 16 DAYS NEONATE CEREBELLUM CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:9630012D08 PRODUCT:UNKNOWN EST, FULL INSERT SEQUENCE || 197336	-8.92984	-9.62752	-6.06884
1439856_AT	|| 1439856_AT || ACTIVIN RECEPTOR IIB || 174176	-4.42757	-3.68924	-3.32907
1441101_AT	|| 1441101_AT || EST AV273951 || 164974	-5.06057	-5.24841	-2.92551
1443238_AT	|| 1443238_AT || TRANSCRIBED SEQUENCES || 166062	-5.26864	-6.36694	-7.26714
1445459_AT	|| 1445459_AT || TRANSCRIBED SEQUENCES || 173987	-3.40817	-3.07889	-2.87662
1446362_AT	|| 1446362_AT || 10 DAYS NEONATE CEREBELLUM CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:6530421N19 PRODUCT:UNKNOWN EST, FULL INSERT SEQUENCE || 181604	-9.39235	-8.13343	-7.17529
1447103_AT	|| 1447103_AT || RIKEN CDNA A330004A13 GENE || 186181	-5.73557	-5.66539	-4.84656

Table 3-3, continued.

Column ID	Name	Fold change		
		BR/Ca-depl.	BR/ NFDM	BR/low Ca/ BCAA/ACEi
1447248_AT	|| 1447248_AT || EXPRESSED SEQUENCE AW046938 || 169265	8.65124	6.67552	8.03085
1449945_AT	|| 1449945_AT || PEROXISOME PROLIFERATIVE ACTIVATED RECEPTOR, GAMMA, COACTIVATOR 1 BETA || 177533	1.49591	1.40401	1.60624
1450547_X_AT	|| 1450547_X_AT || DEUBIQUITINATING ENZYME 2 || 176587	-8.40261	-4.62682	-4.92194
1450552_AT	|| 1450552_AT || ATPASE, H+ TRANSPORTING, V1 SUBUNIT E-LIKE 2 ISOFORM 2 || 185482	-1.79494	-1.45664	-1.611
1450759_AT	|| 1450759_AT || BONE MORPHOGENETIC PROTEIN 6 || 202224	1.99759	3.01482	1.99564
1451607_AT	|| 1451607_AT || KALLIKREIN 6 || 201006	3.61267	2.78221	2.24994
1453337_AT	|| 1453337_AT || RIKEN CDNA 1700013E18 GENE || 170853	-5.96078	-5.72438	-3.36659
1453376_AT	|| 1453376_AT || RIKEN CDNA 4921524J06 GENE || 167329	-4.22151	-3.08852	-4.54265
1455797_X_AT	|| 1455797_X_AT || LIGATIN || 182450	-1.45902	-1.61028	-1.69425
1456144_AT	|| 1456144_AT || NEURON NAVIGATOR 3 || 167220	1.27034	1.36313	1.29699
1457465_AT	|| 1457465_AT || 0 DAY NEONATE KIDNEY CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:D630003C04 PRODUCT:UNKNOWN EST, FULL INSERT SEQUENCE || 179065	1.43248	1.52273	1.60869
1457994_AT	|| 1457994_AT || RIKEN CDNA 4930563J15 GENE || 187806	-11.4323	-12.926	-8.58853
1459012_AT	|| 1459012_AT || RIKEN CDNA A730041O05 GENE || 169694	-30.385	-15.3028	-11.0091
1459160_AT	|| 1459160_AT || TRANSCRIBED SEQUENCES || 206099	4.92788	3.48275	5.6296

Table 3-4: List of genes commonly altered among all three diet groups compared to the low calcium basal restricted group (BR) in muscle (step-up FDR corrected $p \leq 0.05$).

Column ID	Name	Fold change		
		BR/NFDM	BR/Ca-depl.	BR/lowCa/ BCAA/ACEi
1415712_AT	|| 1415712_AT || DNA SEGMENT, CHR 7, WAYNE STATE UNIVERSITY 87, EXPRESSED || 187001	-1.35335	-1.41134	-1.49227
1416383_A_AT	|| 1416383_A_AT || PYRUVATE CARBOXYLASE || 171605	-2.00201	-1.82452	-1.56093
1416851_AT	|| 1416851_AT || SUPPRESSION OF TUMORIGENICITY 13 || 194327	-6.63426	-4.76242	-4.5287
1417169_AT	|| 1417169_AT || UBIQUITIN SPECIFIC PROTEASE 2 || 184062	-1.28938	-1.39951	-1.40193
1417475_AT	|| 1417475_AT || ATPASE TYPE 13A || 185179	-2.36991	-2.22073	-2.51043
1417679_AT	|| 1417679_AT || GROWTH FACTOR INDEPENDENT 1 || 172182	-4.44749	-7.02771	-11.2293
1418028_AT	|| 1418028_AT || DOPACHROME TAUTOMERASE || 178332	-8.46549	-14.4285	-5.23316
1418399_AT	|| 1418399_AT || POTASSIUM CHANNEL TETRAMERISATION DOMAIN CONTAINING 9 || 176253	1.29951	1.44645	1.34111
1418853_AT	|| 1418853_AT || DNA SEGMENT, CHR 10, UNIVERSITY OF CALIFORNIA AT LOS ANGELES 2 || 177725	1.92403	3.63223	2.74606
1419148_AT	|| 1419148_AT || ADVILLIN || 175347	7.52673	2.68323	1.51062
1419490_AT	|| 1419490_AT || EXPRESSED SEQUENCE AW049604 || 187294	-7.12415	-6.3702	-4.59859
1420144_X_AT	|| 1420144_X_AT || RIKEN CDNA D930043C02 GENE || 205205	6.26498	3.90203	4.06337
1420355_AT	|| 1420355_AT || G PROTEIN-COUPLED RECEPTOR KINASE 2, GROUCHO GENE RELATED (DROSOPHILA) || 185080	11.7003	3.16774	1.85416
1420503_AT	|| 1420503_AT || SOLUTE CARRIER FAMILY 6 (NEUROTRANSMITTER TRANSPORTER), MEMBER 14 || 176389	-8.88834	-6.28409	-5.65696
1420522_AT	|| 1420522_AT || RIKEN CDNA 2610529H08 GENE || 197009	1.24124	1.65767	1.32168
1420556_AT	|| 1420556_AT || OXYTOCIN || 199913	1.29612	1.49924	1.86433
1420669_AT	|| 1420669_AT || ARYL HYDROCARBON RECEPTOR NUCLEAR TRANSLOCATOR 2 || 167270	-3.20953	-4.90921	-5.87866
1421554_AT	|| 1421554_AT || LIM HOMEOBOX TRANSCRIPTION FACTOR 1 ALPHA || 197332	2.44334	8.32438	6.88502
1422588_AT	|| 1422588_AT || KERATIN COMPLEX 2, BASIC, GENE 6B || 190636	3.80457	5.64882	8.11482
1422618_X_AT	|| 1422618_X_AT || CDNA CLONE MGC:74306 IMAGE:6774630, COMPLETE CDS || 191307	-7.37698	-7.25632	-7.63256
1422846_AT	|| 1422846_AT || RETINOL BINDING PROTEIN 2, CELLULAR || 169611	4.79857	4.12604	4.03147
1423310_AT	|| 1423310_AT || TROPHOBLAST GLYCOPROTEIN || 202787	4.21745	4.09507	3.99069

Table 3-4, continued.

Column ID	Name	Fold change		
		BR/NFDM	BR/Ca-depl.	BR/LowCa/ BCAA/ACEi
1423751_AT	|| 1423751_AT || ZINC FINGER PROTEIN 162 || 167252	2.97858	4.22042	7.72445
1423788_AT	|| 1423788_AT || NUCLEOPORIN 133 || 172999	3.15111	3.45462	6.85933
1423828_AT	|| 1423828_AT || FATTY ACID SYNTHASE || 183818	-1.88581	-2.42808	-2.23346
1424183_AT	|| 1424183_AT || ACETYL-COENZYME A ACETYLTRANSFERASE 1 || 203679	1.17905	1.2067	1.23353
1424265_AT	|| 1424265_AT || N-ACETYLNEURAMINATE PYRUVATE LYASE || 188016	1.76472	1.90653	1.90852
1424550_AT	|| 1424550_AT || ZINC FINGER, FYVE DOMAIN CONTAINING 27 || 198523	-8.28198	-3.51495	-8.837
1425997_A_AT	|| 1425997_A_AT || PHOSPHATIDYLINOSITOL GLYCAN, CLASS N || 189756	-7.8173	-3.08041	-4.89823
1426218_AT	|| 1426218_AT || GLUCOCORTICOID INDUCED TRANSCRIPT 1 || 203962	-4.27178	-4.08387	-3.68334
1427331_AT	|| 1427331_AT || ADENOSINE A1 RECEPTOR || 193753	2.36482	4.46957	3.79598
1427346_AT	|| 1427346_AT || OVARY TESTIS TRANSCRIBED || 169585	-5.24395	-7.63352	-6.70744
1427605_AT	|| 1427605_AT || HOMEO BOX B3 || 186655	6.98819	5.27117	9.90109
1427617_AT	|| 1427617_AT || FUCOSYLTRANSFERASE 10 || 197429	-5.73184	-7.50161	-6.26794
1427715_A_AT	|| 1427715_A_AT || 5'-NUCLEOTIDASE, CYTOSOLIC IB || 180067	6.93914	5.58697	6.35728
1427781_AT	|| 1427781_AT || UPREGULATED DURING SKELETAL MUSCLE GROWTH 2 || 164172	-4.33035	-7.89799	-6.05322
1427812_AT	|| 1427812_AT || IDURONATE 2-SULFATASE || 183142	5.50039	8.83638	5.86947
1427814_AT	|| 1427814_AT || ONCOFETAL ANTIGEN || 183782	-4.55377	-7.5122	-3.40696
1427831_S_AT	|| 1427831_S_AT || ZINC FINGER PROTEIN 260 || 171405	1.92638	2.30292	1.65186
1428543_AT	|| 1428543_AT || PHOSPHORIBOSYL PYROPHOSPHATE AMIDOTRANSFERASE || 206672	-1.67048	-1.85049	-1.69324
1429254_AT	|| 1429254_AT || AQUAPORIN 11 || 165817	-4.93817	-8.75124	-9.23874
1429675_AT	|| 1429675_AT || RIKEN CDNA 1700023A16 GENE || 194188	-9.04583	-7.57058	-4.07078
1430340_AT	|| 1430340_AT || RIKEN CDNA 4930431B11 GENE || 198460	7.2684	12.7997	6.61272
1430430_AT	|| 1430430_AT || RIKEN CDNA 6130401L20 GENE || 172694	-18.5265	-20.3426	-4.13402
1430885_AT	|| 1430885_AT || RIKEN CDNA 1110012N22 GENE || 165466	1.62095	1.47514	2.40984
1431501_AT	|| 1431501_AT || RIKEN CDNA 1700037C18 GENE || 162790	3.48469	6.19102	8.07013
1431558_AT	|| 1431558_AT || RIKEN CDNA 2310016D03 GENE || 172264	2.03088	4.85741	9.25755
1431561_A_AT	|| 1431561_A_AT || DEAH (ASP-GLU-ALA-HIS) BOX POLYPEPTIDE 34 || 180508	1.58416	1.82636	1.4558
1431981_AT	|| 1431981_AT || HYPOXIA INDUCIBLE FACTOR 1, ALPHA SUBUNIT || 194391	7.79947	9.29417	4.41806
1432404_AT	|| 1432404_AT || RIKEN CDNA 4930505A04 GENE || 170421	-5.65147	-4.75364	-5.96371
1432707_AT	|| 1432707_AT || || 170589	3.60213	8.90617	3.94438

Table 3-4, continued.

Column ID	Name	Fold change		
		BR/NFDM	BR/Ca-depl.	BR/LowCa/ BCAA/ACEi
1432914_AT	|| 1432914_AT || RIKEN CDNA 4930413P14 GENE || 203657	10.7795	3.70208	16.217
1433550_AT	|| 1433550_AT || CHECKPOINT WITH FORKHEAD AND RING FINGER DOMAINS || 197566	1.58106	1.28237	1.45881
1433744_AT	|| 1433744_AT || EXPRESSED SEQUENCE AI841794 || 202908	-9.1872	-7.17254	-6.35024
1433918_AT	|| 1433918_AT || APG4 (ATG4) AUTOPHAGY-RELATED HOMOLOG D (S. CEREVISIAE) || 178397	-1.68975	-1.47852	-1.63827
1433958_AT	|| 1433958_AT || RIKEN CDNA 9830165K03 GENE || 175166	1.99615	1.4539	2.07115
1434355_AT	|| 1434355_AT || ZINC FINGER PROTEIN 617 || 203264	-1.33503	-1.83736	-1.547
1434535_AT	|| 1434535_AT || RIKEN CDNA 6330509G02 GENE || 196799	-4.78067	-4.33883	-6.56723
1434639_AT	|| 1434639_AT || KELCH REPEAT AND BTB (POZ) DOMAIN CONTAINING 9 || 190078	2.63319	3.1387	2.16413
1434689_AT	|| 1434689_AT || HYPOTHETICAL PROTEIN LOC232337 || 167554	-1.28084	-1.43372	-1.19468
1434908_AT	|| 1434908_AT || EXPRESSED SEQUENCE AI480556 || 204410	-2.968	-1.99812	-2.09165
1435301_AT	|| 1435301_AT || RIKEN CDNA 1110004E09 GENE || 186107	-2.97259	-3.58944	-2.35746
1436552_AT	|| 1436552_AT || ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4930402I24 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE || 199556	-10.8883	-5.17481	-3.09718
1436719_AT	|| 1436719_AT || SOLUTE CARRIER FAMILY 35, MEMBER F1 || 181695	-4.94134	-4.0419	-5.91077
1436973_AT	|| 1436973_AT || CHAPERONIN SUBUNIT 8 (THETA) || 168680	-8.59297	-9.9767	-5.21228
1437145_S_AT	|| 1437145_S_AT || RIKEN CDNA 2310002J15 GENE || 197482	3.05395	3.93001	6.96323
1437227_AT	|| 1437227_AT || RIKEN CDNA 2900073H19 GENE || 161902	-1.51708	-2.20731	-1.41817
1437642_AT	|| 1437642_AT || HIV-1 REV BINDING PROTEIN-LIKE || 165187	-6.79194	-9.40643	-6.26239
1437977_AT	|| 1437977_AT || SMALL GLUTAMINE-RICH TETRATRICOPEPTIDE REPEAT (TPR)-CONTAINING, BETA || 206408	-7.59997	-6.80973	-6.39455
1438819_AT	|| 1438819_AT || TRANSCRIBED SEQUENCES || 194263	-6.32152	-5.2748	-8.36567
1439052_AT	|| 1439052_AT || RIKEN CDNA 5430433G21 GENE || 205079	3.47652	3.55648	5.80473
1439218_AT	|| 1439218_AT || ADULT MALE PITUITARY GLAND CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:5330429P03 PRODUCT:UNKNOWN EST, FULL INSERT SEQUENCE || 179605	-4.11129	-5.75808	-3.10785
1439468_AT	|| 1439468_AT || TRANSCRIBED SEQUENCES || 201372	-3.79125	-5.01008	-4.64575
1439931_AT	|| 1439931_AT || 190481 Glycogen synthase kinase 3 beta	-3.94473	-4.34706	-4.19949
1440208_AT	|| 1440208_AT || TRANSCRIBED SEQUENCES || 205613	3.73907	5.68418	1.92974
1440689_AT	|| 1440689_AT || RIKEN CDNA 6230415M23 GENE || 206005	1.64773	3.59789	4.2833

Table 3-4, continued.

Column ID	Name	Fold change		
		BR/NFDM	BR/Ca-depl.	BR/LowCa/ BCAA/ACEi
1440741_AT	|| 1440741_AT || 5-HYDROXYTRYPTAMINE (SEROTONIN) RECEPTOR 1D || 168815	-8.61564	-8.08385	-8.69232
1441037_AT	|| 1441037_AT || ADULT MALE CORPORA QUADRIGEMINA CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:B230218K11 PRODUCT:UNKNOWN EST, FULL INSERT SEQUENCE || 168486	5.23709	4.99003	7.62859
1441372_AT	|| 1441372_AT || RIKEN CDNA 5930405F01 GENE || 162884	9.2002	7.30613	5.17671
1441568_AT	|| 1441568_AT || CDNA SEQUENCE BC010311 || 168842	3.84438	7.33895	5.66456
1441954_S_AT	|| 1441954_S_AT || RIKEN CDNA 1700026P10 GENE || 195127	-3.63389	-5.617	-3.36055
1441996_AT	|| 1441996_AT || RIKEN CDNA E030034P13 GENE || 162754	-3.75646	-4.24349	-2.54038
1442349_AT	|| 1442349_AT || RIKEN CDNA C630028N24 GENE || 177471	1.47798	9.356	3.95694
1444156_AT	|| 1444156_AT || RIKEN CDNA 9230112E08 GENE || 175138	-6.84308	-8.35765	-16.5006
1444555_AT	|| 1444555_AT || || 203863	6.09179	5.43162	6.70045
1445032_AT	|| 1445032_AT || TRANSCRIBED SEQUENCES || 204718	-4.30004	-2.67333	-3.35349
1445379_AT	|| 1445379_AT || TRANSCRIBED SEQUENCES || 165129	-13.4979	-5.30494	-10.9984
1445381_AT	|| 1445381_AT || POLY(RC) BINDING PROTEIN 2 || 165762	1.50243	1.51154	1.33718
1445822_AT	|| 1445822_AT || SIMILAR TO RIKEN CDNA 5730509K17 GENE (LOC212369), MRNA || 162080	3.89108	10.3419	6.41618
1446691_AT	|| 1446691_AT || PLATELET-DERIVED GROWTH FACTOR, D POLYPEPTIDE || 182116	4.17534	5.61025	7.33304
1446795_AT	|| 1446795_AT || || 168115	3.53002	8.61071	1.80826
1447068_AT	|| 1447068_AT || || 175672	2.74191	3.93817	2.26111
1447265_AT	|| 1447265_AT || EXPRESSED SEQUENCE AI848615 || 189238	7.9215	15.3203	13.3661
1447890_AT	|| 1447890_AT || RETICULOCALBIN || 196714	-5.36061	-6.01039	-8.76593
1448235_S_AT	|| 1448235_S_AT || HIGH MOBILITY GROUP BOX 1 || 192396	1.18006	1.28624	1.73583
1448313_AT	|| 1448313_AT || CEROID-LIPOFUSCINOSIS, NEURONAL 2 || 193335	-1.18153	-1.21734	-1.44428
1448365_AT	|| 1448365_AT || EXOSOME COMPONENT 7 || 163427	1.49578	1.45723	1.40844
1448731_AT	|| 1448731_AT || INTERLEUKIN 10 RECEPTOR, ALPHA || 197545	4.02862	2.17129	2.27003
1449856_AT	|| 1449856_AT || REGULATOR OF G-PROTEIN SIGNALING 18 || 194579	-3.89487	-6.36523	-6.81835
1449948_AT	|| 1449948_AT || RIKEN CDNA 1300013B24 GENE || 178487	12.9982	6.72961	10.8192
1451138_X_AT	|| 1451138_X_AT || RIKEN CDNA 2700087H15 GENE || 173904	1.68277	2.13296	1.98595
1451262_A_AT	|| 1451262_A_AT || JTV1 GENE || 188119	-1.24376	-1.25444	-1.21807
1451849_A_AT	|| 1451849_A_AT || LAMIN B2 || 167835	-4.67795	-6.52493	-3.12547

Table 3-4, continued.

