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Calorie and Protein Restriction in Mammals, a Review and Comparison on Respect to Aging

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I am grateful that I was given the chance to choose calorie restriction (CR) and protein restriction (PR) on respect to aging as the topic for this thesis. The precise mechanisms behind the human aging process and how it can be manipulated, remains one of the biggest mysteries of human biology, and I have been following this complex field of research with high curiosity for a long time. Along with my interest for nutrition, health and molecular biology, this topic provided a very interesting challenge.

I would like to express my biggest gratitude to both my supervisors Harald Carlsen and Anders Kielland, for great help and vital support during this work. Their guidance have repeatedly helped me to take the right decisions in times of hesitancy.

I would also like to thank my family and friends for their encouragements, and especially my fiancée Ingvild, whose support has been crucial.

"Do not grow old, no matter how long you live. Never cease to stand like curious children before the Great Mystery into which we were born"

– Albert Einstein

Ås, August, 2016

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Abstract

Calorie restriction (CR), a 10-50% (typically 30%) reduction in calorie intake with adequate nutrients, is the most widely accepted intervention to delay aging and increase lifespan in many model organisms. Recently, there has been an emerging interest for the possibility that protein restriction (PR) can provide the same effects on aging and lifespan. This literature thesis aims to explore if the positive effects and knowledge of CR from model organisms are relevant for mammals and especially humans, and if the same benefits could be achieved with PR. A non-systematic search was done to explore the effects and relevancy of CR to mammals including humans, while in evaluating the effects of PR, a systematic search was performed.

The evaluation of studies uncovered in this thesis, support that much of the knowledge of CR from model organisms seem to be relevant also for mammals and humans. In addition, there is strong evidence that CR in rodents can lead to many of the same effects as seen in other model organisms, including increased lifespan. For CR in non-human primates, high quality prospective studies have uncovered a strong trend of increased lifespan and health, although results are not consistent. Several randomized controlled trials up to two years of length in humans have also uncovered multiple positive effects of CR. It is therefore possible that CR could lead to many of these same effects in both non-human primates and humans.

Findings from the systematic search on effects of PR show a strong trend for increased median lifespan in rodents, and this is also the case for selective amino acid restriction (AA-R) of methionine or tryptophan. Collectively, PR, including AA-R, seem to result in increased lifespan and improved health in rodents. For humans, findings indicate that PR may lead to many of the same positive effects on lifespan and health at old age, but the PR regimen may have a negative contribution after a certain age, and should perhaps not be lifelong.

Findings indicate that PR seems to provide many of the same effects as seen with CR, but these effects appear to have a lower impact. This might occur because both PR and CR can lead to downregulated GH/IGF-1 and mTOR signaling, while only CR appears to result in an activation of sirtuin and AMPK signaling. In this context, PR can possibly be the cause, or contributor to some, but not all the effects seen with CR. More research is needed before any of these two dietary interventions can be recommended for healthy individuals.

Sammendrag

Energi- og kalorirestriksjon (CR), en 10-50% (normalt 30%) reduksjon i energiinntak uten underernæring, er den mest aksepterte intervensjonen for å utsette aldring og øke levealder i en rekke modellorganismer. Nylig har det også blitt en økende interesse for om proteinrestriksjon (PR) kan gi de samme positive effektene på aldring og levealder. Denne litteraturoppgaven tar sikte på å undersøke om de positive effektene og kunnskapen om CR fra modellorganismer også er relevante for pattedyr og spesielt mennesker, og om de samme effektene kan oppnås ved PR. Det ble valgt en ikke-systematisk metode for litteratursøk for å vurdere effekten av CR i pattedyr inkludert mennesker, mens det ble satt opp et systematisk litteratursøk for å undersøke effekten av PR.

Funn fra studier som er gjennomgått i denne oppgaven, underbygger at mye av kunnskapen om CR fra modellorganismer også er relevant for pattedyr og mennesker. I tillegg er det sterke bevis for at CR kan lede til mange av de samme effektene som observeres i andre modellorganismer, inkludert økt levealder. For ikke humane primater har prospektive studier av høy kvalitet avdekket en positiv trend for CR i form av økt levealder og bedre helse, men resultatene er ikke entydige. Flere kontrollerte randomiserte studier med opptil to års varighet på mennesker har også avdekket flere positive helseeffekter av CR. Det er derfor mulig at CR kan gi mange av disse positive effektene både i primater og i mennesker.

Funn fra det systematiske litteratursøket på PR avdekket en sterk tendens til økt median levealder hos gnagere, og dette er også tilfellet for selektiv aminosyre restriksjon (AA-R) av metionin eller tryptofan. PR, inkludert AA-R kan derfor trolig lede til økt levealder og bedre helse ved økt alder i gnagere. Funn indikerer at PR kan lede til mange av disse positive effektene også for mennesker, men det er mulig at PR bidrar negativt etter en viss alder, og derfor ikke bør opprettholdes livet ut.

Funn diskutert i denne masteroppgaven indikerer at PR kan gi mange av de samme effektene som observeres ved CR, men med en svakere effekt. Dette kan muligens forklares ved at både PR og CR kan gi nedregulert aktivitet i GH/IGF-1 og mTOR signalveiene, mens kun CR ser ut til å gi en aktivering av sirtuin og AMPK signalering. Det er derfor mulig at PR kan være årsak, eller bidrag til noen, men ikke alle effektene som observeres ved CR. Mer forskning er nødvendig før noen av disse to kostholdsintervensjonene kan anbefales for friske individer.

Abbreviations

- 4E-BP - Eukaryotic translation initiation factor 4E-binding protein
- AC - Adenylate cyclase
- *Ad lib* – *Ad libitum* (Freely/"at one's pleasure")
- ADP - Adenosine diphosphate
- AGEs - Advanced glycation end-products
- AD - Alzheimer's disease
- AMP - Adenosine monophosphate
- AMPK - 5' adenosine monophosphate-activated protein kinase
- AA-R – (Selective) Amino acid restriction
- (AA)s – (Amino acid)s
- BCAA - Branched-chain AA
- BMI - Body mass index
- CALERIE research program - The Comprehensive Assessment of the Long-term Effects of Reducing Intake of Energy research program
- CON – Control group
- CI - Confidence interval
- CR – Calorie/caloric restriction
- CRP - C-reactive protein
- CVD - Cardiovascular disease
- DHA – Docosahexaenoic acid
- DR – Dietary restriction
- eIF4E - eukaryotic translation initiation factor 4E
- EPA – Eicosapentaenoic acid
- FOXO - Forkhead box O
- GH - Growth hormone
- GH/IGF-1 pathway - Growth hormone/Insulin-like growth factor-1 signaling pathway
- HDL – High-density lipoprotein
- HR - Hazard ratio
- IGF-1 - Insulin-like growth factor-1
- IGFBP – Insulin like growth factor binding protein
- IL-6 - Interleukin-6
- Kcal - kilocalories
- LDL – Low-density lipoprotein
- Max lifespan/survival – Maximal lifespan/survival
- Meth-R – Methionine restriction
- mTOR - mammalian target of rapamycin
- NAD^+ - Nicotinamide adenine dinucleotide
- NIA - National Institute on Aging
- P-C ratio - Protein to carbohydrate ratio
- PGC-1α - Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
- PI3K - Phosphoinositide 3-kinase
- PKA - Protein kinase A
- PKB/Akt - Protein kinase B/Akt
- PR – Protein restriction
- RDA – Recommended daily allowance
- ROS – Reactive oxygen species
- S6K - Ribosomal protein S6 kinase
- SASP - Senescence associated secretory phenotype
- SNPRC - Southwest National Primate Research Center
- T3 – Triiodothyronine
- TNF-α - Tumor necrosis factor- α
- Trypt-R – Tryptophan restriction
- WNPRC - Wisconsin National Primate Research Center

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1. Introduction

A highly appreciated feature of a good life is a long healthy lifespan. However, with increased age follows an increased susceptibility of morbidities and death. More focus is given to the idea that there could be fundamental mechanisms of aging contributing to many of the health problems associated with increased age (Kaeberlein et al. 2015; Longo et al. 2015). Today's health system often aims to treat diseases individually (Kaeberlein et al. 2015). An enormous potential lies in the added approach to also prevent or delay aging related disease pathologies by interventions that could act on fundamental mechanisms of aging.

Calorie restriction[1] (CR), also known as energy restriction or dietary restriction[2], is in many model organisms the most widely accepted intervention to both improve health at old age and increase lifespan (Armanios et al. 2015; de Cabo & Le Couteur 2015; Masoro 2005). CR refers to a reduction in calorie intake of 10-50% (typically 30%) compared to normal calorie intake while maintaining an adequate intake of micronutrients. In this context, a normal calorie intake would either be the *ad libitum* (*ad lib*) intake for sedentary subjects or a fixed calorie intake, that for humans would be equal to approximately 2000 kilocalories (kcal) for females and 2500 kcal for males. CR has been reported to be able to substantially increase lifespan in model organism such as yeast, worms and flies, sometimes in the range of a 2- to 3- fold increase (de Cabo & Le Couteur 2015; Fontana et al. 2010). Whether these positive effects of CR also are obtainable in humans is still uncertain.

Over the recent years, there have been an increased interest for the possibility that restriction of dietary protein (PR) including restriction of specific amino acids (AA-R) also can provide the same effects as CR (Longo et al. 2015; Mirzaei et al. 2014). PR refers to a reduction of protein intake expressed either as percentage of energy contribution or diet

[1] A 2016 PubMed search revealed that in relation to aging, the term "calorie/caloric restriction" is used with a frequency of about 20 times more than the synonym term "energy restriction" (Appendix 6.1). Based upon this fact, CR seems to be a widely used expression, and will therefore be the chosen term in this thesis, despite the fact that "energy restriction" would be more formally correct according to the international system of units (SI) terminology.

[2] The term dietary restriction is also widely used, usually in reference to either CR, PR or AA-R. For clarity to the reader of this thesis, it was decided to mainly use the terms calorie/caloric restriction (CR), protein restriction (PR), and amino acid restriction (AA-R).

weight. In experimental settings, the protein intake are often reduced in the range of 30-85%. This reduction is in comparison with *ad lib* subjects, or a fixed protein and calorie intake with around 10-30% energy from protein. If not specifically corrected for, most CR protocols also implies a restriction of proteins, and it has been speculated that this could be the true cause of the positive effects seen with CR. If this is true, PR could provide a much more viable dietary intervention to improve health and increase lifespan.

1.1 Thesis aims

This thesis seeks to explore and provide preliminary answers to the following questions:

- Thesis aim 1: Are the positive effects and knowledge of CR on lifespan and health at old age in model organisms relevant for mammals and especially humans?
- Thesis aim 2: Can PR (including AA-R) provide increased lifespan or improved health at old age in mammals and especially humans?
- Thesis aim 3: Can PR provide the same effects as seen with CR, and if so can the concomitant PR that often follows with CR studies cause the effects reported by CR?

A literature review was performed to provide answers to these three aims. To retrieve relevant literature, two different approaches were used. In exploring the effects of CR in mammals and humans in chapter 3.1, a non-systematic search was done, while in evaluating the effects of PR (including AA-R) in mammals and humans in chapter 3.2, a systematic search was performed.

As described by Longo et al. (2015) a major challenge with respect to intervention studies aimed to delay aging is the lack of accurate biomarkers to evaluate early effects. Biomarkers that exist today are merely markers of overall health such as maximal oxygen uptake, insulin resistance, fasting blood glucose, lipid profiles, inflammatory markers and hormone levels to mention a few (Longo et al. 2015). Normally, all these biomarkers are strongly influenced by the aging process, but they do not precisely assess the biological age of an individual.

Due to this problem described above, this thesis will preferably scrutinize studies with data on lifespan as this may be the most accurate measure of the effects by CR or PR. Apart from lifespan, other outcomes expected to be relevant to the aging process are also evaluated.

1.2 Thesis structure

During the next two subchapters of this introduction a brief background to the field of aging research is given. Chapter 1.3 gives an introduction to the various hypothesis expected to be the fundamental mechanisms behind the aging process. Next, chapter 1.4 describes four nutrient sensing signaling pathways expected to regulate longevity and aging, most likely through interactions with several of the fundamental mechanisms of aging described in chapter 1.3. Thus, chapter 1.3 and 1.4 explains the theoretical background to how macronutrient intake could directly influence the aging process. Next, chapter 2 describes the two separate search strategies used to retrieve literature for the results in chapter 3.1 and 3.2 respectively.

In chapter 3 the results from the identified literature are presented and discussed. Chapter 3.1 seeks to explore and answer thesis aim number one by presenting and discussing findings on CR in rodents, non-human primates and humans respectively. Chapter 3.2 tries to explore and answer thesis aim number two by presenting the results from the systematic search on effects of PR in rodents and humans respectively. Next chapter 3.3 seeks to explore and answer thesis aim number three by comparing and discussing the previously presented results on effects of CR and PR. As part of thesis aim number three, chapter 3.3.1 tries to compare the signaling mechanisms through which CR and PR induces its effects. Finally, chapter 4 gives an overview of the conclusions that can be drawn from the findings in this thesis, followed by references and appendices in chapter 5 and 6 respectively.

1.3 Fundamental mechanisms of aging

From an evolutionary point of view, it has been suggested that mechanisms and pathways contributing to growth and reproductive success resulting in increased fitness would be strongly selected for, while those affecting health after the reproductive period of an organism is likely to only be minimally influenced through selective pressure (Goodell & Rando 2015). In this regard, the aging process might be explained by the lack of selective pressure on the ability to maintain the same cellular and systemic functions with increased age, which can result in a gradual loss of an individual's health at old age.

Knowledge about the fundamental mechanisms of aging is still at its youth. However, increasing efforts have been initiated to understand what precise mechanisms that are the

most dominant ones and how these mechanisms could contribute or interact with typical age related diseases including cancer, diabetes, neurodegenerative disease and cardiovascular disease (CVD). Increasing regard to this field of research and its applications for human disease pathologies was highlighted with the 2009 Nobel prize in medicine for discoveries in telomere biology, a molecular mechanism strongly expected to contribute to human aging (Blackburn 2010; Greider 2010; Szostak 2010).

An important question yet to be explored is exactly how the mechanisms of aging interact with pathologies at increased age. One possibility is that a few fundamental cellular or systemic mechanisms occurring with increased age, lead on to several pathologies or dysfunctions. Another possibility is that each of these pathologies develop independently or partially independently from each other through distinct mechanisms. In support of the former are observations that many of these seemingly unrelated diseases occur together in the same old individual with a higher frequency than what could be expected by chance alone (Blackburn et al. 2015).

Figure 1 briefly presents some of the theories on the fundamental mechanisms of aging. Some of these might be proven negligible, and additional mechanisms could be discovered in the future. However, it is likely that much of the human aging process could be explained by the complex interactions between many of these mechanisms. The nutrient sensing pathways described in chapter 1.4 are expected to influence these fundamental mechanisms of aging through complex two-way interactions.

Figure 1: Theories of the driving forces behind the human aging process (Blackburn et al. 2015; Childs et al. 2015; de Cabo & Le Couteur 2015; Goodell & Rando 2015; Kaushik & Cuervo 2015; Underwood 2015).

1.4 Nutrient sensing pathways

Some signaling pathways reported to be strongly implicated in the aging process will be briefly described here. These pathways function as cellular “switches” controlling a wide range of cellular functions. They are widely complex, interacting both with each other and with other pathways, and integrating inputs on nutrient and energy availability. In mammals, these pathways are:

- Mammalian/mechanistic target of rapamycin (mTOR) pathway.
- Growth hormone (GH)/insulin-like growth factor-1 (IGF-1) pathway (GH/IGF-1 pathway).
- 5’ adenosine monophosphate-activated protein kinase (AMPK) signaling.
- Sirtuin signaling.

Several additional nutrient sensing pathways have also been described (Efeyan et al. 2015). The four pathways described here are those expected to influence longevity (de Cabo & Le Couteur 2015; Longo et al. 2015; Masoro 2005). The strongest links between these pathways and the aging process are provided by genetic studies in model organisms.

The idea that these nutrient sensing pathways could have a direct influence on longevity might be somewhat surprising at first sight, but could be understood by the mechanisms of evolution. From an evolutionary point of view, the main goal of an organism would be to grow and successfully reproduce. This growth and reproduction requires a surplus of nutrients and energy. Through periods of lower food availability, the second best option is to induce cellular and systemic protection systems to increase the chance of surviving until periods of higher food availability and enough energy for growth and reproduction. These longevity regulating nutrient sensing pathways appear to be partially evolutionary conserved across most species (de Cabo & Le Couteur 2015; Fontana et al. 2010).

mTOR pathway

Both mTOR and the downstream ribosomal protein S6 kinase (S6K) are believed to be activated by cellular amino acid (AA) levels, especially leucine, and also by other inputs such as levels of glucose, insulin and growth factors (Efeyan et al. 2015; Fontana et al. 2010; Mirzaei et al. 2014). In other words, high nutrient availability is expected to be one of the major activator of this pathway that further stimulates anabolic processes. S6K is believed to regulate different transcription factors influencing gene expression of a huge number of genes. Active mTOR and S6K normally stimulate most steps of protein synthesis, whereas reduced activity in this pathway appears to induce a shift towards reduced protein synthesis and increased autophagy through the mechanism describes next, and by other mechanisms not yet understood. mTOR also regulates the activity of eukaryotic translation initiation factor 4E-binding protein (4E-BP). 4E-BP normally inhibits eukaryotic translation initiation

factor 4E (eIF4E). mTOR- dependent phosphorylation of 4E-BP ceases this inhibition and allows eIF4E to stimulate protein translation (Bjedov et al. 2010). As the name of the pathway implies mTOR is strongly inhibited by the pharmacological agent rapamycin, which also has been shown to extend lifespan in many animals, although often with severe side effects (Longo et al. 2015).

GH/IGF-1 pathway

The GH/IGF-1 pathway is the mammalian equivalent to the insulin/insulin growth factor (Ins/IGF) pathway in lower eukaryotes. In mammals, binding of both IGF-1 and GH to cell surface receptors such as IGF-1 receptor (IGF-1R) and GH receptor (GHR) can activate this pathway (Alberts et al. 2015; Fontana et al. 2010; Mirzaei et al. 2014). Growth hormone produced from the anterior pituitary gland induces the liver to produce and secrete IGF-1. Systemic energy and nutrient levels affect both steps. Energy and nutrient availability also influence the production and secretion of insulin-like growth factor binding proteins (IGFBP), which bind to GH and IGF-1 and hence prevent them from activating their receptors. Thus, the ratio of GH/IGF-1 to IGFBP is expected to be a good measure of biological activity of these hormones; increased ratio lead to higher activity and pathway activation. Activation of GH/IGF cell surface receptors activates phosphoinositide 3-kinase (PI3K) and protein kinase B also known as Akt (PKB/Akt). PKB/Akt is also believed to activate mTOR. The forkhead box O (FOXO) transcription factor appears to be one of the major downstream effectors of the GH/IGF-1 pathway identified so far, with PKB/Akt normally working to inhibits FOXO. Activation of Ras, adenylate cyclase (AC) and protein kinase A (PKA) by this pathway also appears to have longevity regulating effects.

AMPK

AMPK functions as a metabolic sensor responding to changes in cellular energy levels (de Cabo & Le Couteur 2015; Finkel 2015). In times of lower energy availability, the cellular levels of adenosine monophosphate (AMP) and adenosine diphosphate (ADP) increase while cellular levels of ATP decreases. Increased ratios of AMP and ADP to ATP activate the AMPK protein. AMPK activation induces a wide range of cellular responses with increased autophagy and mitophagy believed to be some of the many strong responses. In addition, activated AMPK is expected to inhibit the activity of the previously described mTOR pathway

(Alers et al. 2012). Metformin, a pharmaceutical agent widely used to treat diabetes type 2, activates AMPK. Metformin's effect on aging and age-related diseases are now being explored (Longo et al. 2015).

Sirtuins

In mammals there are seven members of this protein family where a number of them have been linked to longevity regulation (Finkel 2015). Sirtuins are a class of protein deacetylases with histone deacetylation and gene silencing as one of many downstream effects (de Cabo & Le Couteur 2015). Sirtuin activity seems to be regulated by nutrient availability, perhaps through cellular levels of the coenzyme nicotinamide adenine dinucleotide (NAD^+) (Verdin 2015). One of several important downstream targets is the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), a master regulator of mitochondrial biogenesis. PGC-1α is also thought to be activated by AMPK. Many different phytochemicals are also expected to activate different members of the sirtuin family. The phytochemical resveratrol, found in high concentrations in grape skin, has for a long time been studied for its proposed beneficial effects on lifespan and health, but its exact potential for this is still uncertain (Longo et al. 2015).

Overview

Reduced activity of the mTOR, and GH/IGF-1 pathways and increased activity of AMPK and sirtuin signaling are associated with longevity promoting effects in most animals studied (de Cabo & Le Couteur 2015; Fontana et al. 2010; Longo et al. 2015; Mirzaei et al. 2014). These nutrient sensing pathways provide a possible link to how CR or PR could possibly influence the fundamental mechanisms of aging described in the previous chapter. Figure 2 shows an overview of these nutrient sensing pathways and how they are expected to regulate longevity.

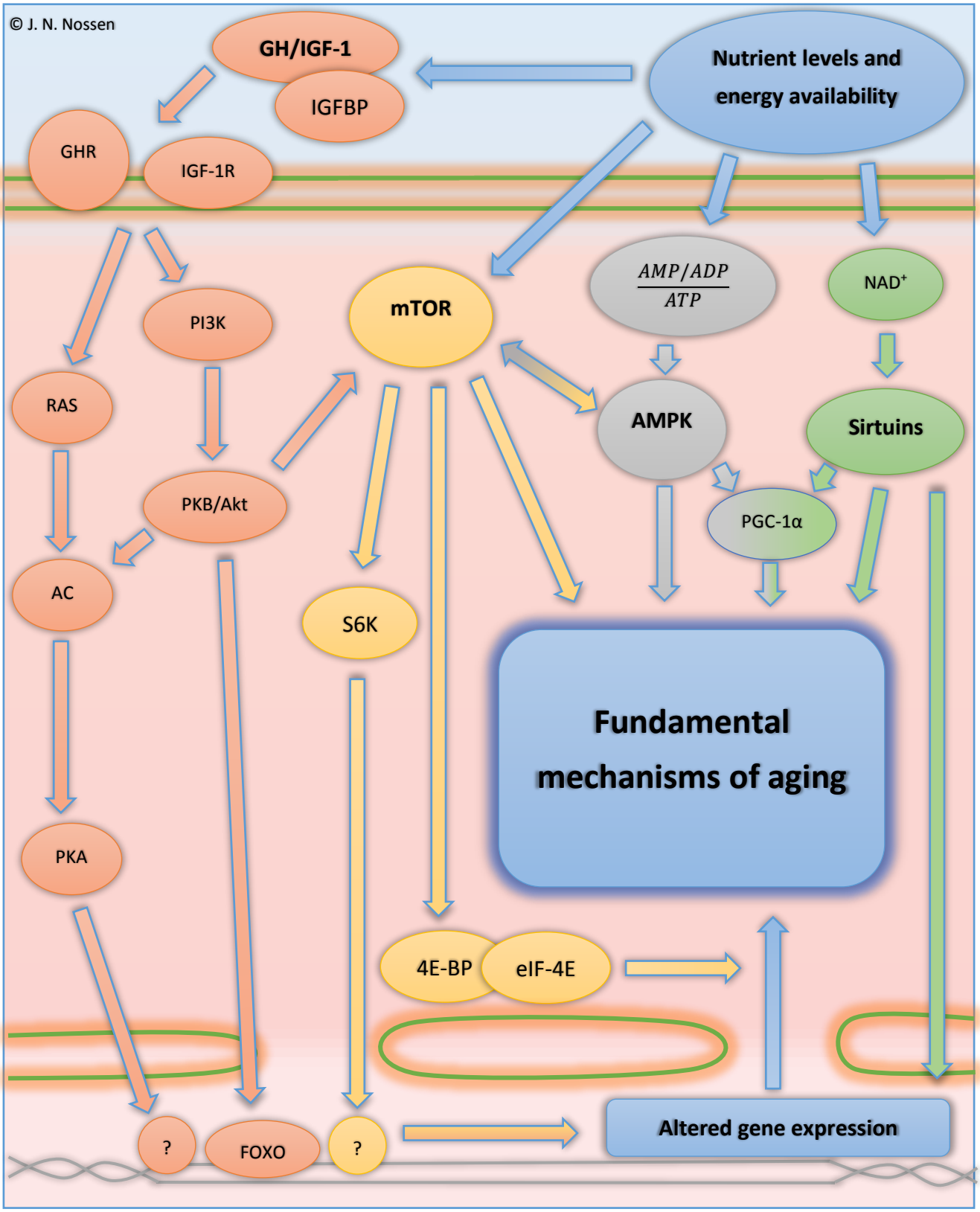


Figure 2: An overview of the nutrient sensing pathways expected to regulate longevity. Colors represents each pathway, GH/IGF-1 pathway in red, mTOR pathway in yellow, AMPK signaling in grey and sirtuin signaling in green. Blue colors represents common upstream activators or endpoint effects. Arrows represent regulatory functions on downstream mediators and crosstalk, independent of regulatory effect (activation/inhibition). This figure does not represent the full complexity and crosstalk between these pathways. Abbreviations: AC, adenylate cyclase; ADP, adenosine diphosphate; AMP, adenosine monophosphate; AMPK, 5' adenosine monophosphate-activated protein kinase; eIF4E, eukaryotic translation initiation factor 4E; FOXO, Forkhead box O; GH, growth hormone; GHR, growth hormone receptor; IGF-1, insulin-like growth factor-1; IGF-1R, insulin-like growth factor receptor; IGFBP, Insulin-like growth factor binding protein; mTOR, mammalian target of rapamycin; NAD+, nicotinamide adenine dinucleotide; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKB/Akt, Protein kinase B/Akt; S6K, ribosomal protein S6 kinase; 4E-BP, eukaryotic translation initiation factor 4E-binding protein.

2. Methods

Non-systematic search on CR

The non-systematic search was carried out from January to August 2016, mainly in PubMed, but also in Medline and Embase. In order to acquire relevant literature, various combinations of the following search words were used: “caloric restriction”, ”CR”, “low caloric diet”, “dietary restriction”, “DR”, “aging”, “longevity”, “lifespan”, “life span”, “life expectancy,” “mechanism”, “pathway”, “rodents”, “primates”, “mammals”, and “human”. Highly relevant papers were identified through subjective quality evaluation with high emphasize on relevancy and study design. Highly relevant literature also included reviews, and studies identified through the reference lists of other papers.

Systematic search on PR

The systematic search was performed in PubMed only. Search words were divided into two different groups. Group 1 was used in order to capture relevant studies exploring the effects of PR or AA-R. Group 1 was restricted to the following words: “ratio of macronutrients”, “protein restriction”, “low protein diet”, “LPHC diet”, “LPHC”, “CPC diet”, “low protein high carbohydrate”, “low protein high carbohydrate diet”, “macronutrient ratio”, “protein carbohydrate ratio”, “amino acid restriction”, “methionine restriction”, "tryptophan restriction", and "leucine restriction". Group 2 was used in order to limit the number of studies to those with an outcome relevant to aging and lifespan. Group 2 was restricted to the following: “aging”, “longevity”, “lifespan” “life span”, “life expectancy” and “survival”.

Search words within each group were combined with the Boolean operator “or” and search words from group 1 and group 2 were combined with each other using the Boolean operator “and”. All search words were restricted to title and abstract in order to limit the number of irrelevant literature. Full PubMed search query is shown in appendix 6.2. The search was performed on June 30th, 2016, resulting in a total of 377 initial articles. The results of the systematic search and the selection of included articles are shown in figure 3.

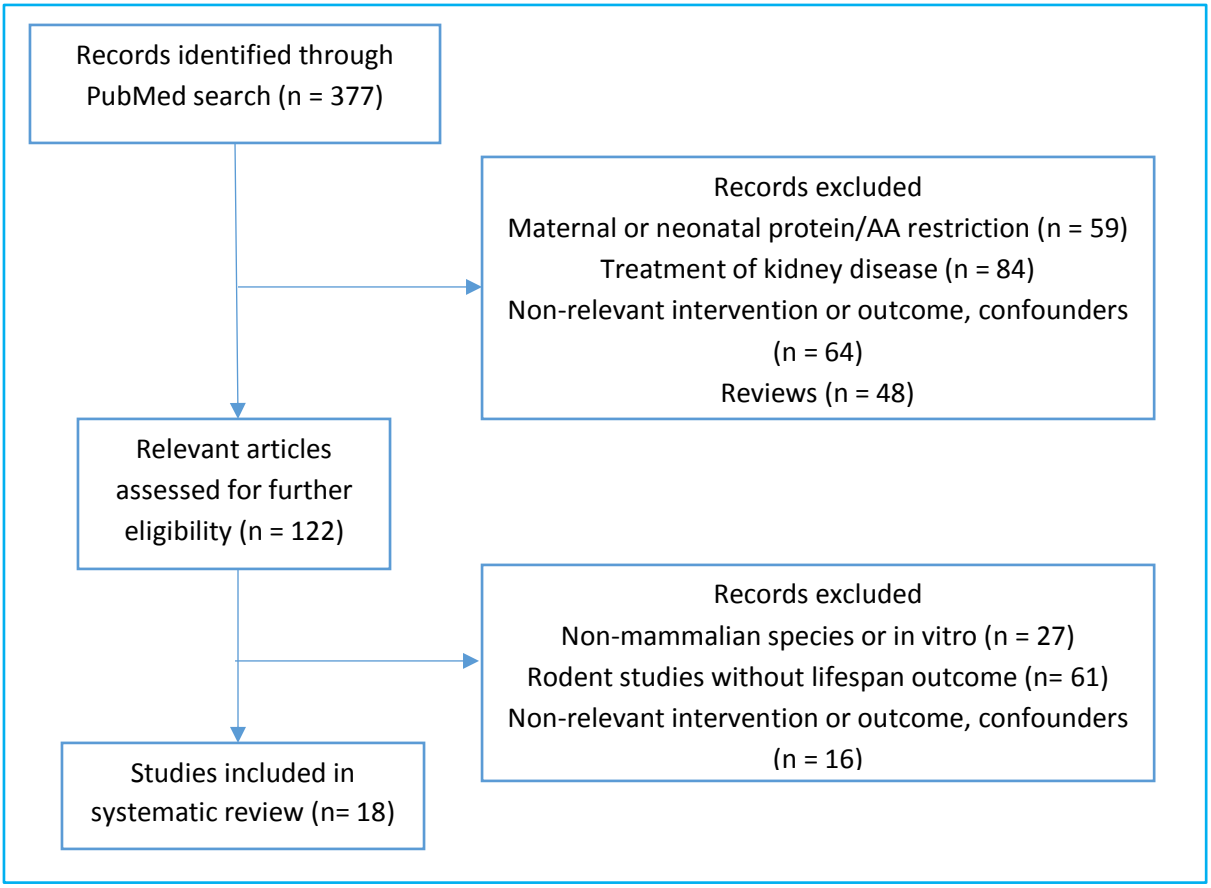


Figure 3: Results of the systematic search on PR and AA-R and selection of studies for inclusion.

Titles and abstracts of all 377 initial articles were read at least once. Based on this, 122 articles were identified to consist of interventional or observational studies with PR or AA-R that had an outcome relevant to aging or lifespan. Exclusion criteria were studies linked to maternal or neonatal restriction, treatment of kidney disease, review articles, interventions not relevant to PR or AA-R, outcomes not relevant to aging, or the presence of various confounders preventing PR or AA-R from being the single intervention examined.

Upon further evaluation of these 122 relevant studies, a number of 18 studies was finally included in accordance with the following inclusion criteria:

- Interventional or observational studies of PR or AA-R in vivo in mammals only, with previously healthy subjects and with a control group (CON) on normal diet, without any induced pathologies such as infections etc.
- For rodents, only articles with lifespan or survival as outcome were included (n = 15).
- For all other mammals, all studies with an outcome relevant to aging were included (n = 3).