Column ID	Name	Fold change		
		BR/NFDM	BR/Ca-depl.	BR/LowCa/ BCAA/ACEi
1452070_AT	|| 1452070_AT || DEATH EFFECTOR DOMAIN-CONTAINING DNA BINDING PROTEIN 2 || 174805	-3.81644	-2.91332	-3.617
1452765_AT	|| 1452765_AT || SOLUTE CARRIER FAMILY 39 (ZINC TRANSPORTER), MEMBER 9 || 194164	-9.24371	-10.7547	-5.57523
1453216_AT	|| 1453216_AT || GLUTAMIC PYRUVIC TRANSAMINASE 1, SOLUBLE || 164902	6.64767	3.17801	5.88132
1454088_AT	|| 1454088_AT || RIKEN CDNA 5330411O13 GENE || 197780	3.12934	3.62557	4.9815
1454169_A_AT	|| 1454169_A_AT || RIKEN CDNA 2310046K10 GENE || 161872	-9.19905	-2.19488	-4.05857
1454188_AT	|| 1454188_AT || RIKEN CDNA 4921508D12 GENE || 182487	-9.16763	-9.20286	-7.5828
1455177_AT	|| 1455177_AT || ABELSON HELPER INTEGRATION SITE || 163090	-5.0662	-5.37209	-5.04347
1455346_AT	|| 1455346_AT || MANNAN-BINDING LECTIN SERINE PROTEASE 1 || 190960	2.3901	4.10574	2.18403
1456186_AT	|| 1456186_AT || TRANSCRIBED SEQUENCES || 164626	1.43038	1.46722	1.68548
1456211_AT	|| 1456211_AT || NACHT, LEUCINE RICH REPEAT AND PYD CONTAINING 10 || 164658	4.62179	7.85207	4.89053
1456230_AT	|| 1456230_AT || TRANSCRIBED SEQUENCE WITH WEAK SIMILARITY TO PROTEIN SP:Q14258 (H.SAPIENS) Z147_HUMAN ZINC FINGER PROTEIN 147 || 185268	-8.09735	-2.59303	-2.78549
1457379_AT	|| 1457379_AT || EXPRESSED SEQUENCE C78549 || 206080	2.33128	1.73971	3.28989
1457497_AT	|| 1457497_AT || ADULT MALE CORPUS STRIATUM CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:C030032K10 PRODUCT:UNCLASSIFIABLE, FULL INSERT SEQUENCE || 166004	8.10728	7.50515	8.47225
1457581_AT	|| 1457581_AT || UBX DOMAIN CONTAINING 3 || 183425	13.4241	5.97589	7.20791
1458854_AT	|| 1458854_AT || RIKEN CDNA 4931400A14 GENE || 188664	9.28783	8.22178	9.15518
1459015_AT	|| 1459015_AT || TRANSCRIBED SEQUENCE WITH WEAK SIMILARITY TO PROTEIN REF:NP_038603.1 (M.MUSCULUS) L1 REPEAT, TF SUBFAMILY, MEMBER 23 [MUS MUSCULUS] || 170647	12.9549	5.11613	4.33649
1460068_AT	|| 1460068_AT || RIKEN CDNA 1110034C04 GENE || 169594	-4.39683	-4.22002	-4.03103
1460330_AT	|| 1460330_AT || ANNEXIN A3 || 172337	1.7872	1.32635	1.46192
1460379_AT	|| 1460379_AT || HOMEO BOX B4 || 179283	-6.78036	-6.39404	-4.81976

Table 3-5: Pathway analysis from genes uniquely altered (step-up FDR corrected $p < 0.05$) in milk diet group in adipose tissue.

Category	Term	Count	%
KEGG_PATHWAY	Pyruvate metabolism	8	0.7
KEGG_PATHWAY	Chronic myeloid leukemia	11	0.9
KEGG_PATHWAY	Insulin signaling pathway	15	1.3
KEGG_PATHWAY	PPAR signaling pathway	10	0.9
KEGG_PATHWAY	Prostate cancer	11	0.9
KEGG_PATHWAY	Endometrial cancer	8	0.7
KEGG_PATHWAY	Glycolysis / Gluconeogenesis	8	0.7
KEGG_PATHWAY	mTOR signaling pathway	8	0.7
KEGG_PATHWAY	Non-small cell lung cancer	8	0.7
KEGG_PATHWAY	Colorectal cancer	10	0.9
KEGG_PATHWAY	Pentose phosphate pathway	5	0.4
KEGG_PATHWAY	Tight junction	13	1.1
KEGG_PATHWAY	Ribosome	10	0.9
KEGG_PATHWAY	Polyunsaturated fatty acid biosynthesis	4	0.3
KEGG_PATHWAY	Acute myeloid leukemia	7	0.6

Table 3-6: Genes involved within the uniquely altered pathways for milk diet group and their fold change compared to the low calcium basal restricted diet group (BR) in adipose tissue (step-up FDR corrected $p \leq 0.05$).

NFDM vs. BR (adipose tissue)		Mean log base 2 value		Fold change (BR/NFDM)
		BR	NFDM	
Insulin signaling pathway (genes)				
1452620_AT	phosphoenolpyruvate carboxykinase 2 (mitochondrial)	6.57352	5.91671	1.5766
1452105_A_AT	tuberous sclerosis 2	9.3192	8.72077	1.51407
1448695_AT	protein kinase c, iota	6.00291	3.97955	4.06532
1421954_AT	v-crk sarcoma virus ct10 oncogene homolog (avian)-like	7.02526	5.95862	2.09454
1452281_AT	son of sevenless homolog 2 (drosophila)	10.7275	10.4635	1.20081
1455967_AT	sorbin and sh3 domain containing 1	9.00249	8.52858	1.38887
1427052_AT	acetyl-coenzyme a carboxylase beta	10.3726	8.11331	4.78753
1446390_AT	elk1, member of ets oncogene family	1.02524	3.15126	-4.36511
1443798_AT	phosphatidylinositol 3-kinase catalytic delta polypeptide	9.99437	9.72786	1.20289
1450196_S_AT	glycogen synthase 1, muscle	6.83221	4.37409	5.49501
1458472_AT	riken cdna 4932417h02 gene	5.19537	2.99517	4.59544
1450457_AT	casitas b-lineage lymphoma	2.91046	6.18569	-9.68146
1417690_AT	protein kinase, amp-activated, gamma 1 non-catalytic subunit	10.5363	10.938	-1.32111
1421324_A_AT	thymoma viral proto-oncogene 2	8.25732	7.03693	2.33009
1450718_AT	adaptor protein with pleckstrin homology and src	9.40873	7.7783	3.09606
1453466_AT	ribosomal protein s6	6.38798	5.56787	1.76554
1415729_AT	3-phosphoinositide dependent protein kinase-1	10.8689	10.6045	1.20113
1434000_AT	v-ki-ras2 kirsten rat sarcoma viral oncogene homolog	10.322	10.5995	-1.21209
1460326_AT	phosphatidylinositol 3-kinase, catalytic, alpha polypeptide	10.6121	10.2147	1.31707

Table 3-6, continued.

NFDM vs. BR (adipose tissue)		Mean log base 2 value		Fold change (BR/NFDM)
		BR	NFDM	
Pyruvate metabolism (genes):				
1424171_A_AT	hydroxyacyl glutathione hydrolase	10.4408	9.57175	1.82642
1452620_AT	phosphoenolpyruvate carboxykinase 2 (mitochondrial)	6.57352	5.91671	1.5766
1448214_AT	pyruvate dehydrogenase (lipoamide) beta	6.92287	6.45589	1.38221
1427052_AT	acetyl-coenzyme a carboxylase beta	10.3726	8.11331	4.78753
1423159_AT	dihydrolipoamide dehydrogenase	11.243	10.3944	1.80084
1430307_A_AT	malic enzyme, supernatant	12.3805	10.1834	4.58568
1422479_AT	acetyl-coa synthetase	10.7544	9.4198	2.52204
1426264_AT	dihydrolipoamide s-acetyltransferase (e2 component of pyruvate dehydrogenase complex)	11.322	10.5461	1.71223
1416617_AT	acyl-coa synthetase short-chain family member 1	9.59437	9.94994	-1.27949
PPAR signaling pathway (genes)				
1452620_AT	phosphoenolpyruvate carboxykinase 2 (mitochondrial)	6.57352	5.91671	1.5766
1435658_AT	solute carrier family 27 (fatty acid transporter), member 1	11.4542	10.5618	1.85625
1449686_S_AT	sterol carrier protein 2, liver	14.2448	13.8489	1.31579
1415965_AT	stearoyl-coenzyme a desaturase 1	12.3516	10.6231	3.31397
1415729_AT	3-phosphoinositide dependent protein kinase-1	10.8689	10.6045	1.20113
1455967_AT	sorbin and sh3 domain containing 1	9.00249	8.52858	1.38887
1416408_AT	acyl-coenzyme a oxidase 1, palmitoyl	12.6655	12.1236	1.45584
1422526_AT, 1460316_AT, 1450643_S_AT, 1423883_AT	acyl-coa synthetase long-chain family member 1	10.4019	9.52782	1.83283
1430307_A_AT	malic enzyme, supernatant	12.3805	10.1834	4.58568
1454373_X_AT	ubiquitin c	9.22671	8.74434	1.39703
1416694_AT	glycerol kinase 2	2.39458	4.80585	-5.31942

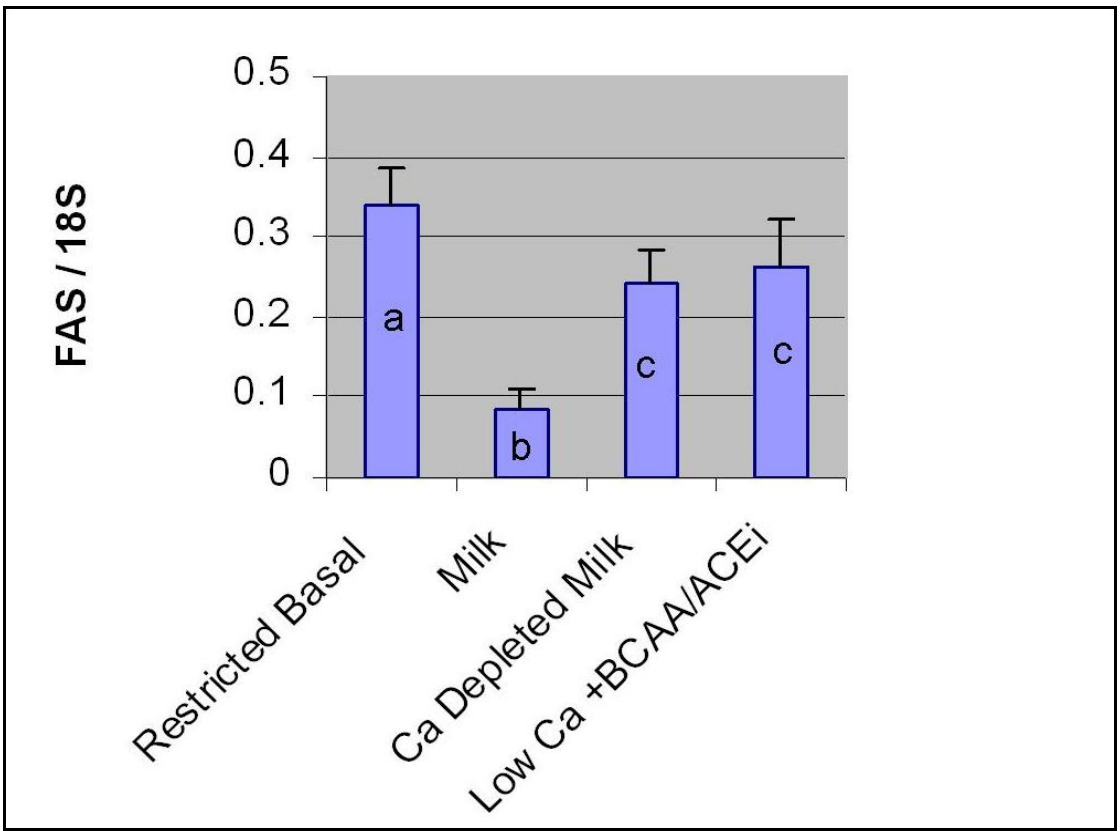

Figure 3-2: Effects of Calcium, ACEi and BCAA on fatty acid synthase (FAS) expression. Non-matching superscripts denote significant difference ($p<0.05$).

Table 3-7: Pathway analysis from genes uniquely altered (step-up FDR corrected $p < 0.05$) in milk diet group in muscle.

Category	Term	Count	%
KEGG_PATHWAY	Wnt signaling pathway	25	1.6
KEGG_PATHWAY	Melanogenesis	18	1.1
KEGG_PATHWAY	Thyroid cancer	8	0.5
KEGG_PATHWAY	Endometrial cancer	11	0.7
KEGG_PATHWAY	Tight junction	19	1.2
KEGG_PATHWAY	Insulin signaling pathway	19	1.2
KEGG_PATHWAY	Adipocytokine signaling pathway	12	0.7
KEGG_PATHWAY	B cell receptor signaling pathway	11	0.7
KEGG_PATHWAY	Basal cell carcinoma	10	0.6
KEGG_PATHWAY	Prostate cancer	13	0.8
KEGG_PATHWAY	VEGF signaling pathway	11	0.7
KEGG_PATHWAY	Citrate cycle (TCA cycle)	6	0.4
KEGG_PATHWAY	Long-term depression	11	0.7
KEGG_PATHWAY	Acute myeloid leukemia	9	0.6
KEGG_PATHWAY	Renal cell carcinoma	10	0.6
KEGG_PATHWAY	TGF-beta signaling pathway	12	0.7
KEGG_PATHWAY	Glyoxylate and dicarboxylate metabolism	4	0.2
KEGG_PATHWAY	Colorectal cancer	11	0.7
KEGG_PATHWAY	Long-term potentiation	9	0.6
KEGG_PATHWAY	Hedgehog signaling pathway	8	0.5
KEGG_PATHWAY	Ubiquitin mediated proteolysis	15	0.9
KEGG_PATHWAY	Chronic myeloid leukemia	10	0.6
KEGG_PATHWAY	MAPK signaling pathway	25	1.6

Table 3-8: Genes involved within the adipocytokine signaling pathway in the milk diet group and their fold change compared to low calcium basal restricted diet group (BR) in muscle (step-up FDR corrected $p < 0.05$).

Genes involved in Adipocytokine signaling		Mean log base 2 value		Fold change (BR vs NFDM)
		BR	NFDM	
1420088_AT	nuclear factor of kappa light chain gene enhancer in b-cells inhibitor, alpha	12.3429	12.0147	1.25539
1423104_AT	insulin receptor substrate 1	11.0837	10.4648	1.53565
1444480_AT	protein kinase, amp-activated, gamma 3 non-catatlytic subunit	13.0262	13.3445	-1.2468
1421324_A_AT, 1424480_S_AT	thymoma viral proto-oncogene 2	10.2967	10.7973	-1.41483
1456156_AT	leptin receptor	7.07321	5.47355	3.03071
1425711_A_AT, 1440950_AT	thymoma viral proto-oncogene 1	8.05	6	4.15
1417291_AT	tumor necrosis factor receptor superfamily, member 1a	10.1378	10.5312	-1.3135
1428386_AT	acyl-coa synthetase long-chain family member 3	8.50904	8.02886	1.39491
1424312_AT, 1451311_A_AT	adiponectin receptor 1	12.17	12.67	-1.42
1433800_A_AT	pro-opiomelanocortin-alpha	8.45222	9.1206	-1.58929
1429463_AT	protein kinase, amp-activated, alpha 2 catalytic subunit	12.4988	12.7497	-1.18995
1457803_AT	protein kinase, amp-activated, gamma 1 non-catalytic subunit	4.46506	3.1242	2.53302

Since the small number of genes in common among all treatment groups could identify only few pathways, we also compared in addition the pathways revealed from using all significant altered genes in each treatment group. In muscle, the LowCa/BCAA/ACEi and Ca-depleted group exhibited significant alterations of the mTOR pathway (Table 3-9). In adipose tissue, pathways found in Ca-depleted and LowCa/BCAA/ACEi were involved in inflammatory or cell regulatory processes such as MAPK signaling, JAK-STAT signaling, and TGF-beta signaling pathway (Table 3-10). The genes involved in these pathways are listed in Appendix Table A1 and A2. In muscle we found involvement of insulin signaling, VEGF signaling, B cell receptor signaling and GnRH signaling pathway among all three treatment groups; the genes are shown in Appendix Table A3 to A6.