3. Results and discussion

3.1 Effects of calorie restriction (CR) in mammals

This chapter seeks to explore if the seemingly consistent results from simple model organisms also could be relevant for mammals and especially humans. The choice of a non-systematic search for chapter 3.1 was based on two key factors:

- The amount of available literature on effects of CR in rodents was considered too comprehensive to be evaluated in detail within the time frame of this thesis.
- Data from high quality prospectively designed CR interventional studies on rhesus monkeys have recently been published (Colman et al. 2014; Mattison et al. 2012) and was expected to be of extraordinary relevance.

This chosen method allowed for an increased focus on specific studies with high relevancy although it should also be noted that this could potentially increase the possibility of a selection bias.

3.1.1 CR in rodents

The first indication of prolonged lifespan by CR was in fact observed in rats almost 100 years ago by Osborne et al. (1917). McCay later backed up this finding in 1935 in a study with stronger study design (McCay et al.). Since then, much data are now available on the effects of CR in rodents (Masoro 2005). Effects of CR in rodents is by far the best-studied dietary restriction method evaluated in any group of mammals with respect to aging. Due to the large amount of studies in rodents, this subchapter will highlight findings from selected studies regarded to be of high scientific value.

An apparently well conducted meta-analysis by Swindell (2012) provides a valuable overview of the effects on lifespan. This meta-analysis included studies with a CR group that also had an *ad lib* or fixed normal caloric control group (CON). A total of 53 studies on rats and 72 studies on mice were found that contained data of total lifespan. Most data on rats existed

from CR on inbred strains of males. An overview of the most important findings[3] from Swindell (2012) are shown in table 1 and both figure 4 and 5.

Table 1: The table shows the main findings from a meta-analysis on effects of CR in rodents by Swindell (2012).

Overview of results from a meta-analysis of CR in rodents (Swindell 2012)		
Species (number of studies)	*Rats (n = 53)*	*Mice (n = 72)*
Average % increased median lifespan from all studies	*30.4%* *Males: 31.3%* *Females: 25.8%*	*14.6%* *Males: 12.3%* *Females: 15.5%*
Average % increased maximal lifespan from all studies	*32.3%* *Males: 32.6%* *Females: 20.4%*	*17.8%* *Males: 14%* *Females: 20%*
Range of % increases in median lifespan of the lower quartile (25%) of sorted results from all studies	*<13.8%*	*<4.1%*
Range of % increases in median lifespan of the two middle quartiles (50%) of sorted results from all studies	*13.8% - 45.4%*	*4.1% - 27%*
Range of % increases in median lifespan of the upper quartile (25%) of sorted results from all studies	*>45.4%*	*>27%*

Figure 4: The figure shows the distribution and magnitude of results from studies examining the effects of median lifespan by CR in rats from the meta-analysis by Swindell (2012).

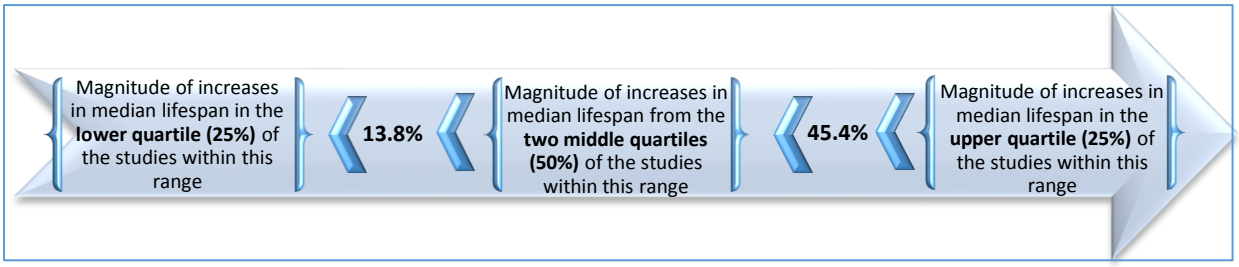
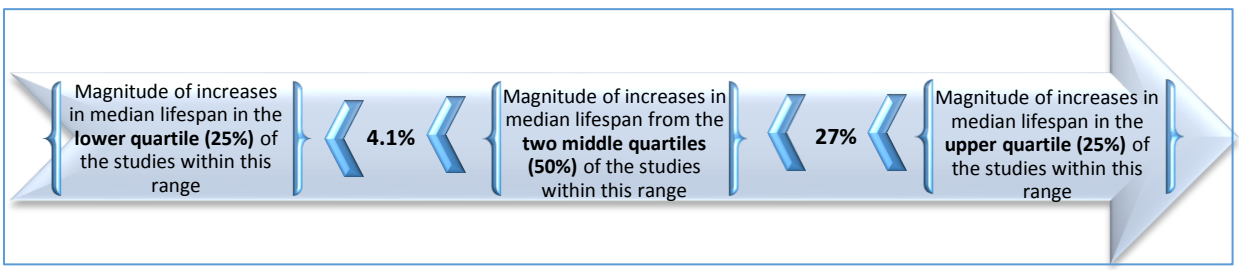


Figure 5: The figure shows the distribution and magnitude of results from studies examining the effects of median lifespan by CR in mice from the meta-analysis by Swindell (2012).



[3] In the findings by Swindell (2012) studies in the ILSXISS strains of mice have been excluded. For these specific strains, CR tends to result in small positive or in some cases negative effects on lifespan.

As seen in table 1 and figure 4 based on the meta-analysis by Swindell (2012), the range of % increases in median lifespan of the lower quartile (25%) of sorted results from all studies on rats was below a 13,8% increase. The two middle quartiles of the sorted results was within the range of 13.8% to 45.4% increases in lifespan, and the top quartile of the sorted results was all resulting in more than 45.4% increases in median lifespan. Average increase in median lifespan from all rat studies was 30.4% with some differences between the sexes, specifically 31.3% for males and 25.8% for females, although the data for females was less comprehensive. Average increase in maximal lifespan (max lifespan)[4] for all rat studies was 32.3%, showing a distinct difference between the sexes with 32.6% for males and 20.4% for females (Swindell 2012).

In this meta-analysis by Swindell (2012), weaker effects of CR on median lifespan were observed for mice as shown in table 1 and figure 5. For mice, the range of percentage increases in median lifespan of the lower quartile (25%) of sorted results from all mice studies was below a 4.1% increase. The two middle quartiles of the sorted results was within the range of 4.1% to 27% increases in lifespan, and the top quartile of the sorted results was all resulting in more than 27% increases in median lifespan. Median lifespan for all mice studies was increased by 14.6% on average, with 12.3% for males and 15.5% for females. Average increase of max lifespan was 17.8% for both sexes together, and 14% for males and 20% for females (Swindell 2012).

The main findings by Swindell (2012) clearly indicate consistent increases in both median and max lifespan with CR for these strains of both rats and mice in the most common laboratory settings, with the only exception being the ILSXISS strains of mice. The ability of an intervention to increase both median and max lifespan is regarded as a clear sign that fundamental mechanisms of aging are involved (Mattison et al. 2012).

Interestingly, the data from Swindell (2012) showed that the average increases in both median and max lifespan for both rats and mice seems to be clearly correlated with each other. In fact, the average increases for median and max lifespan for both sexes together

[4] Max lifespan represents the lifespan of the longest living animals of a specific cohort. The number of the longest living animals used to generate max lifespan varies between studies but usually this is generated from the longest living 1-3 subjects. In studies where max lifespan is generated from the single longest living animal, this is specified as absolute max lifespan further on in this thesis.

within each species only varies with a maximal 3.2%. This further supports the assumption that CR might increase lifespan by delaying some of the fundamental mechanisms of aging, and that some of these mechanisms have an important impact on both median and max life span. The effects of CR is on average consistently greater for males in rats, but contradictory consistently greater for females in mice. These differences between males and females do not seem to follow a clear pattern and are likely to be caused by chance due to the few number of studies on female rats.

Not surprisingly, the observed differences in increased lifespan between different studies vary greatly. This could to some extent be a result of differences in study design resulting in falsely stronger effects. However, failure to provide adequate intake of vitamins and minerals for CR groups, or increased exposure to pathogens are likely to result in falsely weaker effects of CR on lifespan. Swindell (2012) also notes the possibility that genetic factors varying among different strains could be responsible for this great variation. The fact that the average percentage increased lifespan between the closely related rats and mice species vary so greatly further supports this possibility. This raises the possibility that gene-diet interactions might be responsible for some strains or species being strong CR responder, only weak CR responder or total CR non-responders. If this proves to be true for rodents, this phenomenon would likely also be conserved among other mammals, including humans as well.

There is no reason to question the validity of the data by Swindell (2012), and the impression is on the contrary that these numbers give a fairly good overview of the effects of CR in rodents from the existing data at the time (2012). Although, these existing data might be affected by result of publication bias and also the low genetic diversity of inbred strains and the low number of strains as mentioned by the author himself. The existing data on the topic of CR in rodents might therefore provide results that are not representative for rodents as a whole group including wild-type animals.

Speakman et al. (2016) also support many of the findings by Swindell (2012) in a recent review on the effects of dietary restriction in rodents. They found that 40% CR generated an average of 30% increased median lifespan. The review by Speakman et al. (2016) also found that different levels of CR in the range of 20-60% resulted in increased median lifespans in the

range of 10-50%. The percentage increased median lifespan was associated with levels of CR but did not appear to show a consistent linear correlation.

Although CR has been shown to increase lifespan in rodents, negative outcomes also clearly exist. CR was found to impair healing of skin wounds and this side effect was reversed by a short period of *ad lib* feeding (Reed et al. 1996). Although CR has been reported to delay the normal age-associated decline in immune function it also increases the acute susceptibility to infections (Kristan 2008).

CR in rodents decrease the activity of the GH/IGF-1 pathway by reducing the serum levels of GH and IGF-1 (Breese et al. 1991; D'Costa et al. 1993). Further, CR reduce the activity of the downstream mediators PI3K, PKB/Akt, AC, PKA of the GH/IGF-1 signaling pathways including activation of the FOXO transcription factor (Cheng et al. 2014). In addition, mTOR and its downstream mediator S6K are downregulated, which appear to be one of the major mechanisms associated with CR in rodents (Cheng et al. 2014). Both increased AMPK and sirtuin signaling have been linked to the mechanisms of CR in some studies, but the influence of CR on AMPK and sirtuin signaling is not entirely clear (Boily et al. 2008; Canto & Auwerx 2011; Lee & Min 2013).

3.1.2 CR in non-human primates

Only two studies prospectively designed to examine the long-term effects of CR in monkeys have been published so far (Kemnitz 2011). This is the Wisconsin National Primate Research Center (WNPRC) CR study (Colman et al. 2014) and the National Institute on Aging (NIA) CR study (Mattison et al. 2012). Both studies are still ongoing and results published so far show some contradicting findings. The WNPRC CR study showed significant positive effects of CR on all-cause mortality and age-related mortality and positive effect of CR on reducing the incidence of many age-related diseases. The NIA study on the contrary did not shown any significant effects of CR on survival, although a trend of positive effect on delaying the onset of age-related diseases was noted. Both are using the rhesus monkey *Macaca mulatta*, which has an average lifespan of approximately 26-27 years in captivity and a max lifespan of around 40 years (Colman et al. 2014; Mattison et al. 2012).

Primal prospective studies of this type are costly and time consuming and these two studies might offer some of the best data existing today to understand the effects of CR and its

relevance to humans. Non-human primates are regarded the best model organism to give findings that are relevant to humans. In addition, the long lifespan compared to other model organisms makes it much more relevant especially when studying prospective interventional effects on aging related mortality or age-related diseases (E.g. in the instance of telomere biology that is less relevant in other model organisms). An extra strength with these two referred studies is the high degree of controlled environment and intervention, making them more similar to randomized controlled trials. This study design limits the possibility of confounders and biases. For these reasons, the WNPRC and NIA studies are here given a detailed evaluation.

WNPRC CR study

Design WNPRC

The WNPRC CR study (Colman et al. 2014) was started in 1989 and the number of animals included in the study was later increased in 1994. A total of 76 animals (61% males) were randomized to either CR or CON. All animals were adults of age 7-14 years old when included. The CR animals were fed a 30% CR diet matched to their individual baseline intake. CON monkeys were fed *ad lib*. Although, specifically 15 years into the study (monkeys age 22 years or more), an age-related decline in energy intake was observed and the difference in caloric intake between CR and CON was only 17% at this specific point in time (Rezzi et al. 2009). The diet used was semi-purified, nutritionally fortified, and composed by energy of 62% carbohydrate, 15% protein and 23% fat for each group (see appendix 6.3 for details). Protein source was animal-based lactalbumin, a constituent of mammalian milk (whey fraction) with a broad spectrum AA composition providing all essential AAs, specifically high in aspartic acid and tryptophan, and low in arginine, methionine and proline (Gordon & Ziegler 1955). Carbohydrate sources were cornstarch (30% of total weight), dextrin (5% of total weight) and a sucrose content of 25-29% of both total weight and total energy. Source of dietary fat was from corn oil and the diet included 5% of total weight from cellulose. Vitamin and mineral mixes were added to the diet in portions to allow recommended daily allowance (RDA). The main aspects of the WNPRC study design are shown in table 2.

Table 2: Study design from the Wisconsin National Primate Research Center (WNPRC) prospective primate CR study.

WNPRC primate CR study design	
Total number of monkeys	*N = 76 (CON; n = 38) (CR; n = 38)*
Males/females	*61% / 39%*
Age of animals included	*7-14 years*
CR intervention	*30% matched to individual baseline intake*
Diet	*Semi-purified, nutritionally fortified*
Diet composition in % of total weight	*61% carbohydrate, 15% protein and 10% fat*
Diet composition in % of total energy	*62% from carbohydrate, 15% from protein and 23% from fat*
Caloric intake CONs	*Ad libitum*
Protein source and AA composition	*Animal-based: Lactalbumin. AA composition: broad-spectrum with all essential AAs, high in aspartic acid and tryptophan, and low in arginine, methionine and proline (Gordon & Ziegler 1955)*
Carbohydrate sources and sucrose content	*Cornstarch: 30% of total diet weight. Dextrin: 5% of total diet weight. Sucrose: 25-29% of both total weight and total energy*
Fat source	*Corn oil: consisting of 13% saturated fatty acids, 59% polyunsaturated fatty acids, high in linoleic acid (ω-6) and small amounts of linolenic acid (ω -3) (Dupont et al. 1990)*
Cellulose	*5% of total diet weight*
Vitamins and minerals	*Both groups given around 100% of RDA*

Results WNPRC

Results published from the study so far show significant reduction in aging-related and all-cause mortality with CR (Colman et al. 2014). Hazard ratio (HR) for death from age related conditions for an animal in the CON group compared to the CR group was 2.89 with a 95% confidence interval (CI) of 1.34-6.25. The same HR for death from all-cause mortality was 1.78 (95% CI: 1.04-3.04). The pathologist categorizing the cause of death was reported to be blinded with respect to the monkey's group. 63% of the CON monkeys died of age-related diseases compared to only 26% in the CR group. The estimated median survival[5] of CON monkeys was 25-26 years for all cause survival and 26-27 years for age-related survival, which is close to the expected average lifespan in captivity. In CR monkeys, the estimated median survival[5] was 27.5-28.3 years for all cause survival, and 30.5-32.2 years for age-related survival. CR significantly increased lifespan both for age related mortality (p= 0.007) and all-cause mortality (p= 0.037).

[5] Survival estimates had some variation depending on statistical model used and inclusion or exclusion of non-age related deaths.

An earlier publication from the WNPRC study compared the incidence of specific age-related diseases between the CR monkeys and CON monkeys (Colman et al. 2009). CR was reported to reduce the incidence of CVD, neoplasia (growth dysfunction that might progress to cancer) and brain atrophy (associated with neurodegenerative diseases). Strikingly, no incidence of diabetes was found in the CR group. Collectively, CR was found to significantly delay the onset of these age-related diseases ($p = 0.008$). These main results are briefly included in table 3.

Table 3: An overview of the results from the Wisconsin National Primate Research Center (WNPRC) prospective primate calorie restriction (CR) study.

WNPRC primate CR study results (Colman et al. 2014)	
Median survival of CONs (expected 26-27 years)	*25-26 years (all-cause survival)* *26-27 years (age-related survival)*
Median survival of CR monkeys	*27.5-28.3 years (all-cause survival)* *30.5-32.2 years (age-related survival)*
% CR after age related decline in energy intake	*17% (monkey age 22 years+)*[(Specific time point only)]
HR for all-cause mortality, CON/CR	*1.78 (95% CI: 1.04-3.04)*
HR for age-related mortality, CON/CR	*2.89 (95% CI: 1.34-6.25)*
Group % died from age-related diseases	*CR: 26%* *CON: 63%*
Onset of age-related disease	*Delayed with CR ($p = 0.008$)*
Diabetes	*CR: zero incidence* *CON: 5 diabetic, 11 pre-diabetic*
Cancer/neoplasia	*Reduced 50% by CR*
CVD	*Reduced 50% by CR*
Brain atrophy	*Reduced by CR ($p < 0.05$)*

NIA CR Study

Design NIA:

The NIA CR study (Mattison et al. 2012) was started in 1987 with 42 males grouped as young (1-5 years), and 20 males grouped as old (16-23 years). In 1992 a cohort of 44 females grouped as young (1-14 years), and 15 females grouped as old (16-21 years) were added. Monkeys of the same sex were matched for age, body size and food intake and assigned to either CR or CON. CON monkeys were fed a diet with caloric content based on age and body weight. Although these CON monkeys was not fed truly *ad lib*, their food intake was considered to be approximately *ad lib* since it was reported that food often had been left uneaten after each meal. CR monkeys were fed 30% less calories than their individually matched CONs. All monkeys were fed a natural ingredient based diet consisting of 67% carbohydrate, 20% protein and 13% fat in energy content (see appendix 6.3 for details).

Protein sources were mainly plant-based with wheat, corn, soybean, alfalfa meal and fish. Carbohydrate sources were wheat and corn with a sucrose content of approximately 5% of total energy. Sources of fat were soy oil and fat from corn, wheat and fish. CR monkeys were given approximately 100% RDA of vitamins and minerals as opposed to CON monkeys that were super supplemented with around 140% of RDA. An age-related decline in food intake was seen for all groups, and by age 26 years CR males only ate 20% less calories than their matched CONs, and 12% less for females (Mattison et al. 2005). Most probable cause of death was classified by necropsy into either age-related deaths or not. An overview of the NIA study design is shown in table 4.

Table 4: Study design from the National Institute on Aging (NIA) prospective primate CR study.

NIA primate CR study design	
Total number of monkeys	*N = 121 (CON; n = 64) (CR; n = 57)*
Males/females	*51% / 49%*
Age and number of animals included	*Young: 1-14 years (n = 86) (CON; n = 46) (CR; n = 40)* *Old: 16-23 years (n = 35) (CON; n = 18) (CR; n = 17)*
CR intervention	*30% based on age and body weight*
Diet	*Natural ingredient based*
Diet composition in % of total weight	*56.9% carbohydrate, 17.3% protein and 5% fat*
Diet composition in % of total energy	*67% from carbohydrate, 20% from protein and 13% from fat*
Caloric intake CONs	*Approximate, but not truly ad libitum, based on age and body weight*
Protein sources and AA composition	*Mainly plant-based: Wheat, corn, soybean, alfalfa meal and fish (exact AA composition unknown, but expected to provide all essential AAs)*
Carbohydrate sources and sucrose content	*Wheat and corn.* *Sucrose: 3.9% of total diet weight, equal to 5% of total energy*
Fat sources	*Soy oil + corn, wheat and fish (likely to be a good source of the ω-3 fatty acids EPA and DHA)*
Crude fiber	*5% of total diet weight (Kemnitz 2011)*
Vitamins and minerals	*CR: 100% of RDA. CONs: super supplemented, 140% of RDA*

Young-onset NIA results

The young-onset CR monkeys from the NIA study are most comparable to the WNPRC study. Contrary to the WNPRC study, the NIA study essentially showed no positive effect of CR on survival (Mattison et al. 2012). Although just less than 50% of the monkeys from the young-onset group was still alive in 2012, estimates have indicated that significant effects by CR on lifespan are unlikely to occur. Immune function of the young-onset NIA monkeys has previously been reported to be improved with better maintenance of both naïve T-cells and T-cell receptor repertoire and reduced secretion of inflammatory cytokines (Messaoudi et al.

2006). No significant effects of CR on fasting glucose or triglycerides were found, although a trend was noted for males with respect to CR mediated reduction in triglycerides ($p = 0.051$). Incidence of diabetes appeared to be lower in the CR group (not reported to be significant), but no effect on CVD was found. With respect to cancer, the CR group had no incidence of neoplasia whereas six monkeys were identified with neoplasia in the CON group. Overall CR caused a slight, but non-significant delay in the onset of age-related diseases ($p=0.06$).

Old-onset NIA results

As for the young-onset CR group, the old-onset CR group from the NIA study did not have increased survival compared to CONs either from all-cause or age-related causes (Mattison et al. 2012). Although no differences were identified between CR and CONs, all males lived significantly longer than the expected 26-27 years average lifespan. Median survival for all CON females was 27.8 years and for CON males 35.4 years. Median survival for the CR monkeys are not highlighted in numbers, although from the attached survival curves this appears to be quite similar to the numbers for CONs. Neoplasia, CVD and traits associated with old age were equally represented in both CR and CON. The lack of CR effect on neoplasia is in contrast to the findings in the young-onset group, possibly indicating that early onset interventions can be important in preventing the early steps on development. However, old-onset CR gave significant positive effects on metabolic health and function. Triglycerides and cholesterol were significantly lowered ($p = 0.026$ and 0.02 respectively). Fasting glucose was lower in both sexes, but only significant in males ($p = 0.04$). The incidence of diabetes was not reported to be significantly lower in the CR group, although a trend was noted (zero incidence in CR compared to two monkeys in CONs). For males, a marker of oxidative stress (isoprostane) was found to be significantly higher in CON compared to CR ($p = 0.009$). A negative side effect of old-onset CR from the NIA study was previously reported to be reduced immune function through decreased T-cell proliferation (Messaoudi et al. 2008). This is contrary to the findings in young-onset monkeys, which could indicate that CR might acutely suppress immune function but might delay the normal age related decline in immune function. A previous report from the NIA study by Smith et al. (2011) reported no significant effects of CR on telomere shortening, despite telomere shortening is expected to play a vital role in human aging. The main results from both young-onset and old-onset groups are briefly included in table 5.

Table 5: Results from the National Institute on Aging (NIA) prospective primate calorie restriction (CR) study.

NIA primate CR study results (Mattison et al. 2012)		
% CR after age related decline in energy intake	*Males: 20% (from 26 years+)* *Females: 12% (from 26 years+)*	
Age of onset	*Young (1-14 years)*	*Old (16-23 years)*
Median survival of CONs (expected 26-27 years)	*Ongoing (results not ready)*	*Females: 27.8 years* *Males: 35.4 years*
Number of monkeys reaching 40 years of age	*Ongoing (results not ready)*	*CR: 4 monkeys* *CON: 1 monkey*
All-Cause mortality	*No effect*	*No effect*
Age related mortality	*No effect*	*No effect*
Group % died from age-related diseases	*CR: 20%* *CON: 24%*	*No effect*
Onset of age-related disease	*Delayed (trend only, p = 0.06)*	*No effect*
Diabetes	*CR: 2 monkeys* *CON: 5 monkeys*	*No effect*
Cancer/neoplasia	*CR: zero incidence* *CON: 6 monkeys*	*No effect*
CVD	*No effect*	*No effect*
Immune function	*Improved*	*Reduced* *(Messaoudi et al. 2008)*
Triglycerides	*Reduced (trend only)*	*Reduced (p = 0.026)*
Cholesterol	*No effect*	*Reduced (p = 0.02)*
Fasting glucose	*No effect*	*Reduced in males only (p = 0.04)*
Oxidative stress	*No effect*	*Reduced in males only (p = 0.009)*
Telomere shortening	*No effect (Smith et al. 2011)*	

Comparison of the WNPRC and NIA studies

The two independent yet quite similar prospective CR studies on primates conducted by WNPRC and NIA show quite conflicting results. The high quality and environmental control conducted in these studies reduce the number of factors that might have influenced the results, and it is likely that the conflicting outcomes might be a result of differences in the study designs, diets or possibly also influences from genetics.

Comparison of outcomes

The WNPRC study showed significant positive effects of 30% CR on all-cause mortality and age-related mortality and positive effects on reducing the incidence of many age-related diseases. The NIA study however had results showing no significant benefits on the same traits, only a positive trend on delaying the onset of age-related diseases (young-onset group only). An overview of comparable results from the two studies are highlighted in table 6. The WNPRC study showed a striking effect of CR on reducing diabetes with zero incidence in the CR monkeys. The young-onset NIA group corroborated to some extent these findings,

although not significantly. The old-onset NIA CR group showed no reduction in diabetes incidence.

Table 6: An overview of comparable results from the Wisconsin National Primate Research Center (WNPRC) and National Institute on Aging (NIA) prospective primate calorie restriction (CR) studies on primates.

	WNPRC primate CR study results	NIA primate CR study results	
% CR after age related decline in energy intake	*17% (From age 22 years+) (Rezzi et al. 2009)*	*Males: 20% (from age 26 years+) Females: 12% (from age 26 years+) (Mattison et al. 2005)*	
Age of onset	*Young (7-14 years)*	*Young (1-14 years)*	*Old (16-23 years)*
Median survival of CONs (expected 26-27 years)	*25-26 years (all-cause survival) 26-27 years (age-related survival)*	*Ongoing (results not ready)*	*Females: 27.8 years Males: 35.4 years*
Median survival of CR monkeys	*27.5-28.3 years (all-cause survival) 30.5-32.2 years (age-related survival)*	*Ongoing (results not ready)*	*Females: ~27.8 years Males: ~35.4 years*
HR for all-cause mortality, CON/CR	*1.78 (95% CI: 1.04-3.04)*	*No effect*	*No effect*
HR for age-related mortality, CON/CR	*2.89 (95% CI: 1.34-6.25)*	*No effect*	*No effect*
Group % died from age-related diseases	*CR: 26% CON: 63%*	*CR: 20% CON: 24%*	*No effect*
Onset of age-related disease	*Delayed (p = 0.008)*	*Delayed (trend only, p = 0.06)*	*No effect*
Diabetes	*CR: zero incidence. CON: 5 diabetic, 11 pre-diabetic.*	*CR: 2 monkeys CON: 5 monkeys*	*No effect*
Cancer/neoplasia	*Reduced 50% by CR*	*CR: zero incidence CON: 6 monkeys*	*No effect*
Cardiovascular disease	*Reduced 50% by CR*	*No effect*	*No effect*

On the incidence of neoplasia, both young-onset NIA group (zero incidence) and WNPRC (50% reduction) clearly benefited from CR, whereas the old-onset NIA group showed no effect. The WNPRC study reports 50% reduced incidence of CVD with CR, but the NIA study reported no reduction in incidence for either the young- or old-onset groups.

In summary, the only pattern of similar results from the WNPRC and the NIA prospective CR primate studies is a significant reduction in incidence of neoplasia with young-onset CR, and possibly also a trend of reduced diabetes incidence and delay of onset of age-related diseases with young-onset CR. None of these findings were reproduced by the NIA old-onset CR group.

An important aspect to consider is the median survival of CONs compared to the expected 26-27 years. This median survival from the WNPRC study was 26 years as expected. However, the old-onset CON monkeys from the NIA study show a median survival longer than expected with 27.8 years for females and a striking 35.4 years for males (31% longer than expected). The increase in median survival for CONs from the NIA study clearly indicates that these monkeys probably had specific factors increasing their lifespan possibly related to genetics, study design or diet. A possible effect of CR would be harder to observe when the CON monkeys also live longer than expected. Median survival of the young-onset monkeys from the NIA study has not been determined yet, possibly because about half of these monkeys were still alive in 2012 at the time of the publication.

Comparison of study designs

A number of differences in the design have been identified between the WNPRC and NIA study, and it is likely that these differences hold the keys to understand the different results in the two studies. The major differences are listed in table 7, and these will be the focus for further discussion. The differences in effects of CR could possibly be linked to the increased median lifespan for NIA CONs compared to WNPRC CONs. In this regard, the possible explanations behind differences in CON lifespans would provide valuable insight. Possible factors that will not be further discussed here are genetics and different household conditions possibly favoring longer lifespan of the NIA CONs.

The first obvious difference is the approach to 100% caloric intake of CONs. The CON monkeys in the WNPRC study were truly fed *ad lib*, whereas CON monkeys in the NIA study were only allowed a caloric intake based on age and body weight. The latter approach was considered approximately *ad libitum*. However, there is a distinction, which is important as most humans in the developed countries have continually *ad lib* access to food. Studies where CON groups are truly fed *ad lib* are therefore more transferable to humans.

Studies with *ad lib* CONs are likely to have a higher energy intake than fixed calorie CONs, possibly leading to reduced overall health. In this regard, studies with *ad lib* CONs are likely to show more significant differences between CR and CONs, compared to studies were CON animals are not fed truly *ad lib.* This could also be influence by decreased health through increased obesity not directly related to the mechanisms of CR. The non *ad lib* feeding and related lower body weight of NIA CONs could positively have improved their health and

lifespan through reduced obesity or a low degree of CR compared to the WNPRC *ad lib* CONs.

Table 7: Comparison of the study design and diets from the Wisconsin National Primate Research Center (WNPRC) and the National Institute on Aging (NIA) prospective calorie restriction (CR) studies in primates. Important differences between the two studies are underlined.

	WNPRC primate CR study design	NIA primate CR study design
Total number of monkeys	*N = 76 (CON; n = 38) (CR; n = 38)*	*N = 121 (CON; n = 64) (CR; n = 57)*
Males/females	*61%*	*51%*
Age of animals included	*7-14 years*	*1-14 years (young; n = 86) or 16-23 years (old; n = 35)*
CR intervention	*30%* matched to individual baseline intake	*30% based on age and body weight*
Diet	*Semi-purified, nutritionally fortified*	*Natural ingredient based*
Diet composition in % of total weight	*61% carbohydrates, 15% protein, and 10% fat*	*56.9% carbohydrates, 17.3% protein and 5% fat*
Diet composition in % of total energy	*62% from carbohydrates, 15% from protein and 23% from fat*	*67% from carbohydrates, 20% from protein and 13% from fat*
CON diet	Ad libitum	*Based on age and body weight*
Protein sources	*Animal-based: Lactalbumin*	*Mainly plant-based: Wheat, corn, soybean, fish and alfalfa meal*
Carbohydrate sources and sucrose content	*30% of total diet weight from corn starch, 5% from dextrin.* *Sucrose: 25-29% of both total diet weight and energy*	*Wheat and corn.* *Sucrose: 3.9% of total diet weight, 5% of total energy*
Fat sources	*Corn oil: consisting of 13% saturated fatty acids, 59% polyunsaturated fatty acids, high in linoleic acid (ω-6) and small amounts of linolenic acid (ω -3) (Dupont et al. 1990)*	*Soy oil + corn, wheat and fish (likely to be a good source of the ω-3 fatty acids EPA and DHA)*
Fiber	*5% cellulose of total diet weight*	*5% crude fiber of total diet weight (Kemnitz 2011)*
Vitamins and minerals	*Both groups given around 100% of RDA*	*CONs: 140% of RDA* *CR: 100% RDA*

Many other differences regarding the diet composition between these two studies have also been underlined in table 7. If CR results in greater health and longevity, the lack of positive results from the NIA study would imply that these CON monkeys also had a healthier diet than the WNPRC CONs. The ratio of macronutrients in the two diets are relatively equal and is unlikely to have a big impact. The WNPRC diet was however semi-purified, compared to the natural ingredient based NIA diet. As suggested by Mattison et al. (2012) this could possibly lead to an increased intake of phytochemicals, trace minerals or other unidentified bioactive components for the NIA monkeys that could contribute to better health.