3.5 Discussion

In this study, two different approaches were used to compare the gene expression changes caused by the three different diet groups: the Venn Diagram, which is a simple but effective procedure to examine the overlap of large gene lists differentially expressed between different sample sets (25), and a comparison of pathways identified by significantly altered genes in each diet group.

The results of both approaches identified several gene expression patterns and pathways consistent with our previous findings. We have demonstrated that dietary calcium stimulates lipolysis and decreases lipogenesis in adipocytes *in vivo* and *in vitro*, with dairy products exerting greater effects than supplemental calcium (4,28,29). These additional effects are partly caused by the BCAA leucine, contained in the whey fraction of dairy products (7). Leucine is

Table 3-9: Genes involved within the mTOR pathway in Ca-depleted and LowCa/BCAA/ACEi diet group and their induction fold change compared to basal restricted diet group (BR) in muscle (step-up FDR corrected p< 0.05).

Genes involved within mTOR pathway (muscle)		Mean log base 2 value		Fold change
		BR	Ca-depl.	(BR/Ca-depl.)
Ca-depleted vs. BR				
1428188_AT	riken cdna 2610019a05 gene	7.34586	8.4323	-2.1235
1437539_AT	protein kinase, amp-activated, alpha 1 catalytic subunit	7.65128	7.17523	1.39093
1444831_AT	thymoma viral proto-oncogene 3	7.79715	5.37593	5.35622
1447940_A_AT	braf transforming gene	5.49872	7.37856	-3.68034
1431981_AT	hypoxia inducible factor 1, alpha subunit	7.41284	4.19651	9.29417
1453281_AT	phosphatidylinositol 3-kinase catalytic delta polypeptide	8.76758	8.02663	1.67127
1455252_AT, 1422043_AT	tuberous sclerosis 1	10.1581	10.7441	-1.50108
1438954_X_AT	c-fos induced growth factor	9.1695	8.58222	1.50242
1429434_AT	phosphatidylinositol 3-kinase, catalytic, alpha polypeptide	8.38313	7.65034	1.66186
		Mean log base 2 value		Fold change
		BR	LowCa/ BCAA/ACEi	(BR/LowCa/ BCAA/ACEi)
LowCa/BCAA/ACEi vs. Br				
1453069_AT	phosphatidylinositol 3-kinase, catalytic, beta polypeptide	5.1568	7.60749	-5.46678
1441311_AT	ribosomal protein s6 kinase, related sequence 1	5.84424	3.4635	5.20803
1442044_AT	ribosomal protein s6	6.70554	4.72864	3.93645
1425514_AT	phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	7.83016	8.52621	-1.62007
1444831_AT	thymoma viral proto-oncogene 3	7.79715	5.0313	6.80146
1415842_AT	g protein beta subunit-like	9.06191	8.24947	1.75617
1431981_AT	hypoxia inducible factor 1, alpha subunit	7.41284	5.26942	4.41806
1453281_AT	phosphatidylinositol 3-kinase catalytic delta polypeptide	8.76758	8.13474	1.55061
1439989_AT	tuberous sclerosis 1	6.65765	3.65317	8.02488
1446185_AT	fk506 binding protein 12-rapamycin associated protein 1	7.97048	6.33475	3.10744
1419568_AT	mitogen activated protein kinase 1	9.26914	8.58738	1.6041
1457562_AT	ribosomal protein s6 kinase, polypeptide 1	4.27528	7.28731	-8.06699

Table 3-10**:** Pathway analysis from genes altered in lowCa/BCAA/ACEi and Ca-depleted compared to the low calcium basal restricted diet group (BR) in adipose tissue (step-up FDR corrected $p < 0.05$).

Category	Term	Count	%
LowCa/BCAA/ACEi vs. BR			
KEGG_PATHWAY	Basal cell carcinoma	12	0.8
KEGG_PATHWAY	Wnt signaling pathway	19	1.2
KEGG_PATHWAY	Dentatorubropallidoluysian atrophy (DRPLA)	5	0.3
KEGG_PATHWAY	MAPK signaling pathway	28	1.8
KEGG_PATHWAY	Adipocytokine signaling pathway	11	0.7
KEGG_PATHWAY	Melanogenesis	13	0.8
KEGG_PATHWAY	Jak-STAT signaling pathway	18	1.1
KEGG_PATHWAY	Toll-like receptor signaling pathway	13	0.8
KEGG_PATHWAY	Pancreatic cancer	10	0.6
KEGG_PATHWAY	Hedgehog signaling pathway	8	0.5
KEGG_PATHWAY	Streptomycin biosynthesis	3	0.2
KEGG_PATHWAY	Ubiquitin mediated proteolysis	15	0.9
KEGG_PATHWAY	Small cell lung cancer	11	0.7
Ca-depleted vs BR			
KEGG_PATHWAY	Endometrial cancer	11	0.6
KEGG_PATHWAY	GnRH signaling pathway	15	0.9
KEGG_PATHWAY	MAPK signaling pathway	31	1.8
KEGG_PATHWAY	Regulation of actin cytoskeleton	26	1.5
KEGG_PATHWAY	TGF-beta signaling pathway	14	0.8
KEGG_PATHWAY	ErbB signaling pathway	13	0.8
KEGG_PATHWAY	Leukocyte transendothelial migration	16	0.9
KEGG_PATHWAY	Long-term potentiation	10	0.6
KEGG_PATHWAY	Folate biosynthesis	7	0.4
KEGG_PATHWAY	Calcium signaling pathway	21	1.2
KEGG_PATHWAY	Olfactory transduction	6	0.3
KEGG_PATHWAY	Long-term depression	11	0.6

well known for its effects on protein synthesis and maintenance of muscle mass (10,11). However, this is an anabolic process and requires the addition of energy. Interestingly, there is evidence that leucine alters energy partitioning between adipose tissue and muscle (8). The decrease in fat storage and the increase in fat oxidation in muscle which is seen after leucine treatment suggest that adipose tissue fat stores are utilized by muscle to fuel the energetically-expensive process of protein synthesis. The results of this analysis support this concept. The insulin and PPAR signaling pathways were highly impacted in adipose tissue by the milk diet group, resulting in downregulation of genes affecting lipogenesis such as ACC, SCD1, and FAS. ACC and FAS are key enzymes of fatty acid synthesis and highly regulated by hormonal and transcriptional control (30,31). The fact that these results are mostly found in the milk diet groups implies that these effects are mainly calcium dependent. In addition, we found in muscle an upregulation of adiponectin receptor I in the milk diet group. AdipoRI is the main adiponectin receptor expressed in skeletal muscle, and adiponectin binding leads to stimulation of AMPK. Both an increase in AdipoRI expression and an increase in AMPK activity are associated with improved insulin sensitivity, enhanced glucose uptake and fatty acid oxidation (32,33). On the other hand, the mTOR pathway was altered in muscle in both the lowCa/BCAA/ACEi and Ca-depleted diet group, suggesting a leucine effect. The fact that AMPK activation results in inhibition of mTOR pathway may explain that the pathway was less impacted in the milk diet group (34).

Other pathways that were altered in adipose tissue in the low Ca/BCAA/ACEi and Ca depleted diet group were JAK-STAT signaling pathway, MAPK signaling pathway, and TGF-beta signaling. These pathways are involved in inflammation, proliferation and cell cycle regulation (35-38). Adipose tissue is a major production site of both pro-inflammatory cytokines

such as TNFα and IL-6, and anti-inflammatory factors such as adiponectin and IL-15. However, excess in body fat, especially visceral adiposity, is associated with a dysregylation of adipocytokines leading to a low-grade systemic inflammation (39.40). In addition, adipose tissue is recognized as a significant source of angiotensin II (41), and the local renin-angiotensin-system (RAS) plays a significant role in the development of inflammatory and atherogenic diseases linking obesity to hypertension, diabetes and CVD (42). Accordingly, ACEi peptides in dairy products may contribute to an anti-inflammatory and antithrombotic effect in these diet groups. We also note a significant downregulation of platelet-derived growth factor D in all three diet groups (PDGF-D). Overactivity of PDGFs has been implicated in conditions characterized by increased cell proliferation such as tumor growth but also in atherosclerosis and fibrotic diseases (43).

Interestingly, we found an upregulation of transformation related protein 53 (p53) in all three treatment groups in adipose tissue. p53, first identified as tumor suppressor gene, regulates cellular response such as apoptosis, cell cycle arrest and senescence to a broad array of stress types (44). However, p53 activation has also been found in adipocytes of obese mice, where it results in negative regulation of SREBP-1 and thereby of lipogenic genes such as FAS (45). Accordingly, the observed downregulation of adipose tissue FAS expression in all three treatment groups may be due to upregulation of p53.

In conclusion, we have identified several roles for dairy components in modulating expression pattern and pathways involved in energy metabolism and inflammation in adipose and muscle tissue. Our results demonstrate calcium-dependent effects as well as calcium-independent effects of BCAAs and ACEis on lipid metabolism, protein synthesis and suppression of inflammation.

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Part Four

Dietary Calcium and Dairy Modulation of Oxidative Stress and Life Span in aP2-Agouti and Wild-type Mice

4.1 Abstract

Dietary Ca reduces oxidative and inflammatory stress, while milk exerts a greater effect than supplemental Ca. To examine the effects on lifespan and related biomarkers, aP2-agouti transgenic (model of diet-induced obesity) and wild-type mice were fed a low Ca (0.4% Ca), high-Ca (1.2% Ca from $CaCO_3$), or milk (1.2% Ca from non-fat dry milk) obesigenic diet until their death. Randomized subgroups were sacrificed at 28, 52 and 78 weeks of age for biomarker analysis. The milk diet attenuated weight and adiposity gain, prevented muscle loss ($p<0.04$), and the age-related rise in reactive oxygen species (ROS) production in adipose tissue ($p=0.05$). This was associated with an increase in SOD3 (in wild-type, $p=0.04$) and glutathione peroxidase gene expression ($p<0.02$) in soleus muscle, and liver SOD activity ($p<0.04$). In male mice, the milk diet decreased IL6 ($p<0.05$) and TNFα ($p<0.03$) gene expression in adipose tissue. The survival analysis showed an overall shorter lifespan in transgenic vs. wild-type mice. Although no diet effect was found on maximal lifespan, the milk diet resulted in a significant increase in the 75% survival rate in wild-type mice ($p<0.05$). These data demonstrate that milk diet attenuates adiposity, protects against muscle loss and reduces oxidative and inflammatory stress. Although these effects seem not to influence the maximum lifespan, they may suppress early mortality.

4.2 Introduction

Oxidative stress and inflammatory stress have been implicated as a cause of tissue damage in multiple organ systems leading to the development of chronic diseases such as

obesity, diabetes, hypertony and atherosclerosis (1-3). They are also recognized as one of the major factors contributing to the physiological process of aging (4, 5). Dietary calcium appears to play a significant role in regulating ROS production and inflammation in adipocytes (6) and may therefore contribute to a reduction of metabolic disorders and a prolonged lifespan.

The major intracellular source of ROS production and free radicals in mammalian cells are mitochondria (7). Under normal physiological conditions, mitochondria generate ROS only at very low levels. However, several conditions, including obesity, hyperglycemia and hyperlipidemia can promote ROS production leading to oxidative damage to DNA, lipids and proteins. The resulting mitochondrial dysfunction can lead to increased ROS production with subsequent further damage and progressive decline of cellular and tissue functions due to energy depletion (7). Supporting evidence shows a strong correlation between aging, increased mitochondrial ROS production and mitochondrial dysfunction (8-11). In addition, inflammatory mediators such as tumor necrosis factor alpha (TNF-α) can induce oxidative stress by up-regulating NADPH oxidase and altering the mitochondrial redox state (12, 13).

As a cellular defense against oxidative stress, mammalian cells have developed an array of antioxidant enzymes and proteins to dispose ROS under normal physiological conditions. These include superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase. However, the age associated decline of the enzyme activities coupled with an increase in intracellular ROS production can overwhelm the capacity of this defense system, resulting in increased oxidative stress (4, 7, 14).

Previous work from our laboratory has demonstrated an anti-obesity effect of calcium rich diets, which we have proposed to be mediated, in part, by calcium suppression of circulating 1α, 25-Dihydroxyvitamin D (Calcitriol) (15-17). Calcitriol promotes glucocorticoid production

in adipocytes, favoring fat storage in visceral rather than subcutaneous depots (18). Visceral adiposity is characterized by a low-grade systemic inflammation and is associated with the development of metabolic disorders (19). Calcitriol also regulates ROS production by adipocytes (6). Consequently, we have shown that suppression of calcitriol by dietary calcium inhibits adipocyte-derived inflammatory cytokine expression (20) and reduces oxidative stress in murine and humane adipocytes (6, 21). We also demonstrated that dairy products exert greater effects than supplemental calcium, and have proposed that this increased effect is due to additional components in dairy such as branched-chain amino acids (BCAA) and angiotensin converting enzyme inhibitor (ACEi) peptides (22, 23).

Considering the protective role of dietary calcium and other dairy components against oxidative and inflammatory stress, which otherwise accelerate the process of aging, the present study was designed to evaluate the effects of dietary calcium from both non-dairy and dairy sources on mouse lifespan and on lifespan-related biomarkers. We utilized aP2-agouti transgenic mice because we have already demonstrated effects of calcium on adiposity, oxidative stress and inflammatory stress in this model and because these mice are susceptible to diet-induced obesity. These mice express normal agouti protein under the control of the aP2 promoter in adipose tissue (24), thereby mimicking the human pattern of agouti expression. They are not obese on a standard AIN-93 G diet but develop mild to moderate obesity when fed high sucrose/high fat diets. In order to gain a more complete understanding of the effect of this genotype, this study was also done in wild type littermates.

4.3 Materials and Methods

4.3.1 Animals and Diets

Six to eight week old aP2-agouti mice of both genders (n=197) and their littermate controls (n=186) from our colony were randomly divided into three diet groups. Group 1 (control group) was fed a modified AIN93G diet with suboptimal calcium (calcium carbonate, 0.4%) and with sucrose as sole carbohydrate source, providing 64% of energy. Fat was increased to 25 % of energy with lard. Group 2 (high calcium group) and group 3 (milk group, NFDM) received the same diet as group 1, however modified to contain 1.2% calcium in form of calcium carbonate or to contain 50% of protein replaced by non-fat dry milk with 1.2% calcium, respectively. Diet composition is shown in Table 4-1. There were similar numbers of both genders and genotypes in each group (31-35 animals/subgroup). Three to four animals of same gender and diet group were kept together in polypropylene cages at room temperature of 22+/- 2^0C and a regime of 12h light/dark cycle until their death. The animals had free access to water and their experimental food throughout the experiment. All animals were checked daily for any signs of diseases or death and moribund animals (as defined by the facility veterinarian) were humanely euthanized. Weight was measured to the nearest gram at the beginning of the experiment and then at the beginning of each month until the death of the animals. Small subgroups (4 males and 4 females) of each diet group and breed (48 animals per age group) were terminated under CO_2 anesthesia at 28, 52 and 78 weeks of age to determine biochemical markers in adipose tissue, liver, muscle and blood. Blood was collected by cardiac puncture. The excised tissues were immediately weighed and used for further studies, as described below. This study was approved from an ethical standpoint by the University of Tennessee Institutional Animal Care and Use Committee.

Table 4-1: Diet composition of the control, high calcium and non-fat dry milk (NFDM) diet groups.

	Diet		
	Control (0.4% Ca)	High Ca (1.2% Ca)	NFDM (1.2% Ca)
Ingredient (gm)			
Casein, 80 Mesh	160	160	0
DL-Methionine	3	3	0
Sucrose	637.9	638.3	429.7
Cellulose	50	50	50
Soybean oil	100	100	41.8
Lard	10	10	65
Mineral Mix S10022B	7	7	7
Calcium carbonate	10	30	17.4
Potassium phosphate, monobasic	8	8	8
Potassium Citrate, 1 H2O	1.6	1.6	1.6
Vitamin Mix V10037	10	10	10
Choline Bitartrate	2.5	2.5	2.5
t-butyl hydroqinone	0.014	0.014	0.014
Milk, Nonfat, Dry	0	0	400
Total	1000.014	1020.414	1033.014
Macronutrients (g/kgdiet)			
Protein	143.8	143.8	144.8
Carbohydrate	647.9	648.3	647.7
Fat	110	110	110
Fiber	50	50	50
Macronutrients (gm %)			
Protein	14.4	14.1	14
Carbohydrate	64.8	63.5	62.7
Fat	11	10.8	10.6
Fiber	5	4.9	4.8

Table 4-1, continued.

	Diet		
	Control (0.4% Ca)	High Ca (1.2% Ca)	NFDM (1.2% Ca)
Macronutrients (kcal/kg diet)			
Protein	575	575	579
Carbohydrate	2592	2593	2591
Fat	990	990	990
TOTAL	4157	4158	4160
Macronutrients (kcal %)			
Protein	14	14	14
Carbohydrate	62	62	62
Fat	24	24	24
TOTAL	100	100	100
Calcium, gm	4.03	12.03	12.03
Phosphorus, gm	3.1	3.1	5.7
Potassium, gm	3.61	3.61	10.81

4.3.2 Measurement of blood chemistry markers

Blood glucose was determined in whole blood by using a glucometer. Insulin and adiponectin plasma levels were assessed by using Mouse Insulin and Adiponectin ELISA Kits, respectively (Linco Research, St. Charles, MO). The levels of TNFα, IGF-I, IL6 and IL15 in plasma were measured by using the specific Mouse TiterZyme ELISA Kits from Assay Designs Inc. (Ann Arbor, MI). Plasma CRP levels were calculated by using the Mouse C-reactive protein ELISA Kit (Life Diagnostics, Inc., West Chester, PA). MDA in plasma was assessed by using the TBARS Assay Kit from ZeptoMetrix Corporation (Buffalo, NY).