Other obvious differences in diet composition are the sources of protein. When looking at the contribution of protein the WNPRC diet had 15% energy from protein versus 20% in the NIA diet. This difference could possibly be enough to have an impact on the results, but current knowledge of PR would imply that an increase from 15% to 20% of energy from protein such as in the NIA diet would result in poorer health, and this does not appear to have happened in the NIA study. A more likely explanation is the sources of proteins. The WNPRC diet was animal-based (lactalbumin from milk) as opposed to the plant-based protein sources in the NIA diet. As reported in a recent study, protein from animal-based sources could have a more negative impact on health and longevity than protein from plant-based sources (Levine et al. 2014). If this is true, it could be expected that the plant-based protein sources from the NIA diet would result in greater health of both CR monkeys and CONs, and the observed differences between the groups would be smaller.

Another difference in the diet composition is the content of sucrose. The WNPRC diet is high in sucrose, contributing to 25-29% of total energy compared to only 5% in the NIA diet. High intake of sucrose has been associated with obesity, and risk of age-related diseases in humans (World Health Organization 2014). Dietary recommendations in most countries suggest a maximum of 10% energy to be from free sugars. It is therefore likely that the high sucrose content of the WNPRC diet had a negative influence on the health and longevity of all monkeys and possible health effects of CR were easier to observe.

NIA sources of dietary fat was from soy oil, corn, wheat and fish, with fish providing a good source of ω-3 fatty acids. Dietary fat from corn oil used in the WNPRC study contains only small amounts of the ω-3 fatty acid linolenic acid (Dupont et al. 1990). Given the health effects of fish and ω-3 consumption (Yashodhara et al. 2009) it is possible that this could be a contributing factor leading to better health of the monkeys in the NIA compared to those in the WNPRC study. Finally, it is possible that the difference between 100% and 140% of RDA of vitamins and minerals for CONs could have influenced the results. Supplementation of more than 100% RDA of vitamin D (Golden & Carey 2016; Muscogiuri et al. 2016) or some B vitamins (Dawson et al. 2016; Kennedy 2016) could possibly improve health. Conversely, increased supplementation of other vitamins and minerals are in general not associated with additional benefits (Bjelakovic et al. 2007; Bjelakovic et al. 2012).

Other non-human primate CR studies

Other studies also exist on the effects of CR in non-human primates (Kemnitz 2011). Most of these studies clearly have a weaker study design than the WNPRC and NIA studies, and the results need to be viewed in that context. Together, findings from these studies are summarized in table 8.

NIA squirrel monkey study:

Together with the initiation of the more comprehensive NIA CR study on rhesus monkeys, a smaller cohort using the squirrel monkeys *Saimiri sciureus* and *Saimiri boliviensis* was also initiated (Ingram et al. 1990; Weindruch et al. 1995). Twelve young, 13 adults and 4 old monkeys were included in the study, which had a similar design to what was used in the NIA CR study on rhesus monkeys (Mattison et al. 2012). The main changes in the study design was an increase to approximately 22% protein and 20% fat by energy in the diet. Only minor reports have been published from this study so far. One report found no significant decrease in the accumulation of advanced glycation end-products (AGEs) in the skin of squirrel monkeys (Sell et al. 2003). AGEs are formed by glycation of simple sugars with proteins or lipids, and their accumulation have been linked to several pathologies (Glenn & Stitt 2009; Semba et al. 2009). Another report found that CR reduced the content of Alzheimer's disease (AD)-type brain amyloidosis associated β-amyloid peptides in the temporal cortex of the brain (Qin et al. 2006). This study reported that this decrease in CR monkeys was inversely correlated with SIRT1 activity in the same brain region. In this study, increased SIRT1 activity is further described to be a key effector through which CR works to reduce AD-type pathologies (Qin et al. 2006).

University of Maryland:

Bodkin et al. (2003) reported findings from a 25-year-old prospective CR primate study conducted at the University of Maryland. This study was originally designed to study diabetes and obesity (Mattison et al. 2002). They found that *ad lib* fed CON rhesus monkeys had a 2.6 fold increase in risk of death, in part related to lower insulin sensitivity compared with monkeys on CR. CR monkeys also had less signs of organ pathologies. Median lifespan was 32 years with CR compared to 25 years in CONs, supporting the findings in the WNPRC study. However, although this is a prospective follow up that increase the quality of the study design, they used a very low number of only eight CR monkeys compared to 109 CONs.

Thus, the observed median lifespan from a small cohort size of only eight subjects should be interpreted with caution since the outcomes could be sensitive to events affected by chance alone.

Wake Forest University:

A 4-year long study from Wake Forest University tested the effect of 30% CR on insulin resistance and atherosclerosis (Cefalu et al. 2004). Thirty-two adult cynomolgus monkeys (*Macaca fascicularis*) were randomized to 30% CR or a moderately atherogenic *ad lib* diet (30% energy from fat, 0.25mg cholesterol/kcal for both groups). Effects of CR were increased insulin sensitivity, but no effects was found on lipid profiles or atherosclerosis.

SNPRC Baboon study:

This study was set up at the Southwest National Primate Research Center (SNPRC) to investigate the effects of maternal 30% CR on fetal growth and metabolism in baboons, with 12 pregnant CR baboons, 20 pregnant *ad lib* CONs and 14 non-pregnant *ad lib* CONs (Schlabritz-Loutsevitch et al. 2007). Results showed no difference in fetal body weight, but showed a reduction in placental weight with reduced capillary surface area suggested to reduce the nutrient transport to the fetus.

Upon studying these effects of maternal CR on fetal baboon kidney Nijland et al. (2007) scrutinized the effect of CR on the mTOR pathway. It was found that downregulated mTOR signaling was a key mediator. Some of the downstream effects of reduced mTOR signaling was found to be downregulated S6K and eIF4E. Findings also indicated that hormones, growth factors, AAs and PKB/Akt, normally activated this pathway, in contrast to AMPK that could indirectly inhibit mTOR.

Additionally, for the GH/IGF-1 pathway, the downstream mediator PI3K was surprisingly found to be upregulated (Nijland et al. 2007). Both pro- and anti-apoptotic genes was downregulated with CR, implying that apoptosis signaling is not a central part of the CR mechanism. In another report, Li et al. (2009) found that this maternal CR reduced fetal IGF-hormone levels and IGF receptor proteins, but also increased IGFBPs, still leading to overall reduced GH/IGF-1 signaling. Li et al. (2009) also reported increased apoptosis and decreased activity of PKB/Akt.

Table 8: Study design and results from non-human primate CR studies other than the NIA- and WNPRC primate CR studies on rhesus monkeys.

Study:	*Study design*	*Main results on effects by CR*
NIA squirrel monkey study (Ingram et al. 1990; Weindruch et al. 1995)	29 squirrel monkeys, *S. sciureus* and *S. boliviensis* (12 young, 13 adults and 4 old). 22% protein and 20% fat by energy. 30% CR, based on age and weight. CONs approximately, but not truly *ad lib*, based on age and body weight. Lifelong follow up	*No significant decrease in accumulation of AGEs (Sell et al. 2003). CR reduced the content of Alzheimer's disease (AD)-type brain amyloidosis associated* beta-*amyloid peptides in the temporal cortex of the brain (Qin et al. 2006)*
University of Maryland (Bodkin et al. 2003)	8 CR monkeys (*M. mulatta*) and 109 *ad lib* CONs, 25 years prospective study. 17% protein and 13% fat by weight. CR regulated by target body weight, effectively 40% CR	*CONs had a 2.6 fold increase in risk of death, in part related to decreased insulin sensitivity. Less signs of organ pathology with CR. Median survival for CONs was 25 years, compared to 32 years for CR. Individual reaching highest age, 40 years was from CON*
Wake Forest University (Cefalu et al. 2004)	32 adult cynomolgus monkeys (*M. fascicularis*) randomized to 30% CR or a moderately atherogenic *ad lib* diet (30% energy from fat, 0.25mg cholesterol/kcal for both groups). 4-years of duration	*CR increased insulin sensitivity, but no effects on lipid profile or atherosclerosis*
SNPRC baboon study (Schlabritz-Loutsevitch et al. 2007)	Maternal 30% CR examining effects on fetal growth and metabolism in baboons with 12 pregnant CR baboons, 20 pregnant *ad lib* CONs and 14 non pregnant *ad lib* CONs	*Reduced IGF-1, and increased IGFBP. PI3k gene expression upregulated and reduced PKB/Akt activity. Reduced mTOR activation, downregulated S6K and eIF4E gene expression*

3.1.3 CR in humans

The recently initiated CALERIE research program has provided several interesting result on effects of CR in humans. These studies generally have a high quality randomized controlled study design, although so far they have only provided data on various outcomes related to aging and no effects on lifespan. Other conducted studies of CR in humans rely on self-imposed CR resulting in an obvious "selection" bias.

CALERIE research program

The comprehensive assessment of the long-term effects of reducing intake of energy (CALERIE) research program was first initiated as short-term phase 1 trials to monitor the safety for the later initiation of a more comprehensive phase 2 study. All the CALERIE studies were designed as randomized controlled trials. One of these CALERIE phase 1 trial studies was a 6-month study of CR in moderately overweight individuals with body mass index (BMI) of 25-30 kg/m^2, age 20-42 years (Meydani et al. 2011). For this study, 34 volunteers were

randomized to either 10% or 30% CR combined with either high glycemic or low glycemic diets. The main aim of this study was to assess whether CR affected markers of oxidative stress and endogenous antioxidants. 30% versus 10% CR significantly increased plasma glutathione (endogenous antioxidant) levels (p=0.04) and decreased protein carbonyl (measure of ROS protein damage). Further, 8-epi-prostaglandin F2α levels (measure of oxidative stress related lipid peroxidation) were non-significantly decreased while the endogenous antioxidants superoxide dismutase and catalase were unaffected. Not surprisingly, as with most interventional studies with CR a reduction of body weight was observed, however not significantly between the groups (Das et al. 2007).

In another CALERIE phase 1 trial study, the effect of 20% CR for 1-year was compared to an interventional group with 20% increased metabolic expenditure from exercise, and a healthy CON group (Fontana et al. 2007). Subjects (n=35) had a mean BMI of 27.3 ±2 kg/m^2, and age of 57 ±3 years. Both CR and exercise provided the same benefits with reductions of CVD risk factors. Low-density lipoprotein (LDL), total cholesterol/high density lipoprotein (HDL) ratio were all found to be significantly decreased with both 20% CR and exercise. 20% CR resulted in significantly lower total cholesterol and non-significantly reduced C-reactive protein (CRP).

In a separate cohort, Tam et al. (2012) tested the effect of 25% CR, compared to 12.5% CR plus exercise (increased energy expenditure 12.5%) and healthy CONs in 25 individuals (BMI 27.7 ±0.7 kg/m^2) for 6 months. Outcomes examined were salivary cortisol levels and markers related to systemic and adipose tissue inflammation. 25% CR resulted in non-significantly improved insulin sensitivity, and lower insulin levels. Serum levels of adiponectin, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and CRP was non-significantly increased, while serum leptin was significantly reduced. No effects were found on salivary cortisol levels (Tam et al. 2014).

CALERIE 2-year long phase 2 study

Later on, a CALERIE phase 2 study was initiated with a larger study group of individuals with normal bodyweight (BMI 22-28 kg/m^2, age 21-50 years) (Rochon et al. 2011). The CALERIE phase 2 study had 225 subjects randomized to either 25% CR or an *ad lib* diet (2:1 ratio on group assignment in favor of CR) maintained for two years. Main findings from this study are that 25% CR significantly increased serum IGFBP-1 by 21% and reduced the ratio of IGF-1: IGFBP-1 by 42%. CR had no significant effects on serum IGF-1 levels or IGF-1: IGFBP-3 ratio

(Fontana et al. 2016). IGFBP-3 is expected to reduce the biological activity of IGF-1 in the same way as IGFBP-1. Although CR reduced IGF-1 compared to baseline, this was also observed for the CON group. Other reported effects from this two year phase 2 study apart from weight loss, is the significant reductions in both plasma triiodothyronine (T3) and TNF-α (Ravussin et al. 2015). According to the depicted exploratory aims of this study, several additional outcomes are expected to be published in the near future. The main findings from the CALERIE research program are shown in table 9.

Table 9: An overview of the results from the comprehensive assessment of the long-term effects of reducing intake of energy (CALERIE) research program studies on effects of CR in humans.

	Study:	Study design	Main results on effects of CR
CALERIE phase 1 trials	Das et al. (2007); Meydani et al. (2011)	Randomized controlled trial, 6 months duration with 10% or 30% CR, combined with high or low glycemic load in subjects with BMI 25-30 kg/m^2	*CR level significantly positively correlated with increased plasma glutathione (endogenous antioxidant) and decreased protein carbonyl levels (measure of ROS protein damage). 8-epi-prostaglandin F2α (measure of oxidative stress related lipid peroxidation) non-significantly decreased*
	Fontana et al. (2007)	Randomized controlled trial of 1-year, 20% CR group, 20% increased energy expenditure from exercise group, and healthy CON. Subjects with BMI of 27.3 ±2 kg/m^2	*Both CR and exercise provided same benefits with reductions of CVD risk factors. LDL and total cholesterol/HDL ratio were significantly reduced. CR also gave significantly lower total cholesterol and non-significantly reduced CRP*
	Tam et al. (2012); Tam et al. (2014)	Randomized controlled trial, 6 months duration. 25% CR, compared to 12.5% CR plus exercise (increased energy expenditure 12.5%) and healthy CON. Subjects with BMI 27.7 ±0.7 kg/m^2	*25% CR resulted in non-significantly improved insulin sensitivity, and lower insulin levels. Serum levels of adiponectin, TNF-α, IL-6 and CRP were non-significantly increased, while serum leptin was significantly reduced. No effects on salivary cortisol levels*
CALERIE 2-year long phase 2 study	Rochon et al. (2011); Fontana et al. (2016); Ravussin et al. (2015)	Randomized controlled trial. Group of 225 subjects with BMI 22-28 kg/m^2 and age of 21-50 years, randomized to 25% CR or an ad lib diet (2:1 ratio on group assignment in favor of CR) maintained for two years	*25% CR gave 21% significant increase in serum IGFBP-1 and 42% significant reduction in IGF-1: IGFBP-1 ratio. No significant effects on serum IGF-1 or IGF-1: IGFBP-3 ratio, although IGF-1 was reduced with CR compared to baseline (also in CON). Also significant reductions in T3 and TNF-α*

Long term voluntarily CR

Studies on voluntarily self-imposed CR provides valuable insight, but needs to be considered in the context of several factors that could bias the CR group towards a healthier lifestyle than the CON group. Correctively in this setting, the CON group is often described as a comparison group rather than CON as it would be quite difficult to control for all possible

factors differing between the groups. As with vegetarian groups used as a measure of protein restriction in chapter 3.2.3 individuals practicing a CR regimen most likely do not represent the general population on several aspects that could influence their health. An overview of studies of self-imposed CR in humans can be seen in table 10.

Fontana et al. (2004) studied a group of 18 individuals that had practiced a CR regimen for an average of 6 years. This group was compared to healthy age-matched CON (comparison group) on respect to CVD risk factors. The CR group had not surprisingly lower BMI and percentage of body fat. In general, several CVD risk factors was reduced in the CR group including lower total cholesterol, LDL, total cholesterol: HDL ratio, systolic and diastolic blood pressure, CRP, fasting glucose and insulin levels as well as significantly higher HDL. Data from 12 of the volunteers followed prospectively from the onset of their CR regimen supported that these results were effects of the CR diet. Most of their CVD risk factors were reduced compared to baseline, and this effect was significant for many, but not all risk factors.

Mercken et al. (2013) reported valuable insight into the mechanisms of CR in humans. This study also used subjects that had practiced CR for a long time. Fifteen middle-aged subjects aged 58.7 ±7.4 years that had practiced a 30% CR regimen for an average of 9.6 years were compared to a CON group of 10 age-matched individuals. Highly relevant findings from this study were significant downregulation of several mediators of the GH/IGF-1 pathway with in the 30% CR group compared to CON. PI3K and PKB/Akt was downregulated 1.7 and 2 folds respectively. In addition, one of the expected endpoint mediators at the transcriptional level of this pathway, FOXO, was found to be upregulated, consistent with decreased activity of the pathway. SIRT2, SIRT4, SIRT5 (sirtuins) as well as two AMPK transcripts were also found to be significantly upregulated. An upregulation of PGC-1α was also observed, and all together, this strengthens the possible influence of sirtuin and AMPK signaling in the mechanisms behind the effects of CR in humans.

Table 10: Result from selected studies on the effects of long-term voluntarily CR in humans.

Voluntarily CR studies	Study design	Main results on effects of CR
Fontana et al. (2004)	18 subjects that had practiced a CR regimen for an average of 6 years. Healthy age-matched CON group	*The CR group had lower BMI and fat percentage. Several CVD risk factors was reduced in the CR group compared CON/comparison group. The CR group had significantly lower total cholesterol, LDL, total cholesterol: HDL ratio, both systolic and diastolic blood pressure, CRP, fasting glucose, insulin and significantly higher HDL. 12 subjects prospectively followed from CR onset supported these effects of CR. Most CVD risk factors reduced by CR compared to baseline*
Mercken et al. (2013)	15 subject aged 58.7 ± 7.4 years that had practiced a 30% CR regimen for an average of 9.6 years compared to age-matched CON group (n = 10)	*Significant downregulated activity of the GH/IGF-1 pathway in the 30% CR group compared to CON. PI3K and PKB/Akt was downregulated 1.7 and 2 folds, respectively. Upregulated FOXO transcription. SIRT2, SIRT4, SIRT5 (sirtuins) as well as two AMPK transcripts significantly upregulated. Also observed upregulated PGC-1α*

Okinawa diet

The Island of Okinawa, southeast in Japan has one of the world's longest living population (Willcox et al. 2008). Okinawa has an isolated population with exceptionally low risk of age related diseases, especially CVD and cancer, and the population seems to preserve physical and cognitive functions also at old ages. The precise explanations behind these observed health benefits is hard to identify. Genetic, social and cultural aspect of an isolated island could be expected to influence the health of the populations, but the Okinawan diet has also been implicated to be a major factor. The Okinawan diet, with low caloric content, has many similarities with the typical CR diet tested in studies and is speculated to provide many of its health benefits by the same mechanisms as CR (Willcox et al. 2014). Other aspects of the typical Okinawan diet are:

- High content of vegetables, legumes, fiber and phytochemicals.
- Low consumption of meat, dairy products, (saturated) fat and low ω-6: ω-3 ratio.
- Low glycemic index food.

PR could also be a factor providing some of the health benefits associated with this diet. Only 9% of energy is derived from protein whereas 89% is provided by carbohydrates (Le Couteur et al. 2016b). From the above list, it also seems obvious that much of the protein consumed would be from plant-based sources, rather than animal-based sources, and as reported in the study by Levine et al. (2014) this could also be an important factor improving health.

3.1.4 Summary and answer to thesis aim one

From chapter 3.1.1 it seems that studies testing the effects of CR in rodents are resulting in consistent increases in lifespan. Although this topic is not reviewed in detail here, data from two independent meta-analyses both show that CR increases median lifespan in rodents by 15-30% on average. Based on this, there is strong evidence that CR can increase lifespan in rodents under normal experimental settings. The magnitude of the effects mediated by CR is however varying to a huge extent both between the rat and mouse species and across different strains. As previously discussed, strain specific gene-diet interactions as well as differences in study design are likely to play a major role in this high degree of varied magnitude of effects.

In chapter 3.1.2 much emphasize have been given to the prospective controlled trials of CR in non-human primates in the NIA and the WNPRC studies respectively. These studies are by far the best data identified in this thesis to evaluate the transferability of CR to humans. Although both studies are ongoing with some monkeys still alive, much of the most important data have already been published. Results from the WNPRC study clearly indicate significant increases in median lifespan by CR. However, the comparable NIA study did not uncover any differences in median lifespan or HR for death between CR and CON, but all monkeys and especially males, lived much longer than expected for the species in captivity. As discussed extensively in chapter 3.1.2 these studies have major differences in their designs possibly contributing to the opposing results. These are especially diet related differences such as caloric intake of CONs, diet purification, protein sources, sucrose content and fatty acid composition, as well as the possibility that also genetic differences between the monkeys used in the two studies could influence the results. Collectively, there is a strong trend for CR to increase lifespan and health in non-human primates, but more long-term high quality studies with consistent results are needed for this association to be confirmed.

With respect to CR in humans (chapter 3.1.3), no interventional studies with effects on lifespan have been carried out yet. Studies of isolated populations with long lifespan, such as on the Island of Okinawa, Japan, could indicate that a low caloric diet plays a major role contributing to long lifespan, although there are several other factors, including low protein intake, that might influence health and lifespan just as much as caloric intake. Studies of

subjects on long term voluntarily CR indicate several positive health outcomes. The up to two-year long studies from the CALERIE research program, all with a strong randomized controlled design supports the multiple benefits CR might provide for humans, and it will be highly interesting to see results from similar studies with longer duration and larger subject groups in the future.

Collectively this summary provides the basis to answer thesis aim number one:

- Are the positive effects and knowledge of CR on lifespan and health at old age in model organisms relevant for mammals and especially humans?
    - Much of the knowledge of CR from model organisms seem to be relevant also for mammals and humans. There is strong evidence that CR can increase lifespan in rodents and provide many of the same effects as seen in other model organisms. It is also likely that CR could provide many of these same effects in both non-human primates and humans.

3.2 Effects of protein restriction (PR) in mammals

Upon following the methods described in chapter 2, a total number of 18 articles were identified. Two of these were later found to consist of the same cohort of animals; still both articles will be included here, but reviewed under the same section as one study. Among these final 17 studies, 14 were exploring the effects of PR or AA-R on lifespan in rodents, and three articles were exploring the same effects on miscellaneous outcomes or lifespan in humans. No studies on PR or AA-R in non-human primates or other mammals were found to meet the inclusion criteria. Table 11 shows the included articles sorted according to species, intervention and measured outcomes. For effects on lifespan in mice, four articles were found on PR, one on methionine restriction (Meth-R), and one on tryptophan restriction (Trypt-R). For effects on lifespan in rats, four articles were found on PR, two on Meth-R, and two on Trypt-R. Articles on other mammals exploring lifespan or miscellaneous outcomes were all three on effects of PR in humans with only one of these three measuring mortality as an outcome.

Table 11: Study characteristics of included studies from the systematic search on PR and AA-R.

Number of studies	Intervention	Species	Measured outcomes
n = 8	PR	Mice (n = 4), rats (n = 4)	Lifespan and miscellaneous outcomes
n = 3	Meth-R	Mice (n = 1), rats (n = 2)	Lifespan and miscellaneous outcomes
n = 3	Trypt-R	Mice (n = 1), rats (n = 2)	Lifespan and miscellaneous outcomes
n = 3	PR	Humans	Miscellaneous outcomes only (n = 2), lifespan and miscellaneous outcomes (n = 1)

The selection of a systematic search for this chapter has the advantage of making the inclusion of studies completely unbiased. The only weaknesses here lie within the creation of the systematic search in the selection of databases and search words. It seems that the systematic search was able to pick up a representative sample of the available studies for rodents. The systematic search did not uncover any studies on PR in non-human primates, and on the topic of PR in humans, only one study was an observational population study with lifespan data. It can be expected that other population studies exist on the topic of PR/protein intake and effects on lifespan. Population studies with data on lifespan and protein intake are hard to pick up in a systematic search due to the fact that other outcomes or areas of focus might be highlighted in title and abstract.

3.2.1 PR in rodents

Included studies on effects of PR in mice (n = 4)

Fernandes et al. (1976)

Fernandes et al. (1976) examined the effect of various diets in the B/W and DBA/2f strains of mice. Eight different diets (only four diets in the DBA/2f strain), varying in protein content, corn oil and total calories were initiated at an age of three weeks and maintained throughout life. Four of the diets tested had a protein content of 22% (by weight) compared to 6% in the remaining four, equivalent to a 73% reduction in protein intake (73% PR). Out of the total 24 specific sex-diet-strain cohorts, each had a total of 11-15 mice. The selection of diet groups for each animal were not reported to be randomized. Data on lifespan for each dietary group were presented as mean, median, 20% survival and max survival. Statistical significance of differences between the results are not given, although it appears to be significant for most parameters commented below.

For the B/W strain, PR increased lifespan compared to CONs on all parameters except median survival in females that was slightly shorter (306 days versus 317 days). Increases in survival were quite impressive for some of the lifespan parameters. For instance, survival of the 20% longest living mice (20% max survival) was in males raised from 400 days in CONs to 600 days in for PR, equivalent to a 50% increase. Among all parameters, the increased lifespan was consistently higher for males than females. Among the four high fat diets tested in the B/W strain, the longest lifespan was seen with PR plus CR, followed by CR alone. High fat PR alone resulted in a slightly decreased lifespan compared to high fat CON.

For the DBA/2f strain, both mean and median lifespan were increased with PR for both sexes. Some of the max survival data for this strain are of low informative value as the study was published while many of the longest living mice were still alive. Additional max lifespan data from this cohort has been searched for without success.

Due to the total number of eight diets tested, this study also offers the opportunity of direct comparison between PR and CR. Although, the degree of CR tested in this study was as much as 50% compared to CONs. For the B/W strain, CR increased all lifespan parameters more than PR alone. Combined PR and CR also extended lifespan, more than PR alone, but not as much as CR alone. For the DBA/2f strain on the contrary, CR resulted in a slight reduction in

lifespan compared to CONs. For the B/W strain, PR combined with CR gave longer lifespan than PR alone, but lower than CR alone. This was exactly the opposite for the DBA/2f strain where PR gave the longest lifespan, followed by combined PR + CR, and lastly CR alone. It should also be noted that the 50% CR tested here is a stronger restriction than what is used in most other CR protocols. Besides assessing lifespan, this study found that both CR and PR retarded early growth from 3 weeks of age, and CR resulted in an increased susceptibility to infections.

Leto et al. (1976)

This study by Leto et al. (1976) tested the effects of 26% protein from casein versus 4% protein from casein (85% PR) on mortality in female C57BL/6 mice. Diet interventions were initiated at an age 30 days with animals randomized to each group with both CONs and PR fed *ad lib*. CON diet had 26% casein, 15% cornstarch, 49% sucrose. In the PR diet, casein was replaced by sucrose. Half of the 140 mice in each diet group were used to establish data on mortality.

Median survival was significantly longer ($p < 0.001$) in PR than controls with 852 days (28 months) compared to 685 days (23.5 months). Max lifespan was 840 for CON and 1167 for PR. Miscellaneous outcomes examined in this study was also a 10% decreased food intake in both grams (g) and calories for the PR group. Decreased food intake induced by PR is surprising because protein has been reported to induce strong feedback mechanisms on food intake (Solon-Biet et al. 2014). Other findings were a consistent higher oxygen consumption and lower rectal body temperatures of the PR mice.

Stoltzner (1977)

This study tested the effects of PR in BALB/c male mice. One hundred mice were placed (not randomly) to one of five different dietary groups with various protein content. CON group was *ad lib* fed with 24% protein by weight from an age of 22 days. Two of the four *ad lib* fed PR groups were fed the same diet as the CONs until they were four months of age, where they switched to either 8% or 4% protein throughout their lives. The last two PR groups were fed lifelong *ad lib* diets of 8% or 4% protein from age of 22 days. No significant differences in mean lifespan were seen with the different diets, but on median lifespan the three diets with the lowest protein content resulted in significantly longer lifespan compared to CON (e.g. 4% protein diet gave a median lifespan of 725 days versus 590 days for CON).

Collectively, Stoltzner (1977) found that PR significantly increased median lifespan compared to CON, and that the level of PR was associated with the level of increase in median lifespan. No other significant effects were observed on lifespan although PR had a trend of increasing all parameters except absolute max lifespan.

Solon-Biet et al. (2014); Solon-Biet et al. (2015)

Solon-Biet et al. (2014) used a promising new approach called geometric framework to examine the effects of different macronutrient ratios on longevity and health in mice. The geometric framework approach has been described as a state-space nutritional modeling method to examine the complex and likely interactive effects of varying macronutrient ratios (Le Couteur et al. 2016a; Lee et al. 2008). A total of 858 (lifespan data for n = 533) male and female mice of the C57BL/6 strain were fed *ad lib* from three weeks of age with 25 different diets varying in ratios of all three macronutrients. Some of the 25 different diets also had equal protein to carbohydrate ratios (P-C ratio) but were different in fat content or energy density. The selection of diet groups for each animal were not specifically mentioned to be randomized. The 25 different diets varied in protein content from 5-60%, carbohydrate from 16-75%, fat from 16-75% (all by total energy) and energy content of either 8, 13 or 17 kJ/g of food.

Strongest increase in lifespan was reported to be with low protein and high carbohydrate intake. This seems to be true for max lifespan, but the same correlation should not be drawn from the median lifespan data. Max lifespan was measured as the average of the longest living 10% of the mice (n = 2-3). The diet resulting in the longest max lifespan of 157 weeks contained 5% protein, 75% carbohydrate and 20% fat (all by total energy), with a protein to carbohydrate (P-C) ratio of 0.07. In fact, all the top four diets resulting in the longest max lifespan had a P-C ratio of 0.25 or lower. However, this was followed by two diets with P-C ratios of 1.45 resulting in max lifespans of 151 weeks.

Collectively, max lifespan appears to increase with low P-C ratio and low protein content, however this correlation does not seem to follow a linear pattern and other factors influencing max lifespan are likely. There is however a trend that the diets with high P-C ratios are resulting in shorter max lifespans. The three shortest max lifespans are for instance seen with diets of P-C ratios of 1.45, 3.00 and 0.61, protein content 42%, 60% and 23%, and max lifespans of 104, 103 and 100 weeks respectively.

The longest median lifespan was reported to be 139 weeks with an energy dense diet with a P-C ratio of 1.45 and 42% protein. For median lifespan, no other diets resulted in more than 130 weeks, but among seven diets that resulted in median lifespan from 120-130 weeks, six of these had a P-C ratio below 0.69.

Solon-Biet et al. (2014) report that the median lifespans in this study increases from approximately 95 to 125 weeks (30%) with reduced P-C ratios. From reviewing the individual median lifespan data and P-C ratios for each group, this statement cannot outright be agreed on as there are several individual diet groups that interferes with this pattern and the p-value for P-C ratio and median lifespan correlation is > 0.05. Protein content or P-C ratios does not seem to be clearly correlated with median lifespan. However, for HR of death correlated with P-C ratio, there was a clear significant correlation of increased ratio of death with increased P-C ratio. Effects of CR was also examined in this study as some of the diets was diluted with non-digestible fiber leading to a lower total caloric intake. Only the highest calorie intakes, but not CR was found to be influencing the HR of death.

Several other interesting findings on other parameters also arose from this study. Food intake was found to increase when the content of protein or carbohydrates in the food were reduced. This effect on reduced food intake by specific macronutrients were strongest for protein, followed by carbohydrates and apparently negligible for fat. As noted by the authors, this point to the fact that nutrient specific feedback mechanisms for fat seem to be considerably overruled by the feedback mechanisms of protein and carbohydrates. Leptin levels were positively associated with both body fat and total macronutrient intake.

Plasma branched-chain AA (BCAA) levels were found to be correlated with chronic protein intake. In addition, insulin levels were found to be affected by both protein and carbohydrate intake, with highest levels measured at high protein intakes and medium (15-25 KJ /day) carbohydrate intake. Hepatic mTOR activation was strongly influenced by circulating glucose and BCAAs thereby supporting the influence of nutrient availability on this pathway. Additionally, low protein intake was associated with increased mitochondrial activity, number and elevated free radical production.

Lastly, macronutrient ratios influenced several parameters of cardiovascular health; low protein, high carbohydrate diets were associated with lower blood pressure, better glucose

sensitivity, reduced LDL, increased HDL and reduced triglycerides. Despite these findings, low P-C ratio was associated with increased body fat and reduced lean body mass.