4.3.3 Measurement of Cortisol release

Perirenal fat tissue was incubated in KRB buffer for 2 hours. Cortisol release in the medium was determined by using the Cortisol Enzyme Immunoassay Kit from Assay Designs, Inc (Ann Arbor, MI).

4.3.4 Determination of intracellular ROS generation in white adipose tissue (perirenal and retroperitoneal fat pads)

Adipose tissue (retroperitoneal and perirenal) digestion and adipocytes preparation was prepared as described in $[Ca^{2+}]_i$ measurement (6). Adipose tissue was washed several times with Hank's balanced salt solution, then minced into small pieces and digested with 0.8 mg/ml type I collagenase in a shaking water bath at 37^{o}C for 30 min. Adipocytes were then filtered through sterile 500-μm nylon mesh and cultured in KRB buffer supplemented with 1% fetal bovine serum (FBS). Cells were cultured in suspension and maintained in a thin layer at the top of culture media for 2h for cell recovery. Intracellular ROS generation was assessed by using 6-

carboxy-2', 7'-dichlorodihydrofluorescein diacetate (H2-DCFDA) as described (25). Cells were loaded with H2-DCFDA (2 μmol/L) for 30 minutes. After washing twice with KRB, Fluorescence (emission 520 nm) and protein content were measured. The intensity of fluorescence is expressed as arbitrary units per μg protein.

4.3.5 Measurement of enzyme activities in liver

The **Superoxide Dismutase activity** (SOD) was measured by using an assay kit from Cayman Chemical Company, Ann Arbor, MI. Tissue samples were prepared according to manufacture's instructions. 200 μl of diluted radical detector (tetrazolium salt) was added to 10 μl of sample or standard. The reaction was started by adding 20 μl of diluted xanthine oxidase. After 20 min of incubation the absorbance was read at 450 nm using a plate reader. One unit of SOD is defined as the amount of enzyme needed to exhibit 50 % dismutation of the superoxide radical. All three types of SODs (Cu/Zn-, Mn-, and Fe-SOD) were measured by the assay.

The **Catalase activity** was measured as described (26). Tissues were rinsed with phosphate buffered saline three times to remove any red blood cells, and then homogenized in 5-10 vol. of cold buffer (50 mM potassium phosphate, pH 7.0). 1% Triton X-100 was added to the stock homogenate (1 + 9) and then further diluted with phosphate buffer (1:200). The reaction was started by adding 1ml H_2O_2 at 20^oC to 2 ml homogenate. The decrease of absorbance at 240 nm against a blank (1ml of phosphate buffer instead of substrate) was recorded for about 30s.

The **Glutathione peroxidase** (GPx) activity was measured using an assay kit from Oxford Biomedical Research, Oxford, MI. Liver tissue was prepared according to manufacture's instructions. 350 μl of Assay buffer, 350 μl of NADPH Reagent and 70 μl of sample were

pipetted into a cuvette. The reaction was started by adding 350 µl of diluted Tert-Butyl Hydroperoxide. The change in absorbance at 340 nm was recorded for three minutes.

The **NADPH oxidase** activity was measured using the modified Quantitative Photometric Assay as described (27). Freshly excised tissue was homogenized in 5 vol. of homogenization buffer (Krebs-Ringer-Phosphate buffer containing 50 mM HEPES, 100 mM NaCl, 5 mM KCl, 1 mM $MgCl_2/H_2O$, 1 mM NaH_2PO_4, 1 mM $CaCl_2$, and 2 mM glucose, pH 7.4). 50 µl of sample was incubated with 50 µl of PBS and 50 µl of phorbol myristate acetate (PMA) (1.625 µmol/l; Sigma Diagnostics) to initiate the respiratory burst. After 10 min of mixing, 50 µl of Nitroblue tetrazolium (NBT) (2.4 mmol/l; Sigma Diagnostics) was added to each individual well. The rate of NBT reduction was monitored at 490 nm for 30 min and optical density was recorded every 5 min by a photometer for microplates, starting immediately after the addition of NBT. The NADPH oxidase activity is expressed as the mean of absorbance (A) values in the 30-min period.

AMPK activity was measured as described in (28). Freshly excised tissue was immediately homogenized in 2 vol. of homogenization buffer (50 mM Tris/HCL, 250 mM Mannitol, 50 mM NaF, 5 mM Sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM Phenylmethylsulphonylfluoride, 1 mM Benzanidine, 1 µg/ml Soybean trypsin inhibitor). Homogenate was centrifuged at 5000 x g for 10 min and supernatant was aliquoted and stored at -80^{o}C until further use. Reaction mixtures were prepared containing 5 µl of 1 mM [γ-^{32}P] ATP, 5 µl of 1 mM AMP in HEPES-Brij buffer, 5 µl of 1 mM SAMS peptide in HEPES-Brij buffer and 5 µl of HEPES-Brij buffer. The reaction was initiated by the addition of 5 µl AMPK sample. After 30 min of incubation at 30^{o}C, 15 µl was removed and spotted onto a P81 paper square. After washing the paper squares twice with 1 % (v/v) phosphoric acid and once with acetone,

they were laid out on a paper towel and allowed to dry. Dried filters were counted after immersing in 5 ml of scintillation cocktail. The AMPK activity is expressed as nanomoles of phosphate incorporated into substrate peptide per minute.

4.3.6 Measurement of mRNA expression

Total RNA extraction: A total cellular RNA isolation kit (Ambion, Austin, TX) was used to extract total RNA from abdominal (inguinal and retroperitoneal) fat (3 animals/ subgroup), liver and soleus muscle (4 animals/ subgroup) according to manufacturer's instruction. The concentration, purity and quality of the isolated RNA were assessed by measuring the 260/280 ratio (1.7 – 2.1) and 260/230 ratio (close to 2.0) by using the ND-1000 Spectrophotometer (NanoDrop Technologies Inc., DE).

Quantitative real time PCR: Gene expression of SOD (1 to 3), Glutathione peroxidase, Catalase, NADPH oxidase, TNFα, IL6, IL15, UCP2, UCP3, Adiponectin, 11β-HSD, NFκB, PPARα, PPARγ, PPARσ, AMPK, Telomerase, SIRT1 and SIRT3 in retroperitoneal fat, soleus muscle and liver was quantitatively measured by using an ABI 7300 Real Time PCR System (Applied Biosystem, Branchburg, NJ) with a TaqMan 1000 Core Reagent Kit (Applied Biosystem, Branchburg, NJ). The primers and probes sets were obtained from Applied Biosystems TaqMan® Assays-on-Demand TM Gene Expression primers and probe set collection according to manufacturer's instruction. Pooled adipocyte total RNA was serial-diluted and used to establish a standard curve; total RNAs of unknown samples were also diluted in the same range. Reactions of quantitative RT-PCR for standards and unknown samples were performed according to the instructions of the ABI 7300 Real Time PCR System and TaqMan

1000 Core Reagent Kit (Applied Biosystem, Branchburg, NJ). The mRNA quantitation for each sample was further normalized using the corresponding 18S quantitation.

4.3.7 Statistical analysis:

Data were evaluated by Multivariate Analysis of Variance (MANOVA) for statistical significant main effects of gender, genotype, diet and age and possible interactions between groups. Statistical significantly different means in subgroups were separated by the least significant difference test ($p \leq 0.05$) using ANOVA. Data points were identified as outliers graphically with box plots and removed when they were extreme (out of 3 IQ range). Survival analysis was done by using the Kaplan Meier survival analysis. All data analysis was done with SPSS software (SPSS inc., Chicago, IL). Data are expressed as mean ± standard error (SE).

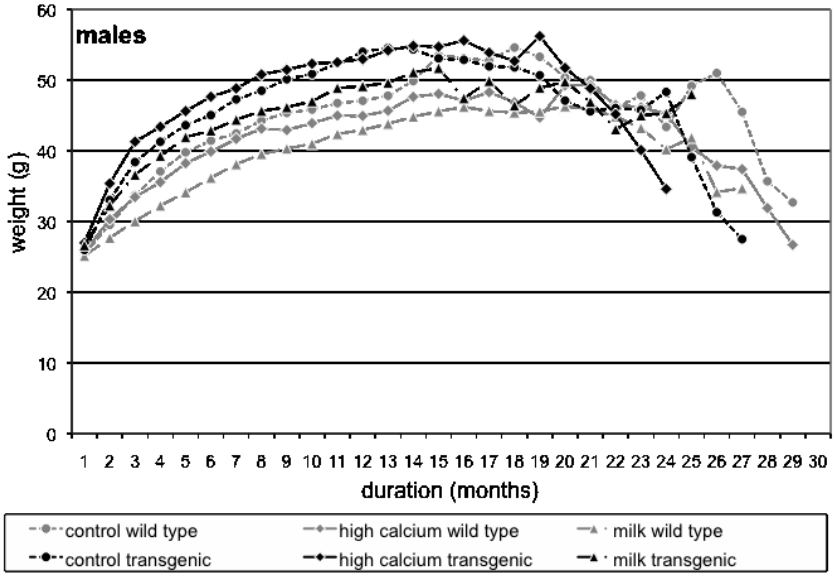
4.4 Results

Because of the complex design of this experiment we used a MANOVA approach in the first step of analysis. This approach permits to assess main effects of diets while holding the other factors (genotype, gender, age) constant as well as to identify interactions between factors. Accordingly, the following section presents the main effects of diet on the combined subgroups, and only presents other main effects and interactions when statistically significant.

4.4.1 Body weight and composition

Figure 4-1 shows the mean of body weight for each subgroup over the study duration. Tables 4-2 and 4-3 list the percentage of total body fat (calculated from the sum of abdominal,

A



B

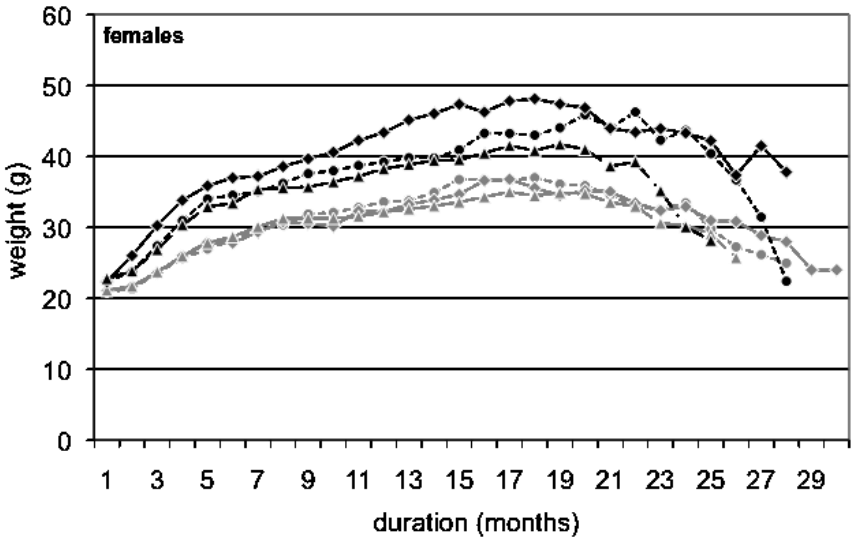


Figure 4-1:

Mean weight distribution (**A:** males, **B**: females of all aP2-agouti transgenic and wild-type mice fed control, high calcium or NFDM diets over the study duration.

Table 4-2: Percentage of total body fat calculated from inguinal, retroperitoneal, perirenal, subscapular and epididymal (in male) fat pads per body weight in aP2-agouti transgenic and wild-type mice fed control, high calcium or NFDM diets at 28, 52 and 78 weeks of age.[1]

			Diet									
			control			high calcium			NFDM			
Gender	Genotype	Age	mean ± SE		n	mean ± SE		n	mean ± SE		n	
female	wild	28 wk	6.53	± 2.89	4	12.10	± 1.57	4	9.24	± 2.22	4	
		52 wk	18.53	± 2.18	4	14.37	± 1.07	4	12.16	± 3.07	4	
		78 wk	19.42	± 1.49	3	17.84	± 3.73	4	12.98	± 1.44	3	
		all	14.41	± 2.26	11	14.77	± 1.44	12	11.32	± 1.39	11	
	transgenic	28 wk	19.27	± 1.69	4	16.90	± 4.72	3	12.99	± 0.65	4	
		52 wk	19.24	± 4.39	4	22.13	± 1.15	4	16.52	± 1.20	4	
		78 wk	23.07	± 2.94	5	23.84	± 4.61	3	18.62	± 3.33	4	
		all	20.72	± 1.76	13	21.07	± 1.98	10	16.05	± 1.29	12	
	both		17.83	± 1.53 [a]	24	17.64	± 1.36 [ab]	22	13.79	± 1.05 [b]	23	
male	wild	28 wk	12.43	± 0.87	4	12.73	± 0.66	4	10.08	± 0.75	4	
		52 wk	15.33	± 1.84	4	15.06	± 0.19	4	13.65	± 0.36	4	
		78 wk	17.47	± 1.96 [a]	4	11.46	± 2.05 [b]	4	13.44	± 1.11 [ab]	4	
		all	15.07	± 1.05 [a]	12	13.08	± 0.79 [ab]	12	12.39	± 0.64 [b]	12	
	transgenic	28 wk	12.54	± 1.11	4	12.38	± 0.49	5	12.87	± 0.94	4	
		52 wk	16.67	± 1.25 [a]	3	12.88	± 1.08 [b]	4	13.18	± 0.51 [b]	4	
		78 wk	19.97	± 0.73	3	13.98	± 4.94	3	13.56	± 0.92	4	
		all	16.00	± 1.23 [a]	10	12.94	± 1.13 [b]	12	13.20	± 0.43 [ab]	12	
	both		15.50	± 0.79 [a]	22	13.02	± 0.68 [ab]	24	12.79	± 0.39 [b]	24	

[1] Values are expressed as mean ± SE. Non-matching superscripts denote significant differences between diet groups, $p < 0.05$.

Table 4-2, continued.

			Diet									
			control				high calcium			NFDM		
Gender	Genotype	Age	mean ± SE			n	mean ± SE		n	mean ± SE		n
both	wild	28 wk	9.48	±	1.79	8	12.42	± 0.79	8	9.66	± 1.09	8
		52 wk	16.93	±	1.46 [a]	8	14.72	± 0.52 [ab]	8	12.91	± 1.46 [b]	8
		78 wk	18.30	±	1.25 [a]	7	14.62	± 2.30 [ab]	8	13.24	± 0.84 [b]	7
		all	14.76	±	1.18 [a]	23	13.93	± 0.83 [ab]	24	11.88	± 0.74 [b]	23
	transgenic	28 wk	15.90	±	1.60	8	14.07	± 1.78	8	12.93	± 0.52	8
		52 wk	18.14	±	2.42	7	17.50	± 1.89	8	14.85	± 0.87	8
		78 wk	21.90	±	1.92	8	18.91	± 3.74	6	16.09	± 1.86	8
		all	18.67	±	1.21 [a]	23	16.64	± 1.38 [ab]	22	14.63	± 0.73 [b]	24
	both	28 wk	12.69	±	1.42	16	13.24	± 0.96	16	11.29	± 0.72	16
		52 wk	17.49	±	1.32 [a]	15	16.11	± 1.01 [ab]	16	13.88	± 0.86 [b]	16
		78 wk	20.22	±	1.24 [a]	15	16.47	± 2.07 [ab]	14	14.76	± 1.09 [b]	15
		all	16.72	±	0.88 [a]	46	15.23	± 0.81 [ab]	46	13.28	± 0.55 [b]	47

[1] Values are expressed as mean ± SE. Non-matching superscripts denote significant differences between diet groups, $p < 0.05$.

Table 4-3: Percentage of total muscle mass calculated from gastrocnemius and soleus muscle per body weight in aP2-agouti transgenic and wild-type mice fed control, high calcium or NFDM diets at 28, 52 and 78 weeks of age.[11]

			Diet									
			control				high calcium			NFDM		
Gender	Genotype	Age	mean ± SE			n	mean ± SE		n	mean ± SE		n
female	wild	28 wk	0.33	±	0.05	4	0.40	± 0.04	4	0.32	± 0.04	4
		52 wk	0.25	±	0.04	4	0.28	± 0.02	4	0.41	± 0.09	4
		78 wk	0.35	±	0.06	3	0.32	± 0.05	4	0.36	± 0.03	3
		all	0.31	±	0.03	11	0.33	± 0.02	12	0.36	± 0.03	11
	transgenic	28 wk	0.28	±	0.02	4	0.35	± 0.10	3	0.38	± 0.05	4
		52 wk	0.22	±	0.02	4	0.24	± 0.04	4	0.30	± 0.04	4
		78 wk	0.18	±	0.02	5	0.27	± 0.08	3	0.33	± 0.08	4
		all	0.22	±	0.02 [a]	13	0.28	± 0.04 [ab]	10	0.34	± 0.33 [b]	12
	both		0.26	±	0.02 [a]	24	0.31	± 0.02 [ab]	22	0.35	± 0.02 [b]	23
male	wild	28 wk	0.29	±	0.05	4	0.28	± 0.05	4	0.43	± 0.06	4
		52 wk	0.13	±	0.01 [a]	4	0.22	± 0.03 [b]	4	0.25	± 0.03 [b]	4
		78 wk	0.15	±	0.05 [a]	4	0.19	± 0.04 [a]	4	0.42	± 0.05 [b]	4
		all	0.19	±	0.03 [a]	12	0.23	± 0.02 [ab]	12	0.36	± 0.04 [b]	12
	transgenic	28 wk	0.23	±	0.05	4	0.26	± 0.02	5	0.23	± 0.01	4
		52 wk	0.15	±	0.02	3	0.16	± 0.03	4	0.24	± 0.03	4
		78 wk	0.19	±	0.005	3	0.29	± 0.04	3	0.23	± 0.08	4
		all	0.19	±	0.02	10	0.24	± 0.02	12	0.24	± 0.03	12
	both		0.19	±	0.02 [a]	22	0.23	± 0.02 [ab]	24	0.30	± 0.03 [b]	24

[1] Values are expressed as mean ± SE. Non-matching superscripts denote significant differences between diet groups, $p < 0.05$.

Table 4-3, continued.

			Diet								
			control				high calcium			NFDM	
Gender	Genotype	Age	mean ± SE			n	mean ± SE		n	mean ± SE	n
both	wild	28 wk	0.31	±	0.03	8	0.34 ± 0.04		8	0.38 ± 0.04	8
		52 wk	0.19	±	0.03 [a]	8	0.25 ± 0.02 [ab]		8	0.33 ± 0.05 [b]	8
		78 wk	0.24	±	0.05 [a]	7	0.25 ± 0.04 [a]		8	0.39 ± 0.03 [b]	7
		all	0.25	±	0.024 [a]	23	0.28 ± 0.02 [ab]		24	0.36 ± 0.02 [b]	23
	transgenic	28 wk	0.25	±	0.02	8	0.29 ± 0.04		8	0.31 ± 0.04	8
		52 wk	0.19	±	0.02 [a]	7	0.20 ± 0.03 [ab]		8	0.27 ± 0.03 [b]	8
		78 wk	0.18	±	0.01	8	0.28 ± 0.04		6	0.29 ± 0.06	8
		all	0.20	±	0.01 [a]	23	0.26 ± 0.02 [ab]		22	0.29 ± 0.02 [b]	24
	both	28 wk	0.28	±	0.02	16	0.32 ± 0.03		16	0.34 ± 0.03	16
		52 wk	0.19	±	0.02 [a]	15	0.23 ± 0.02 [a]		16	0.30 ± 0.03 [b]	16
		78 wk	0.20	±	0.02 [a]	15	0.27 ± 0.03 [ab]		14	0.34 ± 0.04 [b]	15
		all	0.23	±	0.01 [a]	46	0.27 ± 0.01 [ab]		46	0.33 ± 0.02 [b]	47

[1] Values are expressed as mean ± SE. Non-matching superscripts denote significant differences between diet groups, $p < 0.05$.

perirenal, subscapular and epididymal (in male mice) fat pads divided by body weight) and the percentage of muscle tissue (calculated from the sum of soleus and gastrocnemius muscle divided by body weight), respectively.

4.4.1.1 Males:

Transgenic. The milk diet group showed a lower weight compared to the high calcium group continuously over the first year and to the control group starting at month 6. Although, no difference in weight was found between high calcium and control group, there was a significant reduction in adipose tissue mass (Table 4-2).

Wild-type. The weight in the milk diet was continuously lower compared to the control and high calcium diet group. This difference was associated with a reduction in adiposity (Table 4-2) and a prevention of muscle loss with age (Table 4-3) which was significantly different from the control group, but not the high calcium group. Although body weight in the high calcium group was not significantly different from the control group, there was a significant reduction in adiposity and a prevention of muscle loss compared to the control group at 78 weeks and 52 weeks of age, respectively.

4.4.1.2 Females:

Transgenic. The high calcium diet group exhibited the highest weight, and this difference reached statistical significance in older animals. There was a trend for lower adiposity and higher percentage of muscle tissue in the milk diet group compared to the control diet group, however, it did not reach statistical significance.

Wild-type. No difference in weight, fat mass and muscle mass was found among the diet groups.

4.4.1.3 Summary:

There was a significant gender–diet interaction reflected in a significant weight difference in the male mice for both genotypes in the milk diet group compared to control and high calcium diet group but not in female mice. In addition, the milk diet prevented the increase in fat mass and the loss in muscle mass with age compared to the control group when genotypes and genders were combined,

4.4.2 Oxidative stress

ROS production in white adipose tissue (Fig. 4-2). The age-related rise in ROS production was significantly blunted in the high calcium and milk diet groups compared to the control group.

GPx gene expression in soleus (Table 4-4). There were no diet interactions found; therefore data are collapsed for genotype, gender and age. The milk diet group had a significantly higher level of GPx gene expression than the control and high calcium diet groups.

SOD 3 gene expression in soleus (Table 4-4). There was a significant diet-genotype interaction. Wild type mice showed increased levels of SOD 3 expression in the milk diet group while no diet effect was found in the transgenic mice.

SOD 3 gene expression and SOD enzyme activity in liver (Table 4-4 and 4-5). There were no diet interactions found; therefore only main effects of diet are reported. There was an overall increase in SOD enzyme activity in liver in the milk diet group compared to high calcium and control group; however, this was not associated with a significant difference in gene expression.

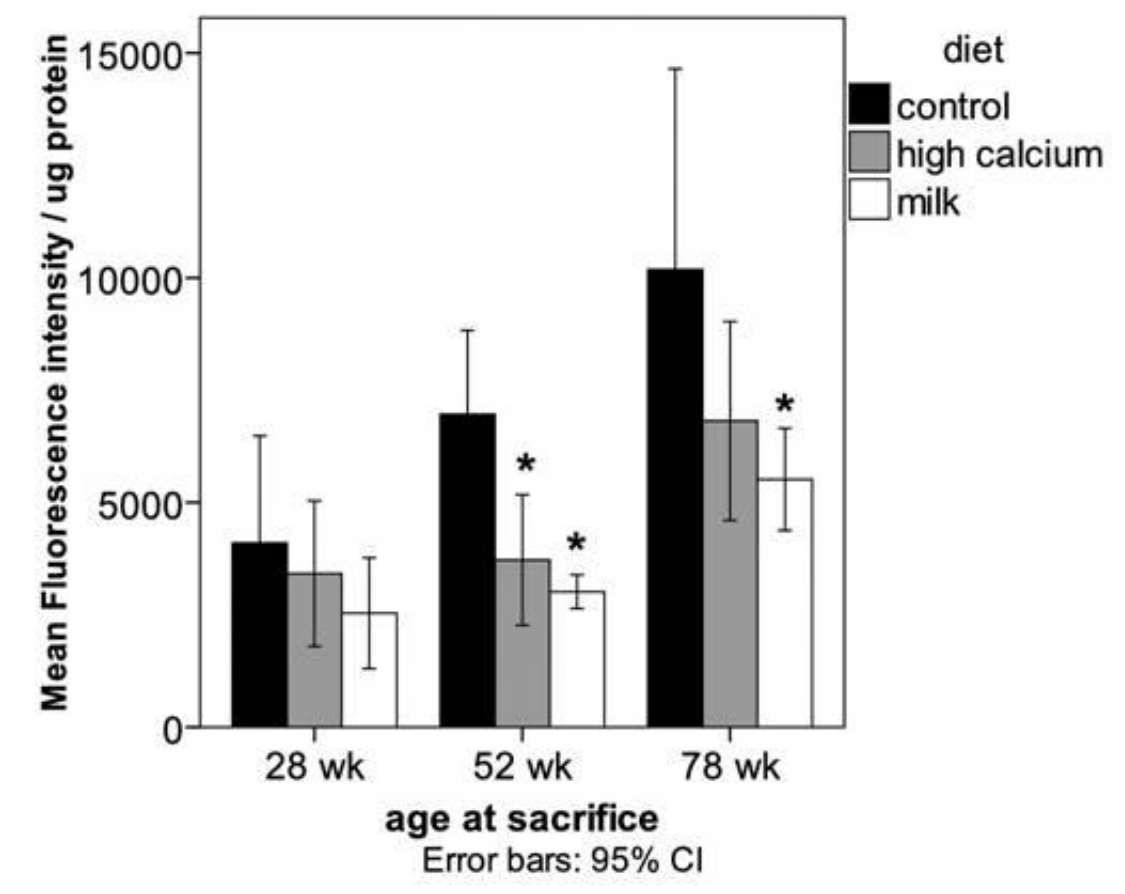


Figure 4-2:

ROS production (expressed as fluorexcence intensity/ μg protein) in white adipose tissue (retroperitoneal and perirenal fat) in aP2-agouti transgenic and wild-type mice (gender and genotype combined) fed control, high calcium or NFDM diets at 28, 52 and 78 weeks of age. Values are expressed as mean ± SE, (n= 4-5). * Significant different from control, $p < 0.05$.

Table 4-4: Glutathione Peroxidase ((GPx), soleus muscle) and Superoxide Dismutase 3 (SOD3) gene expression (soleus muscle and liver) of aP2-agouti transgenic and wild-type mice fed control, high calcium or NFDM diets, all three age groups combined.[1]

			Diet					
			control		high calcium		NFDM	
Gene expression	Gender	Genotype	mean ± SE	n	mean ± SE	n	mean ± SE	n
GPx soleus	female	wild	0.77 ± 0.06 [a]	12	0.93 ± 0.09 [ab]	12	1.06 ± 0.09 [b]	12
		transgenic	0.81 ± 0.08 [a]	12	0.84 ± 0.08 [a]	11	1.20 ± 0.14 [b]	12
		both	0.79 ± 0.05 [a]	24	0.89 ± 0.06 [a]	23	1.14 ± 0.08 [b]	24
	male	wild	0.83 ± 0.07	11	0.74 ± 0.07	12	1.22 ± 0.20	12
		transgenic	0.83 ± 0.14	11	0.81 ± 0.10	13	0.82 ± 0.11	13
		both	0.83 ± 0.07	22	0.77 ± 0.06	25	1.01 ± 0.12	25
	both	wild	0.80 ± 0.05 [a]	23	0.83 ± 0.06 [a]	24	1.14 ± 0.10 [b]	24
	both	transgenic	0.82 ± 0.08	23	0.83 ± 0.07	24	1.02 ± 0.10	25
	both	both	0.81 ± 0.04 [a]	46	0.83 ± 0.05 [a]	48	1.07 ± 0.07 [b]	49
SOD3 soleus	female	wild	0.73 ± 0.09 [a]	12	0.88 ± 0.12 [a]	12	1.31 ± 0.19 [b]	12
		transgenic	0.81 ± 0.10	12	0.84 ± 0.13	11	0.88 ± 0.10	12
		both	0.77 ± 0.07 [a]	24	0.86 ± 0.09 [ab]	23	1.09 ± 0.12 [b]	24
	male	wild	0.96 ± 0.08	11	0.90 ± 0.17	12	1.27 ± 0.26	12
		transgenic	0.80 ± 0.07	11	0.89 ± 0.09	13	0.85 ± 0.14	13
		both	0.88 ± 0.06	22	0.90 ± 0.10	25	1.06 ± 0.15	25
	both	wild	0.84 ± 0.07 [a]	23	0.89 ± 0.11 [ab]	24	1.30 ± 0.16 [b]	24
	both	transgenic	0.81 ± 0.06	23	0.87 ± 0.08	24	0.87 ± 0.09	25
	both	both	0.82 ± 0.05	46	0.88 ± 0.07	48	1.07 ± 0.09	49

[1] Gene expression data are normalized to 18S expression. Values are expressed as mean ± SE. Non-matching superscripts denote significant differences between diet groups, $p < 0.05$.

Table 4-4, continued.[1]

			Diet					
			control		high calcium		NFDM	
Gene expression	Gender	Genotype	mean ± SE	n	mean ± SE	n	mean ± SE	n
SOD3 liver	female	wild	0.48 ± 0.07	12	0.79 ± 0.22	12	0.86 ± 0.27	12
		transgenic	0.62 ± 0.11	12	0.52 ± 0.13	11	0.78 ± 0.26	12
		both	0.56 ± 0.07	24	0.66 ± 0.13	23	0.83 ± 0.19	24
	male	wild	0.48 ± 0.10	11	0.50 ± 0.12	12	0.97 ± 0.52	11
		transgenic	0.52 ± 0.13	11	0.38 ± 0.06	12	0.41 ± 0.08	13
		both	0.51 ± 0.08	22	0.44 ± 0.07	24	0.67 ± 0.25	24
	both	wild	0.49 ± 0.06	23	0.65 ± 0.13	24	0.92 ± 0.28	23
	both	transgenic	0.58 ± 0.09	23	0.45 ± 0.07	23	0.59 ± 0.14	25
	both	both	0.53 ± 0.05	46	0.55 ± 0.08	47	0.75 ± 0.15	48

[1] Gene expression data are normalized to 18S expression. Values are expressed as mean ± SE. Non-matching superscripts denote significant differences between diet groups, $p < 0.05$.

Table 4-5: Liver SOD enzyme activity of aP2-agouti transgenic and wild-type mice fed control, high calcium or NFDM diets, all three age groups combined.[1]

			Diet								
			control			high calcium			NFDM		
Enzyme activity	Gender	Genotype	mean ± SE		n	mean ± SE		n	mean ± SE		n
SOD liver	female	wild	0.59 ± 0.07 [ab]		12	0.35 ± 0.08 [a]		12	0.83 ± 0.17 [b]		12
		transgenic	0.55 ± 0.16		12	0.65 ± 0.14		11	0.63 ± 0.12		12
		both	0.57 ± 0.90		24	0.49 ± 0.08		23	0.73 ± 0.10		24
	male	wild	0.54 ± 0.16		11	0.48 ± 0.10		12	0.76 ± 0.18		11
		transgenic	0.34 ± 0.08		11	0.37 ± 0.07		12	0.59 ± 0.14		13
		both	0.44 ± 0.09		22	0.42 ± 0.06		24	0.68 ± 0.11		24
	both	wild	0.56 ± 0.09 [ab]		23	0.42 ± 0.06 [a]		24	0.79 ± 0.12 [b]		23
	both	transgenic	0.45 ± 0.09		23	0.50 ± 0.08		23	0.61 ± 0.09		25
	both	both	0.50 ± 0.06 [a]		46	0.46 ± 0.05 [a]		47	0.70 ± 0.07 [b]		48

[1] Enzyme activity is normalized to protein content. Values are expressed as mean ± SE. Non-matching superscripts denote significant differences between diet groups, $p < 0.05$.

4.4.2.1 Summary:

The age-related rise in ROS production was significantly attenuated by calcium rich diets. This was associated in the milk diet group with an increase in GPx and SOD3 gene expression in soleus muscle as well as an increase in liver SOD enzyme activity. We also examined other markers such as Catalase and NADPH oxidase gene expression and liver activity, AMPK liver activity, and serum MDA levels; however, no significant differences among the diet groups were found (data not shown).

4.4.3 Inflammatory Stress

There was a statistically significant diet-gender interaction for adipocytokines (Table 4-6). Male mice showed lower levels of TNFα and IL6 gene expression in abdominal fat tissue in the milk diet, consistent with previous data from our laboratory. Levels in females were quite low compared to males and were unaffected by diet. Cytokines and other markers in serum were unchanged (data not shown).

4.4.4 Survival

The survival analysis showed a longer lifespan in wild-type compared to transgenic mice for the milk diet group (Fig. 4-3B). There was no overall diet effect on maximal lifespan (Fig. 4-3A), although the milk diet significantly reduced the early mortality rate in wild-type mice as demonstrated by a significant increase in 75% survival rate (Fig. 4-3C).

Table 4-6: Gene expression of IL6, IL15, TNFα and NFκB in inguinal fat tissue in aP2-agouti transgenic and wild-type mice fed control, high calcium or NFDM diets, all three diet groups combined.[1]

			Diet									
			control				high calcium				NFDM	
Gene expression	Gender	Genotype	mean ± SE			n	mean ± SE			n	mean ± SE			n
IL6	female	wild	0.21	±	0.04	8	0.40	±	0.13	8	0.42	±	0.16	9
		transgenic	0.38	±	0.08	11	0.38	±	0.08	9	0.26	±	0.32	8
		both	0.31	±	0.05	19	0.39	±	0.07	17	0.35	±	0.08	17
	male	wild	1.45	±	0.86	8	2.18	±	0.67	10	0.57	±	0.22	8
		transgenic	6.23	±	2.12	8	2.87	±	1.15	8	0.51	±	0.07	9
		both	3.84	±	1.27 [a]	16	2.49	±	0.62 [a]	18	0.53	±	0.48 [b]	17
	both	wild	0.83	±	0.44	16	1.39	±	0.43	18	0.49	±	0.13	17
	both	transgenic	2.85	±	1.09	19	1.56	±	0.61	17	0.39	±	0.05	17
	both	both	1.93	±	0.64	35	1.47	±	0.36	35	0.44	±	0.07	34
TNFα	female	wild	0.33	±	0.06	8	0.69	±	0.30	8	0.24	±	0.04	9
		transgenic	0.47	±	0.08	11	0.68	±	0.16	9	0.50	±	0.16	9
		both	0.41	±	0.05	19	0.69	±	0.16	17	0.37	±	0.08	18
	male	wild	1.49	±	0.47	8	2.39	±	0.39	10	0.73	±	0.13	9
		transgenic	3.83	±	0.74 [a]	6	2.53	±	0.64 [ab]	9	1.17	±	0.20 [b]	9
		both	2.49	±	0.51 [a]	14	2.46	±	0.35 [a]	19	0.95	±	0.13 [b]	18
	both	wild	0.91	±	0.27 [ab]	16	1.64	±	0.32 [a]	18	0.49	±	0.09 [b]	18
	both	transgenic	1.65	±	0.47	17	1.60	±	0.39	18	0.84	±	0.15	18
	both	both	1.29	±	0.28	33	1.62	±	0.25	36	0.66	±	0.09	36

[1] Gene expression data are normalized to 18S expression. Values are expressed as mean ± SE. Non-matching superscripts denote significant differences between diet groups, $p < 0.05$.

Table 4-6, continued.