Another paper by Solon-Biet et al. (2015) reanalyzed lifespan data from the same cohort on respect to sexes, and reported effects on reproductive function. Analyses of median lifespan revealed that only carbohydrate intake significantly influenced this parameter. Interestingly this paper finds that reproductive function is optimized with diets with P-C ratios of 1.00 indicating that lifespan and reproductive function is not optimized with the same diet. On the contrary, diets optimizing max lifespan and reproductive function seem to contrast each other on respect to P-C ratios.

Overall, findings by Solon-Biet et al. (2014) and (2015) indicate that PR increases lifespan in mice. Max lifespan seems to increase with low P-C ratio and low protein content. On the contrary, protein content or P-C ratios do not seem to be clearly correlated with median lifespan, but HR for death correlated with P-C ratio shows a significantly increased risk with high P-C ratio. Findings from all the studies on effects of PR in mice are shown in table 12.

Table 12: An overview of results from studies on effects of PR in mice.

Author (year)	Study design	Miscellaneous outcomes	Effects on lifespan
Fernandes et al. (1976)	B/W strain (8 diets) DBA/2f strain (4 diets) CON diet: 22% protein PR: 6% protein (73% PR) CR: 50%	*Moderately retarded growth from 3 weeks of age by both CR and PR. Increased infection susceptibility with CR*	*Both CR and PR alone increased most lifespan parameters. Cumulative CR + PR effects only for high fat diets. Strain-specific dietary responses*
Leto et al. (1976)	Female C57BL/6 strain. CON: 26% casein *ad lib* PR: 4% casein (85% PR)	*PR decreased food intake by 10%, increased O_2 consumption and increased body temperature*	*PR significantly increased median survival. Max lifespan increased with PR*
Stoltzner (1977)	Male BALB/C mice. CON: *ad lib*. PR: four different groups with 8% or 4% protein with mixed interventional onset-ages (22 days or 4 months)	*No effect on levels of kidney catalase*	*Median lifespan significantly increased with 3 out of four low protein diets, associated with the degree of PR. No significant effects on other lifespan parameters*
Solon-Biet et al. (2014); Solon-Biet et al. (2015)	C57BL/6 strain. 25 *ad lib* diets different on macronutrient ratios and calories	*Low protein, high carbohydrate diet improved cardiovascular health parameters, lower serum BCAA and glucose, lower hepatic mTOR activity and reduced reproductive function*	*PR increased max lifespan but not median lifespan. PR reduced HR for death. No effect of CR on lifespan*

Included studies on effects of PR in rats (n = 4)

Anantharaman (1983)

This study by Anantharaman (1983) tested the effects of CR and PR both isolated and combined in Sprague-Dawley rats. Separate groups were used to examine the effects related to pregnancy, lactation and adult onset restrictions. Mainly the results on adult-onset restriction will be discussed here, with only minor notice of effects on pregnancy and lactation as miscellaneous outcomes. Adult-onset restrictions were initiated at 58 weeks of age, after receiving *ad lib* standard lab chow up to this point. Rats were placed in 5 different diet groups. Group one (CON) continued to receive standard lab chow *ad lib*, group two were fed 22% protein *ad lib*, group three had 22% protein with 30% CR, group four (PR) were given a fixed low dose of a high protein diet but allowed to feed *ad lib* of a non-protein diet. Finally, the fifth group was restricted of non-protein energy diet but allowed to eat *ad lib* of a high protein diet. Protein source in all diet groups was lactalbumin.

PR (group 4) increased median survival in males compared to CONs fed standard lab chow but not compared to those fed 22% protein *ad lib*. For females, median survival for PR was strongly reduced compared to CONS fed standard lab chow, but slightly increased compared to 22% protein *ad lib*. For males, survival at age 120 weeks was slightly reduced with PR (28.6%) compared to lab chow CONs (32.1%) but equal to 22% protein *ad lib* (28.6%). For females the same 120 weeks of age survival was lower with PR (9.1%) compared to standard lab chow (27.3%) and 22% protein *ad lib* (13.7%).

In summary, both longest median survival and survival at 120 weeks were achieved with the 22% protein CR diet, with highest effect in males. PR did not increase median survival or survival at 120 weeks of age. In some cases, PR actually reduced lifespan.

Davis et al. (1983)

This study tested the effects of various combinations of CR and PR in male Wistar rats with interventional onset at 32 days of age (Davis et al. 1983). Three restricted groups were fed 18%, 30% or 42% casein with 30% CR compared to their *ad lib* paired fed groups with 12%, 20% and 28% casein respectively. With this design, each of the paired groups had the same total intake of protein, categorized as high protein, medium protein or low protein. Survival was only monitored until two years of age.

Survival was decreased with PR in both *ad lib* and CR groups. After two years, the *ad lib* fed rats had 19% survivors in the low protein group, 31% survivors in the medium protein group, and 33% survivors in the high protein group. The CR groups followed basically the same pattern with 31%, 61% and 53% survivors on low, medium or high protein diet, respectively. Thus, dietary protein levels were in this study positively associated with increased survival after two years ($p < 0.05$). However, addition of CR significantly increased the two-year survival rate compared to *ad lib* fed groups ($p < 0.0005$). Interestingly, survival at one year of age was high, 86% or more for all groups, but highest for both individual and combined CR and PR. Additional results from this study was that both CR and PR were found to have positive effects on renal function.

Yu et al. (1985)

Yu et al. (1985) tested the effects of PR and CR in male Fischer 344 rats. Other findings from this same cohort on various outcomes other than aging have been published in a separate report, and will be briefly included here (Maeda et al. 1985). Five different dietary intervention groups were used for this study. One group consisted of CONs with 21% casein, and three other groups had a 40% CR with differences in age of onset and duration of the CR regimen. Specifically, the CR regimen was either maintained from age 6 weeks to death, from age 6 weeks to age 6 months, or from age 6 months to death. The fifth group was fed with a PR diet consisting of 12.6% casein. Both CON and PR diets were fed *ad lib*.

The PR group had significantly longer median lifespan of 810 days, compared to the CON group with 701 days. 10% longest lifespan was slightly increased, but not significantly in PR compared to CON. Max lifespan for PR was 969 days versus 941 days for CON.

40% CR gave strong increases in lifespan in this study. Even the CR regimen only maintained from between age 6 weeks to age 6 months had positive effects on all lifespan parameters. The effects of any of the lifelong CR regiments initiated from age 6 weeks or age 6 months had consistently stronger effects. Both the two lifelong CR interventions resulted in significantly longer median and 10% lifespan than either CON or PR. The lifespan reported here with lifelong CR is in some cases as much as up to 50% more than CON.

Of other outcomes examined from this cohort published by Maeda et al. (1985) it was found that PR delayed the occurrence of chronic nephropathy and cardiomyopathy, with no effect

on the occurrence of neoplasia. CR delayed the occurrence of chronic nephropathy and cardiomyopathy more than PR, and CR delayed the occurrence of neoplasia.

Horakova et al. (1988)

This study by Horakova et al. (1988) tested the effects of various PR and CR protocols on the lifespan of male Fischer 344 rats. CON group was fed a diet consisting of 21% casein, and 44% dextrin. PR diet was 12.6% casein and 52% dextrin. Four groups received the interventional diets with either lifelong PR from age 6 weeks, lifelong 40% CR from age 6 weeks age, PR between age 6 weeks to 6 months or in the last group PR from age 6 weeks to age 6 months followed by lifelong 40% CR only. Both CON and PR groups were fed *ad lib*.

Lifelong PR did significantly increase median lifespan from 715 days in CON to 835 days. 10% max lifespan was also increased but not significant. PR, only from between age of 6 weeks to age of 6 months obtained similar results as lifelong PR, with significantly longer median lifespan than CON, and not significant but still longer 10% max lifespan.

Lifelong CR resulted in both median lifespan and 10% max lifespan significantly longer than CON or any PR groups. CR did in fact increase both median and 10% max lifespan by around 50% compared to CON. PR from age of 6 weeks to age of 6 months followed by lifelong CR resulted in slightly shorter lifespans than CR alone, but still significantly longer compared to CON or any of the PR groups.

The study design used for the CON, PR and CR groups in this study by Horakova et al. (1988) is fairly identical to what was used by Yu et al. (1985). Most results from the present study summarized above are also in agreement with the findings reported by Yu et al. (1985). Table 13 shows an overview of all studies on effects of PR in rats.

Table 13: The main results from studies on effects of PR in rats.

Author and (year)	Study design	Miscellaneous outcomes	Effects on lifespan
Ananthara man (1983)	Sprague-Dawley strain. Adult onset at 58 weeks of age. CON: standard lab chow *ad lib* or 22% protein *ad lib* PR: restricted protein diet and *ad lib* non protein diet CR: 22% protein, 30% CR	*Pregnant and weaning cohorts showed reduced gestational and lactation performance, reduced birth weight and weaning weight with both CR and PR alone*	*PR had no effect on median survival or survival at age 120 weeks (in some cases reduced). CR increase these same lifespan parameters (with up to 50% in some cases)*
Davis et al. (1983)	Male Wistar strain. Onset at 32 days of age. 6 groups. Three 30% CR groups with 18%, 30% or 42% casein compared with *ad lib* pair fed groups with 12%, 20% or 28% casein	*Both CR and PR improved renal function*	*PR significantly decreased two-year survival. CR increased two-year survival. Increased survival with medium or high protein vs low protein. Cumulative positive effect of medium/high protein and CR*
Yu et al. (1985)	Male Fischer 344 strain. CON: 21% casein *ad lib* CR: 40%, various onset ages and durations. PR = 12.6% casein *ad lib*	*PR and CR delayed nephropathy and cardiomyopathy. Neoplasia occurrence delayed by CR, and not PR*	*PR significantly increased median lifespan but not 10% max lifespan. Lifelong CR gave significantly longer median and 10% max lifespan compared to PR or CON*
Horakova et al. (1988)	Male Fischer 344 strain. CON: 21% casein *ad lib*. CR: 40% PR: 12.6% casein *ad lib*	*None*	*PR significantly increased median lifespan but not 10% max lifespan. CR gave significantly longer lifespan on both parameters compared to CON or PR*

3.2.2 Selective amino acid restriction (AA-R) in rodents

Included studies on effects of methionine restriction (Meth-R) in mice (n = 1)

Sun et al. (2009)

This study used male hybrid mice between C57BL/6 and BALB/c (CB6F1) mice to study the effects of Meth-R in comparison with CR with respect to lifespan (Sun et al. 2009). In addition, the study assessed effects on mRNA expression, cellular stress proteins and mTOR pathway enzymes. They included four groups in this study; CON for the Meth-R group (CON-Meth) with a diet of 0.43% methionine by weight, methionine restricted group (Meth-R) with a diet of 0.15% methionine. For the comparison of Meth-R with CR on cellular mechanisms, both an *ad lib* fed CON group (CON-CR) and a CR group was also created. The CR group received a diet with a 40% reduced energy content compared to CON-CR. All

interventions were initiated at age of 1 year, but only mice in the CON-Meth and Meth-R groups were maintained throughout their lives to obtain lifespan data. This study also had a cohort exploring effects of early life (first 20 days) CR, found to increase lifespan, that will not be further discussed here.

Meth-R increased median lifespan compared to CON-Meth but this increase was not significant (1 011 days compared to 948 days). Nonetheless, survival of Meth-R was significantly better than for CON-Meth (by log-rank test, p = 0.02). Also, survival at age 1 175 days (10% max lifespan of pooled population) was significantly higher (p= 0.01) for Meth-R, with 19% still alive compared to CON-Meth with only 2% still alive. No lifespan data on the CR groups exist for comparison as these were only maintained to 18 months of age.

Many of the various outcomes examined in this study are signaling pathways and mechanisms linked to the aging process. Meth-R was found to result in many of the same characteristics as CR in terms of lower serum IGF-1, insulin, thyroxin, glucose levels and increased resistance to hepatic acetaminophen toxicity.

The mRNA expression in liver was examined to reveal potential similarities or differences between CR and Meth-R. Differences in expression between CON-Meth and Meth-R were compared to those of CR and CON-CR. No common effects on gene expression were identified, indicating that the mechanisms of effect is mostly distinct. Only three genes were expressed significantly different between Meth-R and CON-Meth, and these effects were not the same as observed with CR. IGFBP1 was significantly lower in Meth-R but significantly higher in CR compared to each respective CON group. Adm2 (involved in cardiovascular homeostasis and anti-diuresis) was significantly higher in Meth-R, but significantly lower in CR. The third gene, Bid (pro-apoptosis) was significantly lower in Meth-R but not affected in CR. Among several other findings with CR, not present in Meth-R, was increased expression of FOXO1, Sirt1 and Brca1.

Phosphorylation levels of Erk, Jnk2 and p38K, three enzymes associated with cellular responses to injury were also compared between Meth-R, CR and their respective CON groups. Phosphorylation levels of all three enzymes were significantly higher with CR (in the 2-fold to 3-fold range), but only Jnk2 was slightly but significantly increased in Meth-R.

Lastly, this study also explored the phosphorylation levels of three key enzymes related to the mTOR pathway. Phosphorylation levels of PKB/Akt were significantly reduced with both CR and Meth-R. Phosphorylation levels of the mTOR protein and the downstream mediator 4E-BP1 were significantly reduced by more than 50% with CR, but not with Meth-R.

Included studies on effects of methionine restriction (Meth-R) in rats (n = 2)

Orentreich et al. (1993)

Orentreich et al. (1993) examined the effects of lifelong Meth-R on lifespan of male Fischer 344 rats. These rats were randomized to Meth-R or CON groups from 5 weeks of age. The Meth-R group received a diet consisting of 0.17% methionine by weight compared to 0.86% methionine for the CON group. For the Meth-R group the restricted methionine was replaced by glutamic acid. Both groups were fed *ad lib*.

Meth-R was found to increase median lifespan from 818 to 1059 days and absolute max lifespan from 1116 to 1252 days. Although none of these differences are mentioned to be significant. Among other findings, Meth-R resulted in severely reduced body weight compared to the CON group. The Meth-R group also had a slightly lower food intake than the CON group when expressed by weight of food, although when expressed by weight of food per unit of body weight, the Meth-R group had a higher food intake. It was also reported that when methionine content was restricted to 0.12% in a pilot study, all rats died within one month.

In summary Orentreich et al. (1993) found that Meth-R increased both median and absolute max lifespan in male rats. This effect was not explained by lower food intake, but body weight was severely reduced by Meth-R.

Zimmerman et al. (2003)

Zimmerman et al. (2003) used a study design fairly similar to what was used in the previous study by Orentreich et al. (1993). Effects of lifelong Meth-R with 0.17% methionine was compared to CON group with a 0.86% methionine diet. This study used various cohorts of male Fischer 344, Brown Norway, Sprague Dawley and Wistar Hannover rats that were randomly assigned to Meth-R or CON groups. Among the Fischer 344 rats was also one CON group pair fed with the same caloric intake as Meth-R. All other dietary groups were allowed to feed *ad lib*. All interventions started from age 6-8 weeks.

Several interesting results can be seen from this study, but most of these are showed by survival curves with few specified survival number, and no testing for statistical significance. Nonetheless, several of these data indicated that lifespan is increased with Meth-R.

Survival of Meth-R was reported to be increased by 42% compared to *ad lib* CONs. Similarly max lifespan was reported to be increased by 44%, although the referred figure by Zimmerman et al. (2003) does seems to contradict this claim. Another cohort of Fischer 344 rats also included a CON group fed the same absolute amount of calories as the Meth-R group. From this cohort it is apparent that Meth-R increased lifespan and there were no major differences in survival between the *ad lib* fed, and isocaloric pair-fed CONs. Meth-R improved survival curves in all four strains of rats tested, to a huge extent in Brown Norway rats but only to a small extent in the three other strains.

In summary, this study by Zimmerman et al. (2003) indicated that Meth-R increase lifespan in all four different strains of rats tested, and that this is not explained by the lower caloric intake in Meth-R rats. It should be noted that none of the findings reported in this study are challenged statistically and few are highlighted with comparable numbers. A summary of all studies on effects of Meth-R is shown in table 14.

Table 14: An overview of results from studies on effects of methionine restriction (Meth-R) in rodents.