			Diet								
			control			high calcium			NFDM		
Gene expression	Gender	Genotype	mean ± SE		n	mean ± SE		n	mean ± SE		n
IL15	female	wild	0.43 ± 0.09		8	0.64 ± 0.14		8	0.69 ± 0.18		9
		transgenic	0.84 ± 0.19		11	0.88 ± 0.26		9	0.93 ± 0.27		9
		both	0.66 ± 0.13		19	0.77 ± 0.15		17	0.81 ± 0.16		18
	male	wild	0.85 ± 0.22	a	7	1.45 ± 0.22	b	10	0.61 ± 0.09	a	9
		transgenic	1.18 ± 0.26		8	1.26 ± 0.31		9	1.07 ± 0.21		9
		both	1.02 ± 0.18		15	1.36 ± 0.18		19	0.84 ± 0.12		18
	both	wild	0.62 ± 0.12		15	1.09 ± 0.16		18	0.65 ± 0.10		18
	both	transgenic	0.98 ± 0.16		19	1.07 ± 0.20		18	1.00 ± 0.17		18
	both	both	0.82 ± 0.11		34	1.08 ± 0.13		36	0.83 ± 0.10		36
NFκB	female	wild	0.84 ± 0.25		8	0.85 ± 0.20		8	0.72 ± 0.17		9
		transgenic	0.86 ± 0.17		11	0.96 ± 0.21		9	1.03 ± 0.23		9
		both	0.85 ± 0.14		19	0.91 ± 0.14		17	0.88 ± 0.15		18
	male	wild	1.27 ± 0.38		8	1.58 ± 0.22		10	0.83 ± 0.16		9
		transgenic	1.49 ± 0.47		7	1.09 ± 0.26		9	1.17 ± 0.22		9
		both	1.38 ± 0.29		15	1.35 ± 0.17		19	1.01 ± 0.14		18
	both	wild	1.05 ± 0.22		16	1.26 ± 0.17		18	0.77 ± 0.12		18
	both	transgenic	1.13 ± 0.23		18	1.03 ± 0.17		18	1.11 ± 0.16		18
	both	both	1.09 ± 0.16		34	1.14 ± 0.12		36	0.94 ± 0.10		36

[1] Gene expression data are normalized to 18S expression. Values are expressed as mean ± SE. Non-matching superscripts denote significant differences between diet groups, $p < 0.05$.

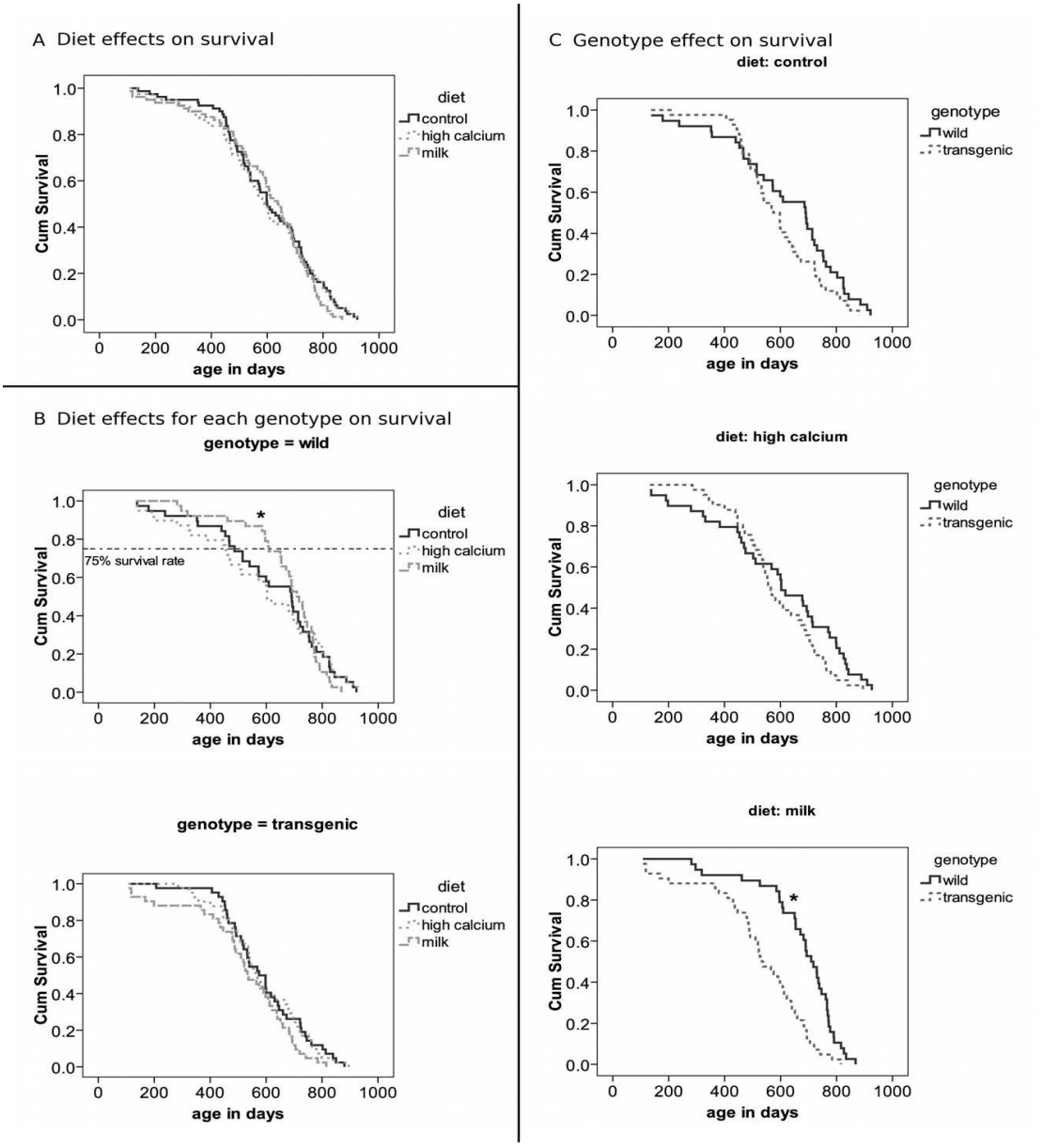


Figure 4-3:

Survival curves of all aP2-agouti transgenic and wild-type mice fed control, high calcium or NFDM diets. **A:** Diet effect on survival independent of gender and genotype. **B:** Genotype effect on survival independent of diet. **C:** Diet effect for each genotype on survival. * Significant different, $p < 0.05$.

4.4.5 Additional findings

Interestingly, we found a reduction of Sirt1 gene expression in abdominal fat (at 78 weeks) and in soleus muscle (Table 4-7). Also liver PPARα gene expression was reduced in the milk diet compared to the control group (Table 4-7).

4.5 Discussion

Our data demonstrate that age-related changes in body composition were blunted by calcium-rich diets. This is consistent with our previous short-term findings, which show that calcium rich diets contribute to a reduction in body fat mass in both humans and mice under eucaloric and hypocaloric conditions (15, 29-31). We have also noted that dairy foods exert significantly greater effects than supplemental sources of calcium, and have attributed this observation to other bioactive components in dairy products, such as branched-chain amino acids (BCAA) and angiotensin-converting enzyme inhibitor (ACEi) peptides (32). Leucine is well recognized to stimulate muscle protein synthesis, and leucine supplementation decreases age-related loss in muscle mass in both rodents and humans (33-35). Our previous data also indicate that leucine alters energy partitioning between adipose tissue and muscle by augmenting lipolysis in adipose tissue and stimulating fatty acid oxidation in muscle to supply the energy demand of the anabolic process of protein synthesis (22). Consistent with these observations, data from this study show a greater effect of the milk diet than of the high calcium diet in preventing the age-related increase in adiposity and the age-related loss of muscle mass.

Table 4-7: Sirt1 (inguinal fat and soleus muscle) and PPARα (liver) gene expression in aP2-agouti transgenic and wild-type mice fed a control, high calcium or NFDM diet.[1]

				Diet					
				control		high calcium		NFDM	
Gene expression	Gender	Genotype	Age	mean ± SE	n	mean ± SE	n	mean ± SE	n
Sirt1 abdominal fat	female	wild	all	0.88 ± 0.28	8	0.97 ± 0.17	8	0.82 ± 0.16	8
		transgenic	all	0.12 ± 0.22	11	1.28 ± 0.25	9	0.91 ± 0.21	9
		both	all	1.02 ± 0.17	19	1.13 ± 0.15	17	0.86 ± 0.13	17
	male	wild	all	1.17 ± 0.38 [a]	8	1.43 ± 0.16 [b]	10	0.77 ± 0.12 [a]	9
		transgenic	all	1.28 ± 0.24	8	1.02 ± 0.25	9	1.10 ± 0.22	9
		both	all	1.23 ± 0.22	16	1.24 ± 0.15	19	0.94 ± 0.13	18
	both	wild	all	1.03 ± 0.23 [ab]	16	1.23 ± 0.13 [b]	18	0.79 ± 0.09 [a]	17
		transgenic	all	1.19 ± 0.16	19	1.15 ± 0.17	18	1.00 ± 0.15	18
		both	28 wk	0.53 ± 0.10	12	0.66 ± 0.13	12	0.69 ± 0.16	11
			52 wk	0.87 ± 0.12 [ab]	12	1.09 ± 0.12 [b]	12	0.56 ± 0.08 [a]	12
			78 wk	2.02 ± 0.19 [a]	11	1.81 ± 0.11 [a]	12	1.43 ± 0.08 [b]	12
	both	both	all	1.12 ± 0.13	35	1.18 ± 0.11	36	0.90 ± 0.09	35
Sirt1 soleus	female	wild	all	0.86 ± 0.12	11	1.02 ± 0.11	11	0.78 ± 0.08	12
		transgenic	all	1.07 ± 0.08 [a]	11	0.86 ± 0.08 [ab]	11	0.82 ± 0.08 [b]	12
		both	all	0.97 ± 0.08	22	0.94 ± 0.07	22	0.80 ± 0.06	24
	male	wild	all	0.96 ± 0.07	10	1.13 ± 0.10	11	0.98 ± 0.13	11
		transgenic	all	0.98 ± 0.11	11	1.01 ± 0.10	11	0.78 ± 0.08	12
		both	all	0.97 ± 0.04 [ab]	21	1.07 ± 0.07 [b]	22	0.88 ± 0.08 [a]	23
	both	wild	all	0.91 ± 0.07	22	1.07 ± 0.07	22	0.88 ± 0.08	23
		transgenic	all	1.03 ± 0.05 [a]	21	0.94 ± 0.06 [ab]	22	0.80 ± 0.05 [b]	24
		both	28 wk	0.79 ± 0.05	14	0.76 ± 0.05	15	0.69 ± 0.06	15
			52 wk	1.04 ± 0.09 [ab]	15	1.20 ± 0.10 [b]	13	0.88 ± 0.07 [a]	16
			78 wk	1.07 ± 0.06	14	1.07 ± 0.07	16	0.93 ± 0.10	16
	both	both	all	0.97 ± 0.04 [a]	43	1.00 ± 0.05 [a]	44	0.84 ± 0.05 [b]	47

[1] Gene expression data are normalized to 18S expression. Values are expressed as mean ± SE. Non-matching superscripts denote significant differences between diet groups, $p < 0.05$.

Table 4-7, continued.

				Diet					
				control		high calcium		NFDM	
Gene expression	Gender	Genotype	Age	mean ± SE	n	mean ± SE	n	mean ± SE	n
PPARα	female	wild	all	0.62 ± 0.11	12	0.65 ± 0.13	12	0.70 ± 0.10	12
liver		transgenic	all	0.83 ± 0.14 [a]	12	0.79 ± 0.14 [a]	11	0.42 ± 0.07 [b]	12
		both	all	0.73 ± 0.09	24	0.72 ± 0.10	23	0.56 ± 0.07	24
	male	wild	all	0.86 ± 0.18	11	0.40 ± 0.08	11	0.41 ± 0.08	11
		transgenic	all	0.86 ± 0.18	11	0.57 ± 0.11	12	0.56 ± 0.07	13
		both	all	0.86 ± 0.12 [a]	22	0.49 ± 0.07 [b]	23	0.49 ± 0.05 [b]	24
	both	wild	all	0.74 ± 0.10	23	0.53 ± 0.08	23	0.56 ± 0.07	23
		transgenic	all	0.84 ± 0.11 [a]	23	0.68 ± 0.09 [ab]	23	0.49 ± 0.05 [b]	25
		both	28 wk	0.88 ± 0.10 [a]	15	0.57 ± 0.09 [b]	16	0.56 ± 0.08 [b]	17
			52 wk	0.47 ± 0.06	16	0.55 ± 0.11	15	0.45 ± 0.07	15
			78 wk	1.04 ± 0.17 [a]	15	0.70 ± 0.12 [ab]	15	0.56 ± 0.07 [b]	16
	both	both	all	0.79 ± 0.08 [a]	46	0.61 ± 0.06 [ab]	46	0.53 ± 0.04 [b]	48

[1] Gene expression data are normalized to 18S expression, enzyme activity to protein content. Values are expressed as mean ± SE.

Non-matching superscripts denote significant differences between diet groups, $p < 0.05$.

The free radical theory of aging, originally proposed by Harman (36), postulates that the aging process is caused by accumulation of free radicals over time, resulting in oxidative damage and progressive deterioration of biological systems. Since mitochondria are the major production site of free radicals, this theory was extended to the mitochondrial theory of aging (37), which proposes that cumulative oxidative damage to mtDNA leads to a decline in bioenergetic function of mitochondria with a progressive decline of cellular and tissue functions. Other major sources of oxidative stress are ROS generating enzymes such as calcium-dependent NADPH oxidase which plays a crucial role in developing vascular diseases (38). Furthermore, there is bidirectional interaction between intracellular calcium signaling and ROS with calcium activating ROS production by cellular enzymes and mitochondria, and ROS increasing intracellular calcium levels by activating calcium channels on plasma membrane and endoplasmatic reticulum (39, 40). Accordingly, our previous data demonstrate that decreasing intracellular $[Ca]^{2+}$ by dietary calcium can diminish ROS production. We have shown that suppression of calcitriol results in an inhibition of cellular NADPH oxidase gene expression (6), and in an increase in UCP production which diminishes mitochondrial membrane potential, thereby resulting in decreased ROS production (41). In addition, dairy products further enhances this anti-oxidant effect (23), most likely mediated by the inhibition of the local adipocyte renin-angiotensin-system (RAS) by ACEi peptides in dairy products (42-44). Data from this long-term study also demonstrate that dietary calcium significantly reduced the age-related rise in ROS, and that dairy exerted a greater effect than calcium carbonate. However, in contrast with our previous data, this reduction was not found in younger mice (28 weeks old) and was not associated with alterations of NADPH oxidase and UCP expression. Instead, we found an increase in antioxidant enzyme activity and associated gene expression in the milk diet. Since

aging is associated with a decline in anti-oxidant enzyme activity (14, 45), our observation that dairy components appear to attenuate this decline is likely to play a role in reducing ROS formation with aging.

Oxidative stress can cause a dysregulation of adipocytokines (2, 46), and data from this study indicate that this effect is attenuated by high calcium diets, as demonstrated by decreases in adipose tissue TNFα and IL6 expression. Consistent with this, we previously demonstrated that calcitriol can stimulate inflammatory cytokine expression in human and murine adipocytes (20) and that suppression of calcitriol by high calcium diets attenuates the inflammatory response in shorter-term studies (23). An additional reduction is seen with dairy products; this is most likely mediated by ACEi peptides (47).

There was a significant gender-diet interaction for body weight and abdominal adipocytokine production. The milk diet significantly reduced body weight gain over the first year and reduced expression of TNFα and IL6 in visceral abdominal fat in male mice, while these effects were not found in females. Multiple studies have shown that sex hormones are responsible for gender specific differences in fat distribution and adipose metabolism (48, 49). Premenopausal females tend to accumulate fat in non visceral areas such as hip and thighs while men develop visceral adiposity with a higher risk for metabolic disorders, as visceral fat is the major production site of adipocytokines (50-52). Moreover, estrogens can cause an anti-inflammatory effect with inhibition of TNFα and IL6 in macrophages (53, 54). We have demonstrated that the response of visceral fat to dietary calcium is higher than that of subcutaneous fat (20). In support of these concepts, the male mice showed markedly higher levels of adipocytokines compared to females.

Since oxidative and inflammatory stress is a major contributor to aging, we hypothesized that a reduction of both by dietary calcium could influence the lifespan in mice. In support of this hypothesis, studies in short-lived animals such as nematodes and flies have shown that a reduction in oxidative stress increased the lifespan (55-57). In addition, the senescence accelerated mouse (SAM) model is associated with many characteristic features of mammalian aging, increased oxidative stress and a shortened lifespan (58), and administration of a free radical scavenger prolonged the lifespan in this model (59). On the other hand, modifying the antioxidant defense system resulted in contradicting findings in some models of aging. While over-expression of catalase in mice extended the life span (60, 61), a reduction in SOD activity increased oxidative stress and cancer incidence but did not accelerate aging in mice (62). Although the age-related increase of oxidative stress was reduced by the milk diet in our animals, we did not find any overall diet effect on mean and maximal survival; however, the milk diet decreased the early mortality in wild-type mice, as demonstrated by a significant increase in 75% survival rate. At this point, it is not clear why this effect is seen only in the wild-type mice. Since the agouti transgene is only locally expressed in adipose tissue, no systemic alterations were expected. However, the survival analysis revealed a shorter survival rate of the transgenic mice compared to the wild-type mice in the milk diet group. Therefore, it is possible that this overall genotype effect could have overwhelmed any possible diet interaction.

Interestingly, we found a decrease of sirtuin 1 (Sirt1) gene expression in abdominal adipose tissue and soleus muscle as well as a reduction of peroxisome proliferation activating receptor (PPAR) α gene expression in liver in the milk diet group. The sirtuins belong to a conserved family of deacetylases and have been linked to prevention of mitochondrial dysfunction, aging and metabolic disease (63, 64). In addition, the NAD+-dependent histone

deacetylase Sirt1 regulates energy metabolism such as glucose homeostasis and fat metabolism (65). PPAR α, a ligand-activated transcription factor and mainly expressed in muscle and liver, is also involved in lipid metabolism and inflammation (66). At this point, it is not clear how to interpret these findings. We speculate that the overall reduction in adiposity, oxidative and inflammatory stress by the milk diet acts as a negative feedback loop and counteracts Sirt1 and PPAR α up-regulation.

In conclusion, data from the present study demonstrate that milk diet attenuates adiposity under eucaloric conditions and protects against age-related muscle loss. Consistent with previous findings, we show that dietary calcium reduces oxidative and inflammatory stress. Although these effects did not to influence the maximum lifespan, they did suppress early mortality.

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Part Five

Summary and Conclusions

The hypothesis that increased oxidative and inflammatory stress contributes to the process of aging, leading to the development of chronic diseases and to reduced life span and quality of life is well accepted. Obesity is associated with increased ROS production and systemic low-grade inflammation, and is consequently one of the major risk factor for the development of metabolic diseases. Dietary calcium and dairy products have been shown to exert anti-obesity effects and to reduce ROS and inflammatory cytokine production in mice and humans. The present studies support this concept. We have demonstrated greater weight and fat loss as well as prevention of age-related changes in adiposity and lean tissue mass for high calcium diets under hypocaloric and eucaloric conditions, respectively. In addition, both studies indicated a role of dietary calcium in reducing oxidative and inflammatory stress. Further, we have elucidated the common and distinct pathways of calcium and other dairy components explaining at least in part the greater efficacy of dairy products on these effects. Although overall these effects were not able to extend absolute lifespan, they did reduce early mortality.

In conclusion, these data demonstrate that dietary calcium, particularly in form of dairy products, is an effective strategy to reduce adiposity, oxidative and inflammatory stress. These effects attenuated age-related changes in body composition and are predicted to maintain physical strength with age, and to reduce morbidity risk. Eventually this may lead to a 'successful aging process' and to a reduction of the early mortality risk.

The finding that Sirt1 gene expression was up-regulated by the milk diet group in the lifespan study was unexpected since sirtuins are linked to longevity, and invites further investigations, especially regarding a possible interaction between situins and oxidative stress. Further, the observed up-regulation of the tumor suppressor gene p53 by dairy components

presents new questions about its possible role in lipid metabolism and may provide a link between obesity and carcinogenesis.

Appendix

Table A1: Genes from Ca-depleted diet group involved in MAPK and TGF-beta signaling pathway in adipose tissue (step-up FDR corrected p≤ 0.05).

Ca-depleted vs BR (adipose tissue)		Mean log base 2 value		Fold change (BR/Ca-depl.)
		BR	Ca-depl.	
MAPK signaling pathway (genes)				
1449901_A_AT	mitogen-activated protein kinase kinase kinase 6	9.16606	8.72352	1.359
1445786_AT	braf transforming gene	6.38478	7.08513	-1.62491
1446390_AT	elk1, member of ets oncogene family	0.459504	2.6056	-4.42628
1441521_AT	calcium channel, voltage-dependent, beta 2 subunit	3.42586	2.48783	1.9159
1422250_AT	mitogen activated protein kinase kinase kinase 2	4.94582	5.86342	-1.88897
1427739_A_AT	transformation related protein 53	6.87725	7.76916	-1.85564
1422078_AT	thymoma viral proto-oncogene 3	6.59676	7.20295	-1.52224
1449545_AT	fibroblast growth factor 18	5.25803	3.47341	3.44527
1450096_AT	guanine nucleotide binding protein, alpha 12	3.85947	6.13253	-4.8335
1452525_A_AT	neurofibromatosis 1	5.25062	6.41262	-2.23768
1448558_A_AT	phospholipase a2, group iva (cytosolic, calcium-dependent)	9.84588	9.55461	1.22372
1421297_A_AT	calcium channel, voltage-dependent, l type, alpha 1c subunit	6.39772	5.40047	1.99619
1443115_AT	transforming growth factor, beta receptor ii	5.06088	5.96757	-1.87474
1439828_AT	map kinase-activated protein kinase 5	6.54279	7.18772	-1.56366
1435196_AT	neurotrophic tyrosine kinase, receptor, type 2	8.51684	7.93628	1.49543
1450698_AT	dual specificity phosphatase 2	7.42911	6.94081	1.40279
1418786_AT	mitogen-activated protein kinase 8 interacting protein 2	4.02391	6.08048	-4.15994
1421376_AT	tnf receptor-associated factor 6	6.72878	7.61592	-1.84951
1456720_AT	protein phosphatase 3, regulatory subunit b, alpha isoform (calcineurin b, type i)	7.16588	8.16064	-1.99275
1427162_A_AT	elk4, member of ets oncogene family	11.2233	10.7157	1.42162
1415974_AT	mitogen activated protein kinase kinase 2	10.1422	9.52584	1.53299
1417542_AT	ribosomal protein s6 kinase, related sequence 1	9.48114	9.2165	1.20134
1427582_AT	fibroblast growth factor 6	3.01589	3.73843	-1.65008
1426108_S_AT	calcium channel, voltage-dependent, beta 1 subunit	4.97692	6.02046	-2.06128

Table A1, continued.

Ca-depleted vs BR (adipose tissue)		Mean log base 2 value		Fold change (BR/Ca-depl.)
		BR	Ca-depl.	
MAPK signaling pathway (genes)				
1447823_X_AT	mitogen-activated protein kinase 12	5.03057	6.62853	-3.02715
1451882_A_AT	fibroblast growth factor 8	1.37588	3.36466	-3.96901
1459029_AT	p21 (cdkn1a)-activated kinase 1	3.00307	1.89113	2.16137
1422999_AT	mitogen-activated protein kinase kinase kinase 14	7.80812	8.39522	-1.50222
1440442_AT	mitogen activated protein kinase kinase 7	3.79031	5.92261	-4.38417
1420596_AT	calcium channel, voltage-dependent, gamma subunit 2	3.13064	3.85915	-1.65692
1449955_AT	calcium channel, voltage-dependent, alpha 1f subunit	6.60535	7.2731	-1.58859
TGF beta signaling pathway (genes)				
1443115_AT	transforming growth factor, beta receptor ii	5.06088	5.96757	-1.87474
1439856_AT	activin receptor iib	5.15205	7.29856	-4.42757
1421822_AT	protein phosphatase 2 (formerly 2a), catalytic subunit, beta isoform	5.33567	6.41794	-2.11737
1440952_AT	mad homolog 7 (drosophila)	7.21287	7.52469	-1.24127
1423259_AT	inhibitor of dna binding 4	9.06812	8.28231	1.72406
1438991_X_AT, 1455929_X_AT	protein phosphatase 2 (formerly 2a), regulatory subunit a (pr 65), alpha isoform	13.5936	13.3733	1.16499
1422058_AT	nodal	6.22734	6.71284	-1.40007
1420033_S_AT	protein phosphatase 2, regulatory subunit b, delta isoform	10.9998	10.8097	1.14087
1433641_AT	mad homolog 5 (drosophila)	9.85841	9.46701	1.31166
1450759_AT	bone morphogenetic protein 6	8.51295	7.51469	1.99759
1453596_AT	inhibitor of dna binding 2	6.31961	7.22302	-1.87048
1419786_AT	latent transforming growth factor beta binding protein 1	2.8265	4.66182	-3.56851
1454852_AT	trans-acting transcription factor 1	11.3672	10.9986	1.29114
1434310_AT	bone morphogenic protein receptor, type ii (serine/threonine kinase)	12.3331	11.9679	1.28804

Table A2: Genes from lowCa/BCAA/ACEi diet group compared to the low calcium basal restricted diet group (BR) involved in MAPK and JAK-STAT signaling pathway in adipose tissue (step-up FDR corrected $p \leq 0.05$).

Low Ca/BCAA/ACEi vs BR (adipse tissue)		Mean log base 2 value		Fold Change (BR/LowCa/ BCAA/ACEi)
		BR	LowCa/ BCAA/ACEi	
MAPK signaling pathway (genes)				
1449901_A_AT	mitogen-activated protein kinase kinase kinase 6	9.16606	8.30664	1.81431
1427739_A_AT	transformation related protein 53	6.87725	7.74499	-1.82481
1422078_AT	thymoma viral proto-oncogene 3	6.59676	7.33573	-1.66899
1425902_A_AT	nuclear factor of kappa light polypeptide gene enhancer in b-cells 2, p49/p100	7.35802	8.40014	-2.05925
1450975_AT	calcium channel, voltage-dependent, gamma subunit 4	5.4792	3.83728	3.12081
1450096_AT	guanine nucleotide binding protein, alpha 12	3.85947	6.18042	-4.99663
1440343_AT	ribosomal protein s6 kinase, polypeptide 5	7.94383	7.28855	1.57492
1435747_AT	fibroblast growth factor 14	2.56013	0.714246	3.59472
1443630_AT	protein phosphatase 3, catalytic subunit, alpha isoform	6.41964	5.65832	1.69505
1453171_S_AT	protein phosphatase 1a, magnesium dependent, alpha isoform	11.0325	10.6321	1.31984
1443115_AT	transforming growth factor, beta receptor ii	5.06088	5.79602	-1.66457
1460296_A_AT	fibroblast growth factor 22	7.13396	4.58583	5.84875
1438325_AT	ecotropic viral integration site 1	8.80928	8.02728	1.71951
1454313_AT, 1457563_AT	epidermal growth factor receptor	7.08212	6.31746	1.69897
1425330_A_AT	protein phosphatase 1b, magnesium dependent, beta isoform	8.20206	8.70774	-1.41979
1447667_X_AT	mitogen activated protein kinase kinase kinase 4	8.0985	7.67661	1.33967
1418943_AT	riken cdna b230120h23 gene	7.0025	7.61462	-1.5285
1449073_AT	filamin c, gamma (actin binding protein 280)	5.4219	7.48902	-4.19049
1426165_A_AT	caspase 3	6.21513	7.01698	-1.74334
1427162_A_AT	elk4, member of ets oncogene family	11.2233	10.8152	1.32692
1438031_AT	ras, guanyl releasing protein 3	6.84792	4.57383	4.83693
1427582_AT	fibroblast growth factor 6	3.01589	5.61369	-6.05361
1449283_A_AT	mitogen-activated protein kinase 12	9.00084	8.27147	1.65792
1442949_AT	nuclear factor of kappa light chain gene enhancer in b-cells 1, p105	2.47938	4.20891	-3.31622
1422999_AT	mitogen-activated protein kinase kinase kinase 14	7.80812	8.24107	-1.34999
1451927_A_AT	mitogen activated protein kinase 14	5.27681	7.39961	-4.35537

Table A2, continued.

Low Ca/BCAA/ACEi vs BR (adipse tissue)		Mean log base 2 value		Fold Change (BR/LowCa/ BCAA/ACEi)
		BR	LowCa/ BCAA/ACEi	
JAK-STAT signaling pathway (genes):				
1416977_AT	signal transducing adaptor molecule (sh3 domain and itam motif) 2	6.92884	7.45337	-1.43847
1450564_X_AT	interferon alpha family, gene 1	4.42874	6.4487	-4.05571
1450565_AT	interleukin 9	4.56361	5.86908	-2.47165
1450033_A_AT	signal transducer and activator of transcription 1	8.81717	9.12516	-1.23799
1442890_AT	suppressor of cytokine signaling 5	7.31111	5.61024	3.25096
1426587_A_AT	signal transducer and activator of transcription 3	10.6534	11.3806	-1.65536
1415874_AT	sprouty homolog 1 (drosophila)	10.0444	9.41377	1.54826
1449026_AT	interferon (alpha and beta) receptor 1	9.33899	9.76613	-1.34456
1448681_AT	interleukin 15 receptor, alpha chain	9.80652	8.85758	1.93045
1425750_A_AT	janus kinase 3	7.15899	7.96689	-1.75067
1422078_AT	thymoma viral proto-oncogene 3	6.59676	7.33573	-1.66899
1450297_AT	interleukin 6	2.73611	5.31157	-5.96061
1438492_AT	suppressor of cytokine signaling 7	6.73446	4.60217	4.38414
1440867_AT	sprouty homolog 4 (drosophila)	7.8384	7.06622	1.70785
1437808_X_AT, 1456173_AT	interleukin 10 receptor, alpha	6.83032	6.08398	1.67754
1450207_AT	leukemia inhibitory factor receptor	6.59455	6.13913	1.37118
1427165_AT, 1451775_S_AT	interleukin 13 receptor, alpha 1	7.8582	8.42551	-1.48177
1433804_AT	janus kinase 1	7.18736	8.07229	-1.84667

Table A3: List of genes in insulin signaling pathway altered in all three diet groups compared to low calcium basal restricted diet group (BR) in muscle (step-up FDR corrected $p \leq 0.05$).

Genes involved in Insulin signaling pathway (muscle)		Mean log base 2 value		Fold change (BR/LowCa/ BCAA/ACEi)
		BR	LowCa/ BCAA/ACEi	
LowCa-BCAA-ACEi vs BR				
1423828_AT	fatty acid synthase	10.7655	11.9248	-2.23346
1447635_AT	protein kinase, camp dependent regulatory, type i, alpha	7.69327	5.1575	5.79889
1453069_AT	phosphatidylinositol 3-kinase, catalytic, beta polypeptide	5.1568	7.60749	-5.46678
1421146_AT	rap guanine nucleotide exchange factor (gef) 1	8.44919	7.14477	2.46985
1445693_AT	v-raf murine sarcoma 3611 viral oncogene homolog	5.10713	7.12827	-4.05902
1453281_AT	phosphatidylinositol 3-kinase catalytic delta polypeptide	8.76758	8.13474	1.55061
1439989_AT	tuberous sclerosis 1	6.65765	3.65317	8.02488
1446185_AT	fk506 binding protein 12-rapamycin associated protein 1	7.97048	6.33475	3.10744
1439931_AT	glycogen synthase kinase 3 beta	5.43039	7.50061	-4.19949
1419568_AT	mitogen activated protein kinase 1	9.26914	8.58738	1.6041
1421275_S_AT	suppressor of cytokine signaling 4	5.9779	3.19968	6.86003
1457562_AT	ribosomal protein s6 kinase, polypeptide 1	4.27528	7.28731	-8.06699
1460336_AT	peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	9.83756	8.90874	1.90372
1433694_AT	phosphodiesterase 3b, cgmp-inhibited	9.73867	10.239	-1.41449
1443436_AT	mitogen activated protein kinase kinase 2	8.17892	6.25341	3.79869
1442044_AT	ribosomal protein s6	6.70554	4.72864	3.93645
1444831_AT	thymoma viral proto-oncogene 3	7.79715	5.0313	6.80146
1425514_AT	phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	7.83016	8.52621	-1.62007
1442147_AT	riken cdna 6330505c01 gene	6.11827	3.40306	6.56692
1426710_AT	calmodulin 1	10.8448	10.3555	1.40374
1430634_A_AT	phosphofructokinase, platelet	6.09196	4.23833	3.6141
1420841_AT	protein tyrosine phosphatase, receptor type, f	5.55126	7.51591	-3.90318

Table A3, continued.

Genes involved in Insulin signaling pathway (muscle)		Mean log base 2 value		Fold change (BR/Ca-depl.)
		BR	Ca-depl.	
Ca-depleted vs BR				
1423828_AT	fatty acid synthase	10.7655	12.0453	-2.42808
1422108_AT	protein phosphatase 1, regulatory (inhibitor) subunit 3a	8.08744	6.73376	2.55563
1440856_AT	mitogen activated protein kinase 8	9.71473	9.39732	1.24609
1437539_AT	protein kinase, amp-activated, alpha 1 catalytic subunit	7.65128	7.17523	1.39093
1427006_AT	rap guanine nucleotide exchange factor (gef) 1	10.3813	10.8088	-1.34494
1447940_A_AT	braf transforming gene	5.49872	7.37856	-3.68034
1421897_AT	elk1, member of ets oncogene family	6.99614	4.30068	6.47763
1453281_AT	phosphatidylinositol 3-kinase catalytic delta polypeptide	8.76758	8.02663	1.67127
1422043_AT, 1455252_AT	tuberous sclerosis 1	10.1581	10.7441	-1.50108
1439931_AT	glycogen synthase kinase 3 beta	5.43039	7.55043	-4.34706
1417308_AT	pyruvate kinase, muscle	14.5938	14.8747	-1.21493
1424132_AT	harvey rat sarcoma virus oncogene 1	10.0517	9.80534	1.18617
1433504_AT, 1445397_AT	brain glycogen phosphorylase	4.7391	6.60962	-3.65665
1443436_AT	mitogen activated protein kinase kinase 2	8.17892	7.61227	1.48107
1444831_AT	thymoma viral proto-oncogene 3	7.79715	5.37593	5.35622
1433691_AT	protein phosphatase 1, regulatory (inhibitor) subunit 3c	15.0011	15.4731	-1.38706
1422414_A_AT	calmodulin 1	12.8256	12.4328	1.3129
1416069_AT	phosphofructokinase, platelet	8.78388	7.85036	1.90993
1429434_AT	phosphatidylinositol 3-kinase, catalytic, alpha polypeptide	8.38313	7.65034	1.66186

Table A3, continued.

Genes involved in Insulin signaling pathway (muscle)		Mean log base 2 value		Fold change (BR/NFDM)
		BR	NFDM	
NFDM vs BR				
1423828_AT	fatty acid synthase	10.7655	11.6807	-1.88581
1425604_AT	v-crk sarcoma virus ct10 oncogene homolog (avian)-like	3.64653	6.37529	-6.62884
1441476_AT	suppressor of cytokine signaling 2	5.91189	7.71666	-3.49372
1438164_X_AT	flotillin 2	9.94438	10.3156	-1.29343
1422315_X_AT	phosphorylase kinase gamma 1	12.1868	12.3306	-1.10479
1429463_AT	protein kinase, amp-activated, alpha 2 catalytic subunit	12.4988	12.7497	-1.18995
1457803_AT	protein kinase, amp-activated, gamma 1 non-catalytic subunit	4.46506	3.1242	2.53302
1439931_AT, 1454958_AT	glycogen synthase kinase 3 beta	5.43039	7.41032	-3.94473
1423104_AT	insulin receptor substrate 1	11.0837	10.4648	1.53565
1443186_AT	pyruvate kinase, muscle	4.6179	6.86393	-4.74378
1444480_AT	protein kinase, amp-activated, gamma 3 non-catatlytic subunit	13.0262	13.3445	-1.2468
1421324_A_AT, 1424480_S_AT	thymoma viral proto-oncogene 2	10.2967	10.7973	-1.41483
1450718_AT	adaptor protein with pleckstrin homology and src	4.16515	7.2041	-8.21891
1415974_AT	mitogen activated protein kinase kinase 2	10.7307	11.2635	-1.44672
1422407_S_AT	harvey rat sarcoma virus oncogene 1	11.166	11.5517	-1.3065
1433504_AT	brain glycogen phosphorylase	8.12467	8.91569	-1.7303
1425711_A_AT, 1440950_AT	thymoma viral proto-oncogene 1	8.0511	5.99692	4.1531
1434518_AT	riken cdna 6330505c01 gene	8.35457	8.87864	-1.43801
1416195_AT	putative phosphatase	9.95815	10.3197	-1.28478
1454060_A_AT	v-ki-ras2 kirsten rat sarcoma viral oncogene homolog	9.09361	9.82235	-1.65718
1417365_A_AT, 1438825_AT, 1455571_X_AT	calmodulin 1	4.07028	6.04404	-3.9279

Table A4: List of genes in VEGF signaling pathway altered in all three diet groups compared to low calcium basal restricted diet group (BR) in muscle (step-up FDR corrected $p \leq 0.05$).

Genes involved in VEGF signaling pathway (muscle)		Mean log base 2 value		Fold change (BR/lowCa/ BCAA/ACEi)
		BR	LowCa/ BCAA/ACEi	
LowCa/BCAA/ACEi vs. BR				
1453069_AT	phosphatidylinositol 3-kinase, catalytic, beta polypeptide	5.1568	7.60749	-5.46678
1426401_AT	protein phosphatase 3, catalytic subunit, alpha isoform	11.9474	12.2035	-1.19423
1452026_A_AT	phospholipase a2, group xiia	9.52965	8.52051	2.01272
1440208_AT	ras-related c3 botulinum substrate 2	7.60771	6.65931	1.92974
1451596_A_AT	sphingosine kinase 1	3.97605	6.87431	-7.45528
1453281_AT	phosphatidylinositol 3-kinase catalytic delta polypeptide	8.76758	8.13474	1.55061
1417814_AT	phospholipase a2, group v	5.38723	3.78561	3.03485
1419568_AT	mitogen activated protein kinase 1	9.26914	8.58738	1.6041
1440426_AT	nuclear factor of activated t-cells, cytoplasmic, calcineurin-dependent 2	7.88457	5.63267	4.7631
1443436_AT	mitogen activated protein kinase kinase 2	8.17892	6.25341	3.79869
1444831_AT	thymoma viral proto-oncogene 3	7.79715	5.0313	6.80146
1425514_AT	phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	7.83016	8.52621	-1.62007
1431278_S_AT	phospholipase a2, group vi	4.40704	7.42211	-8.08403
1426104_AT	mitogen activated protein kinase 14	6.80826	3.16278	12.5141
1423380_S_AT	nuclear factor of activated t-cells, cytoplasmic, calcineurin-dependent 4	6.74705	8.45292	-3.26224

Table A4, continued.

Genes involved in VEGF signaling pathway (muscle)		Mean log base 2 value		Fold change (BR/Ca-depl.)
		BR	Ca-depl.	
Ca-depleted vs. BR				
1423478_AT, 1460419_A_AT	protein kinase c, beta 1	4.34773	6.29499	-3.85641
1440208_AT	ras-related c3 botulinum substrate 2	7.60771	5.$10076	5.68418
1451596_A_AT	sphingosine kinase 1	3.97605	6.35566	-5.20395
1453281_AT	phosphatidylinositol 3-kinase catalytic delta polypeptide	8.76758	8.02663	1.67127
1417814_AT	phospholipase a2, group v	5.38723	4.0432	2.53859
1453914_AT	heat shock protein 1	3.49707	6.45746	-7.78334
1418987_AT	phospholipase a2, group iid	8.08689	7.09444	1.98955
1443436_AT	mitogen activated protein kinase kinase 2	8.17892	7.61227	1.48107
1424132_AT	harvey rat sarcoma virus oncogene 1	10.0517	9.80534	1.18617
1444831_AT	thymoma viral proto-oncogene 3	7.79715	5.37593	5.35622
1416703_AT	mitogen activated protein kinase 14	11.4772	11.7312	-1.19245
1429434_AT	phosphatidylinositol 3-kinase, catalytic, alpha polypeptide	8.38313	7.65034	1.66186
1438999_A_AT	nuclear factor of activated t-cells 5	8.75395	7.71459	2.05531

Table A4, continued.

Genes involved in VEGF signaling pathway (muscle)		Mean log base 2 value		Fold change (BR/NFDM)
		BR	NFDM	
NFDM vs. BR				
1448558_A_AT	phospholipase a2, group iva (cytosolic, calcium-dependent)	8.24018	7.28951	1.93277
1440208_AT	ras-related c3 botulinum substrate 2	7.60771	5.70503	3.73907
1447084_AT	nuclear factor of activated t-cells, cytoplasmic, calcineurin-dependent 1	7.30594	6.09695	2.31175
1453914_AT	heat shock protein 1	3.49707	6.92506	-10.7629
1425990_A_AT	nuclear factor of activated t-cells, cytoplasmic, calcineurin-dependent 2	6.7824	7.4935	-1.63705
1421324_A_AT, 1424480_S_AT	thymoma viral proto-oncogene 2	10.2967	10.7973	-1.41483
1426085_A_AT	paxillin	6.01678	8.32559	-4.95475
1425711_A_AT, 1440950_AT	thymoma viral proto-oncogene 1	8.0511	5.99692	4.1531
1415974_AT	mitogen activated protein kinase kinase 2	10.7307	11.2635	-1.44672
1422407_S_AT	harvey rat sarcoma virus oncogene 1	11.166	11.5517	-1.3065
1431278_S_AT	phospholipase a2, group vi	4.40704	6.91574	-5.69107
1454060_A_AT	v-ki-ras2 kirsten rat sarcoma viral oncogene homolog	9.09361	9.82235	-1.65718
1420743_A_AT	protein phosphatase 3, catalytic subunit, gamma isoform	9.42015	10.0058	-1.50076
1416703_AT	mitogen activated protein kinase 14	11.4772	11.7035	-1.1698
1432821_AT	nuclear factor of activated t-cells, cytoplasmic, calcineurin-dependent 4	3.61167	5.19948	-3.00593

Table A5: List of genes in B-cell receptor signaling pathway altered in all three diet groups compared to low calcium basal restricted diet group (BR) in muscle (step-up FDR corrected $p \leq 0.05$).

Genes involved in B cell receptor signaling pathway (muscle)		Mean log base 2 value		Fold change (BR/LowCa/ BCAA/ACEi)
		BR	LowCa/ BCAA/ACEi	
LowCa/BCAA/ACEi vs. BR				
1456694_X_AT	protein tyrosine phosphatase, non-receptor type 6	9.7332	8.98414	1.6807
1453069_AT	phosphatidylinositol 3-kinase, catalytic, beta polypeptide	5.1568	7.60749	-5.46678
1426401_AT	protein phosphatase 3, catalytic subunit, alpha isoform	11.9474	12.2035	-1.19423
1440208_AT	ras-related c3 botulinum substrate 2	7.60771	6.65931	1.92974
1425902_A_AT	nuclear factor of kappa light polypeptide gene enhancer in b-cells 2, p49/p100	5.56488	7.14272	-2.98522
1444831_AT	thymoma viral proto-oncogene 3	7.79715	5.0313	6.80146
1425514_AT	phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	7.83016	8.52621	-1.62007
1453281_AT	phosphatidylinositol 3-kinase catalytic delta polypeptide	8.76758	8.13474	1.55061
1425797_A_AT	spleen tyrosine kinase	7.5153	4.06872	10.9025
1439931_AT	glycogen synthase kinase 3 beta	5.43039	7.50061	-4.19949
1423380_S_AT	nuclear factor of activated t-cells, cytoplasmic, calcineurin-dependent 4	6.74705	8.45292	-3.26224
1440426_AT	nuclear factor of activated t-cells, cytoplasmic, calcineurin-dependent 2	7.88457	5.63267	4.7631

Table A5, continued.

Genes involved in B cell receptor signaling pathway (muscle)		Mean log base 2 value		Fold Change (BR/Ca-depl.)
		BR	Ca-depl.	
Ca-depleted vs. BR				
1423478_AT, 1460419_A_AT	protein kinase c, beta 1	7.12077	7.63148	-1.42475
1440208_AT	ras-related c3 botulinum substrate 2	7.60771	5.10076	5.68418
1453281_AT	phosphatidylinositol 3-kinase catalytic delta polypeptide	8.76758	8.02663	1.67127
1439931_AT	glycogen synthase kinase 3 beta	5.43039	7.55043	-4.34706
1456694_X_AT	protein tyrosine phosphatase, non-receptor type 6	9.7332	8.79087	1.92163
1435244_AT	vav2 oncogene	5.04971	3.08612	3.90031
1435476_A_AT, 1455332_X_AT	fc receptor, igg, low affinity iib	7.81359	8.69101	-1.83709
1424132_AT	harvey rat sarcoma virus oncogene 1	10.0517	9.80534	1.18617
1444831_AT	thymoma viral proto-oncogene 3	7.79715	5.37593	5.35622
1418262_AT	spleen tyrosine kinase	5.03188	7.06925	-4.10498
1446718_AT	nuclear factor of kappa light chain gene enhancer in b-cells inhibitor, beta	5.6978	3.65303	4.12606
1429434_AT	phosphatidylinositol 3-kinase, catalytic, alpha polypeptide	8.38313	7.65034	1.66186
1438999_A_AT	nuclear factor of activated t-cells 5	8.75395	7.71459	2.05531

Table A5, continued.

Genes involved in B cell receptor signaling pathway (muscle)		Mean log base 2 value		Fold Change (BR/NFDM)
		BR	NFDM	
NFDM vs. BR				
1420088_AT	nuclear factor of kappa light chain gene enhancer in b-cells inhibitor, alpha	12.3429	12.0147	1.25539
1421324_A_AT, 1424480_S_AT	thymoma viral proto-oncogene 2	10.2967	10.7973	-1.41483
1435477_S_AT	fc receptor, igg, low affinity iib	9.34774	8.49187	1.80986
1422407_S_AT	harvey rat sarcoma virus oncogene 1	11.166	11.5517	-1.3065
1425711_A_AT, 1440950_AT	thymoma viral proto-oncogene 1	8.0511	5.99692	4.1531
1440208_AT	ras-related c3 botulinum substrate 2	7.60771	5.70503	3.73907
1447084_AT	nuclear factor of activated t-cells, cytoplasmic, calcineurin-dependent 1	7.30594	6.09695	2.31175
1454060_A_AT	v-ki-ras2 kirsten rat sarcoma viral oncogene homolog	9.09361	9.82235	-1.65718
1420743_A_AT	protein phosphatase 3, catalytic subunit, gamma isoform	9.42015	10.0058	-1.50076
1439931_AT, 1454958_AT	glycogen synthase kinase 3 beta	10.1668	9.67241	1.4087
1432821_AT	nuclear factor of activated t-cells, cytoplasmic, calcineurin-dependent 4	3.61167	5.19948	-3.00593
1425990_A_AT	nuclear factor of activated t-cells, cytoplasmic, calcineurin-dependent 2	6.7824	7.4935	-1.63705

Table A6: List of genes in GnRH signaling pathway altered in all three diet groups compared to low calcium basal restricted diet group (BR) in muscle (step-up FDR corrected p≤ 0.05).

Genes involved in GnRH signaling pathway (muscle)		Mean log base 2 value		Fold change (BR/LowCa/ BCAA/ACEi)
		BR	LowCa/ BCAA/ACEi	
Low Ca/BCAA/ACEi vs. BR				
1443540_AT	mitogen activated protein kinase kinase kinase 1	6.3317	5.06658	2.40348
1452026_A_AT	phospholipase a2, group xiia	9.52965	8.52051	2.01272
1452453_A_AT	calcium/calmodulin-dependent protein kinase ii alpha	7.54691	5.2291	4.98573
1424932_AT	epidermal growth factor receptor	8.33145	9.02714	-1.61966
1417814_AT	phospholipase a2, group v	5.38723	3.78561	3.03485
1434653_AT	ptk2 protein tyrosine kinase 2 beta	7.56944	5.29283	4.84538
1442679_AT	mitogen activated protein kinase kinase 4	7.01674	8.27152	-2.3863
1416572_AT, 1440920_AT	matrix metallopeptidase 14 (membrane-inserted)	8.5956	7.73907	1.81068
1419568_AT	mitogen activated protein kinase 1	9.26914	8.58738	1.6041
1425739_AT	phospholipase d1	7.28787	4.07639	9.26297
1443436_AT	mitogen activated protein kinase kinase 2	8.17892	6.25341	3.79869
1455729_AT	guanine nucleotide binding protein, alpha q polypeptide	9.01062	9.98417	-1.96366
1431278_S_AT	phospholipase a2, group vi	4.40704	7.42211	-8.08403
1423941_AT	calcium/calmodulin-dependent protein kinase ii gamma	11.4742	11.9042	-1.34725
1426710_AT	calmodulin 1	10.8448	10.3555	1.40374
1426104_AT	mitogen activated protein kinase 14	6.80826	3.16278	12.5141
1421416_AT	mitogen activated protein kinase kinase 7	8.19301	7.31861	1.83324
1449955_AT	calcium channel, voltage-dependent, alpha 1f subunit	5.50168	7.93108	-5.38671

Table A6, continued.

Genes involved in GnRH signaling pathway (muscle)		Mean log base 2 value		Fold change (BR/Ca-depl.)
		BR	Ca-depl.	
Ca-depleted vs. BR				
1417279_AT	inositol 1,4,5-triphosphate receptor 1	10.6323	10.1688	1.37882
1421297_A_AT	calcium channel, voltage-dependent, l type, alpha 1c subunit	6.72737	4.49144	4.71068
1440856_AT	mitogen activated protein kinase 8	9.71473	9.39732	1.24609
1423478_AT, 1460419_A_AT	protein kinase c, beta 1	7.12077	7.63148	-1.42475
1421897_AT	elk1, member of ets oncogene family	6.99614	4.30068	6.47763
1417814_AT	phospholipase a2, group v	5.38723	4.0432	2.53859
1418987_AT	phospholipase a2, group iid	8.08689	7.09444	1.98955
1416572_AT, 1440920_AT	matrix metallopeptidase 14 (membrane-inserted)	8.5956	7.87546	1.64733
1437113_S_AT	phospholipase d1	8.27096	9.02738	-1.68929
1424132_AT	harvey rat sarcoma virus oncogene 1	10.0517	9.80534	1.18617
1443436_AT	mitogen activated protein kinase kinase 2	8.17892	7.61227	1.48107
1445112_AT	adenylate cyclase 1	8.08973	6.95162	2.20093
1422414_A_AT	calmodulin 1	12.8256	12.4328	1.3129
1416703_AT	mitogen activated protein kinase 14	11.4772	11.7312	-1.19245
1451714_A_AT	mitogen activated protein kinase kinase 3	12.3122	12.7175	-1.32437
1439364_A_AT	matrix metallopeptidase 2	10.6965	10.1032	1.50874

Table A6, continued.

Genes involved in GnRH signaling pathway (muscle)		Mean log base 2 value		Fold change (BR/NFDM)
		BR	NFDM	
NFDM vs. BR				
1448558_A_AT	phospholipase a2, group iva (cytosolic, calcium-dependent)	8.24018	7.28951	1.93277
1442707_AT	calcium/calmodulin-dependent protein kinase ii alpha	8.98407	9.9863	-2.0031
1434653_AT, 1442437_AT	ptk2 protein tyrosine kinase 2 beta	4.79118	2.87368	3.77769
1422847_A_AT	protein kinase c, delta	9.025	9.44725	-1.34002
1437112_AT	phospholipase d1	4.48281	6.93238	-5.46254
1415974_AT	mitogen activated protein kinase kinase 2	10.7307	11.2635	-1.44672
1422407_S_AT	harvey rat sarcoma virus oncogene 1	11.166	11.5517	-1.3065
1431278_S_AT	phospholipase a2, group vi	4.40704	6.91574	-5.69107
1423941_AT	calcium/calmodulin-dependent protein kinase ii gamma	11.4742	12.1699	-1.6197
1454060_A_AT	v-ki-ras2 kirsten rat sarcoma viral oncogene homolog	9.09361	9.82235	-1.65718
1441531_AT	phospholipase c, beta 4	5.98812	7.60448	-3.066
1417365_A_AT, 1438825_AT, 1455571_X_AT	calmodulin 1	4.07028	6.04404	-3.9279
1450186_S_AT	gnas (guanine nucleotide binding protein, alpha stimulating) complex locus	14.7867	15.2217	-1.35187
1416703_AT	mitogen activated protein kinase 14	11.4772	11.7035	-1.1698
1451714_A_AT	mitogen activated protein kinase kinase 3	12.3122	12.5296	-1.16263

Vita

Antje Bruckbauer, maiden name Eerenstein, was born on October 22, 1965 in Essen, Germany. After graduating from high school, she attended the Medical School at the Heinrich-Heine Universität of Düsseldorf, Germany, from 1986 to 1992. She worked as a resident doctor in radiation oncology at St. Josef-Hospital Bochum, Germany, from 1992 to 1994, and received her doctoral degree for medicine from the Ruhr- Universität in Bochum, Germany, in 1996. After moving to the United States, she decided to pursue a complimentary degree in Nutrition Science. She started her doctoral work in the spring of 2005 at the University of Tennessee, Knoxville and defended her dissertation in June 2009.

She was honored as America's Outstanding Graduate Students in the 'Chancellor's List 2005/2006'.