	Author and (year)	Study design	Miscellaneous outcomes	Effects on lifespan
Mice	Sun et al. (2009)	Male hybrid mice between C57BL/6 and BALB/c (CB6F1). Meth-CON: 0.43% methionine. Meth-R: 0.15% methionine. CR: 40% CR-CON: *ad lib*	*Meth-R and CR reduced serum IGF-1, insulin, thyroxin, glucose and acetaminophen toxicity. mTOR signaling weakly inhibited by Meth-R and strongly inhibited by CR*	*Meth-R significantly increased overall survival and 10% max survival, but not median survival*
Rats	Orentreich et al. (1993)	Male Fischer 344 rats. Onset from age 5 weeks. Meth-R: 0.17% methionine *ad lib*. CON: 0.86% methionine *ad lib*	*Effects of Meth-R not explained by food intake. Abolished growth with Meth-R. Early death with Meth-R levels of 0.12%*	*Meth-R increased median and max lifespan (not significant)*
	Zimmerman et al. (2003)	Cohorts of male Fischer 344, Brown Norway, Sprague Dawley and Wistar Hannover rats. Meth-R: 0.17% methionine *ad lib*. CON: 0.86% methionine *ad lib*. Another CON group isocaloric pair-fed with Meth-R	*Effects of Meth-R not explained by decreased food intake*	*Meth-R increased lifespan in all four strains of rats (not reported to be significant)*

Included studies on effects of tryptophan restriction (Trypt-R) in mice (n=1)

De Marte and Enesco (1985)

This study examined the effects of Trypt-R in male Swiss albino mice. Interventions were started at an age of 4 weeks when the mice were randomly placed in either Trypt-R or CON groups. CON diet contained 0.47% tryptophan compared to 0.08% for the Trypt-R group. Both groups were allowed to feed *ad lib*.

The survival curves from this study indicate that Trypt-R increased survival percentage at most ages. This effect was consistent from 300 days of age to 850 days of age. From an age of between 850 days and 1000 days, the survival percentages among the groups were fairly equal and sometimes higher for CON. Median survival was increased for the Trypt-R group with 683 days compared to 616 days for the CON group. Max 10% survival was slightly reduced in Trypt-R compared to CON. Absolute max lifespan was observed for Trypt-R with 1097 days compared to 1038 days for CON. None of these numbers are backed up by any statistical testing to highlight significance, although statistical testing is provided for other parameters. This study also found no differences in food consumption by food weight among the two groups, but Trypt-R animals weighted significantly less than CON.

All together, findings provided here by De Marte and Enesco (1985) indicate that Trypt-R increases lifespan in mice, but none of the findings are reported as significant. Trypt-R did increase both median and absolute max lifespan, but not 10% max survival.

Included studies on effects of tryptophan restriction (Trypt-R) in rats (n=2)

Segall (1977)

Segall (1977) tested the effects of Trypt-R in rats (strain is not reported) on various cohorts with different ages of onset and durations of the intervention. From this study, it would have been appreciable to have the design of the study described in more details (dietary tryptophan levels, CON diet etc.), although the results will still be briefly included here. Most of the cohorts used appears to have been on a Trypt-R diet only for a duration of 6-22 months, and not maintained throughout their lives.

Survival curves were generated from combined values for interventional onset of 21 days and 3 months with a fairly low number of animals (n=8) compared with a similarly small CON group (n=12). Average lifespan was reported to be (not significantly) increased with Trypt-R,

as average lifespan for Trypt-R was 36.31 months compared to 30.25 months CON. Absolute max lifespan for the Trypt-R rats was 1387 days compared to 1266 days for CON. Among other outcomes mentioned with Trypt-R, were a delay in the onset of tumors and improved stress-induced thermoregulatory abilities.

Ooka et al. (1988)

In this study, Ooka et al. (1988) tested the effects of Trypt-R in female Long-Evans rats. The rats were placed (not mentioned to be randomized) in either of four different dietary groups at 21 days of age. Two CON groups were used, one of these received regular (Purina) rat chow *ad lib*, and the other received a fixed caloric intake of a low tryptophan diet supplemented with additional tryptophan to obtain normal levels. The two Trypt-R groups were fed diets restricted by 60% or 70% tryptophan compared to the fixed calorie CON group. No differences between any parameters on the two CON diets were found and these groups were therefore submerged into one single CON group.

Survival was increased with the degree of Trypt-R for both median, 25%, 10% and max survival. Median survival was 712, 850 and 1050 days for CON, 60% Trypt-R and 70% Trypt-R respectively. Similar numbers for 10% survival were 954, 1125 and 1330 days and max lifespan were 1246, 1347 and 1527 days for CON, 60% Trypt-R and 70% Trypt-R respectively. These numbers are reported with the exclusion of all rats dying before one year of age. This is interesting as Trypt-R actually decreased survival at both one, and two years of age. Both one-year survival and two-year survival were highest in the CON group and lowest in the 70% Trypt-R group. From three years of age, this trend was completely reversed, with CONs having the lowest survival and 70% Trypt-R the highest. None of the survival data referred to above were specifically mentioned to be significant.

This study also found that both growth and body weight were substantially reduced in the Trypt-R groups, with the reduced growth rate corresponding to the level of tryptophan in the diet. In addition, histological biomarkers of aging were reported to be delayed with Trypt-R in liver, heart, uterus, ovary, spleen and adrenal gland, but not in kidney, lung and aorta. Brain serotonin levels were also found to be lower in the Trypt-R groups. The main findings from of all studies on effects of Trypt-R are highlighted in table 15.

Table 15: Results from studies on effects of tryptophan restriction (Trypt-R) in rodents.

	Author (year)	Study design	Miscellaneous outcomes	Effects on lifespan
Mice	De Marte and Enesco (1986)	Male Swiss albino mice. Trypt-R: 0.08% tryptophan *ad lib*. CON: 0.47% tryptophan *ad lib*	*Trypt-R did not affect food consumption, but Trypt-R mice weighted significantly less*	*Trypt-R increased median and max lifespan (not reported to be significant) but not 10% max lifespan*
Rats	Segall (1977)	Trypt-R: 6-22 months duration, not lifelong, compared to CON group. Several aspects of study design missing	*Delay in onset of tumors*	*Trypt-R increased average lifespan and max lifespan (not reported to be significant)*
	Ooka et al. (1988)	Female Long-Evans rats. CON: *ad lib* group and fixed calorie group. Trypt-R groups of 60% or 70% restriction compared to CON	*No differences between ad lib and fixed caloric fed CON. Decreased growth rate and body weight with Trypt-R, but also delaying of histological biomarkers of aging in several organs*	*Trypt-R increased median, 25%, 10% and max lifespan directly related to the level of restriction (not significant). Trypt-R decreased survival up to two year of age*

An overview of effects on lifespan for all studies on rodents can be seen in table 16 and table 17. Among these studies on rodents 75% (6 out of 8) showed overall positive effects of PR on lifespan, but only 50% (4 out of the total 8) reported PR to significantly increase median lifespan. Among these was also one resulting in negative effects on lifespan. For both Meth-R and Trypt-R, 100% of the studies (3 of 3 for each intervention), reported overall positive effects on lifespan, but none of these were reported as significant for median lifespan.

Table 16: An overview of overall effects on lifespan in all included studies of PR or AA-R in rodents.

		Author (year)	Overall effects of PR/AA-R on lifespan	Overall significant increase in median lifespan
PR	**Mice**	Fernandes et al. (1976)	*Positive*	*No*
		Leto et al. (1976)	*Positive*	*Yes*
		Stoltzner (1977)	*Positive*	*Yes*
		Solon-Biet et al. (2014); Solon-Biet et al. (2015)	*Positive*	*No*
	Rats	Anantharaman (1983)	*No effect*	*No*
		Davis et al. (1983)	*Negative*	*No*
		Yu et al. (1985)	*Positive*	*Yes*
		Horakova et al. (1988)	*Positive*	*Yes*
Meth-R	**Mice**	Sun et al. (2009)	*Positive*	*No*
	Rats	Orentreich et al. (1993)	*Positive*	*No*
		Zimmerman et al. (2003)	*Positive*	*No*
Trypt-R	**Mice**	De Marte and Enesco (1986)	*Positive*	*No*
	Rats	Segall (1977)	*Positive*	*No*
		Ooka et al. (1988)	*Positive*	*No*

Table 17: Percentage of studies reporting overall positive effects or significantly increased median lifespan with PR or AA-R in rodents.

	% of studies reporting overall positive effects of on lifespan	% of studies reporting overall significant increase in median lifespan
PR	75% (6 of 8)	50% (4 of 8)
Meth-R	100% (3 of 3)	0% (0 of 3)
Trypt-R	100% (3 of 3)	0% (0 of 3)

3.2.3 PR in humans

Only three studies on effects of PR or AA-R in non-rodent mammals were identified in the systematic search described in chapter 2. These were all studies exploring the effects of PR in humans, and only one of these was a population study with lifespan as an outcome. Table 18 gives an overview of the results from these three studies of PR in humans.

Blum et al. (1989)

This study by Blum et al. (1989) explored the relationship between protein intake and kidney function in humans. As mentioned in chapter 2, an exclusion criteria used in the systematic review, was the treatment of kidney disease as outcome. As this study by Blum et al. (1989) was exploring the effects on respect to kidney function as a measure of normal aging in previously healthy individuals it does not fall within the mentioned exclusion criteria.

In this observational cross-sectional study, a group of healthy vegetarians, considered protein restricted was compared to a healthy CON group mainly made up of medical staff. The vegetarian PR group reported by dietary assessment a diet of maximum 30g protein (plant-based) per day, estimated to provide 5-6% energy from protein. Subjects had maintained the diet for an average of 13.3 years. The CON group reported intakes of minimum 100g protein per day, estimated to provide 16-20% of energy. Kidney function was measured as creatinine clearance, but no significant differences were found among the groups.

It should be expected that the choice of vegetarians as a measure of PR and medical staff as CONs in this study, could result in severe selection biases. Yet, both groups could be expected to have a healthier diet and lifestyle compared to the general population, perhaps leading to equal biases in the same positive direction for both groups.

Overall, Blum et al. (1989) showed that PR was not associated with any effects on kidney function when examined in healthy subjects with vegetarian diet as a measure of PR.

Fontana et al. (2008)

This study reported the effects of both individual CR and PR on serum concentrations of IGF-1 and IGFBP-3 in humans (Fontana et al. 2008). Thus, this study tried to explore the effects of CR and PR on the upstream activators of the GH/IGF-1 signaling pathway. These data consisted of reports from two separate long-term studies on CR, and CR compared to PR respectively. The CALERIE research program previously mentioned in chapter 3.1.3 provided some of the data on effects of CR.

The first study was the one-year long CR study by Fontana et al. (2007) described in chapter 3.1.3. In this randomized controlled trial, no significant differences in IGF-1 or IGF-1: IGFBP-3 ratio were found within or among any groups. A slight increase in IGF-1 was seen for CR compared to baseline, while both the exercise and CON groups achieved slightly decreased IGF-1 levels. Differences for IGFBP-1: IGFBP-3 ratios were barely visible for CR and CON group but slightly increased for the exercise group.

The second study was an observational cross-sectional study with both a CR, PR and CON group. The CR group consisted of participants (86% males) recruited through a CR society, that had practiced CR for an average of 6 years. PR group consisted of vegetarians recruited through a vegetarian society and these had maintained a vegetarian diet for an average of 2 years, with a low protein diet from plant-based sources only. Last group consisted of healthy non-obese volunteers. All groups were age-matched and consisted of individuals aged 53 $\pm$ 12 years. Reported average dietary intakes for the PR, CR and CON groups were 1980 kcal, 1772 kcal and 2502 kcal and 9.6%, 23.5% and 15.9% of the energy from protein respectively. The macronutrient data show that the PR group was also caloric restricted as their energy intake did only differ significantly from CON and not the CR group. For the CR group, the average degree of CR was 30% compared to CONs.

For this cross-sectional study, both serum IGF-1 and IGF-1: IGFBP-3 ratio were significantly lower for the PR group compared to either CR or CON. Both insulin and CRP were also significantly reduced in both PR and CR groups compared to CON, with reductions of more than 50%. At the end of the cross-sectional study, six CR participant also volunteered to reduce their protein intake from 1.67g / kg body weight per day to 0.95g / kg body weight per day for three weeks. This did in fact lead to a 25% reduction in IGF-1, although not specified to be significant.

It should also be noted here that the participants of both the CR group and the vegetarian group are likely to be biased towards a healthier lifestyle and diet (apart from only total calories and protein intake), especially compared to the CON group, but perhaps not on respect to each other.

All together, these findings by Fontana et al. (2008) indicate that 20% CR does not affect IGF-1: IGFBP-3 ratio. Although, a separate study showed that PR subjects had significantly reduced IGF-1: IGFBP-3 ratio, while both PR and CR (30%) subjects had significantly reduced serum insulin and CRP.

Levine et al. (2014)

This publication examined the effects of protein intake on mortality and aging related diseases in an observational prospective study on humans and explored these same effects with cohorts of mice (Levine et al. 2014).

In the observational prospective study, the population was a sample of more than 6000 individuals from the NHAMES III populational study. These subjects had an average intake of approximately 1800 kcal per day, with the energy contribution being 51% from carbohydrate, 33% from fat and 16% from protein. 11% of the total energy was from animal-based protein sources. Protein intakes were classified as high, medium or low, with high being more than 20% energy from protein, and low being less than 10% energy from protein. All subjects were above 50 years of age with an average of 65 years. Nutrient intakes were reported by 24-hour dietary recall. These individuals were prospectively followed for 18 years.

For individuals in the range of 50 to 65 years of age, the HR of death from all-cause mortality was 1.34 for the moderate protein group and 1.74 for the high protein group compared to the low protein group. This HR was only significantly higher for the high protein group. For the age groups above 66 years of age the association was reversed with both medium and high protein intakes significantly associated with decreased HR for death. HR for death was 0.79 and 0.72 for the moderate and high protein groups respectively. When adjusted for protein from animal-based sources, none of the associations mentioned above remained significant. This is highly interesting as it indicates that much of the association between protein intake and mortality could be explained by intake of animal-based protein alone.

No significant associations were found for effects of protein intake on HR for CVD mortality, but a trend for the old age group was that high protein intakes also here decreased the HR. For cancer mortality, the HR for death was significantly higher for both moderate and high protein intake. This association for increased cancer mortality was reversed in the over 65 years of age group with high protein intake being associated with significantly reduced HR. All these three significant associations remained after adjustment for animal-based protein. Diabetes mortality followed a trend of increased HR with increased protein intake across both age groups, although this association was only significant for high protein intake in the above 66 years of age group.

Serum IGF-1 levels were also compared between the three protein groups and across the two age groups from a smaller cohort of individuals. IGF-1 levels were significantly higher in the high protein group compared to the low protein group, but only in the 50-65 age group. No other significant associations were found on respect to IGF-1 levels, although a consistent trend was increased IGF-1 levels with increased protein intake across both age groups.

The effects of various dietary protein levels were also tested in mice to see if these result replicated those associations that were found in humans. Various strains of mice received either a diet with 18% or 4% protein from animal-based sources. None of these mice cohorts were maintained throughout life to assess mortality data. These mouse studies still confirmed a trend of increased tumor incidence, tumor volume, higher IGF-1 and lower IGFBP-1 with high protein. These mouse studies also explored the effects of protein source on IGF-1 and IGFBP-1 levels. Both high casein and high soy protein diets significantly increase IGF-1 and decreased IGFBP-1 levels compared CON mice. No differences were found between the high soy and high casein diets, suggesting perhaps that any possible effects on mortality from animal-based proteins might not be mediated through biological activity of IGF-1.

All together, this comprehensive study by Levine et al. (2014) provided several interesting findings. PR was significantly associated with lower all-cause mortality in the 50-65 year age group. Oppositely, for the 66+ age group, PR was significantly associated with increased all-cause mortality. These associations of protein intake on all-cause mortality were especially linked to intake of animal-based protein alone. PR was significantly associated with protection against cancer mortality in the 50-65 age group, but oppositely associated with

increased mortality in those aged 66+. High IGF-1 activity was linked to high protein intake, but in mice, IGF-1 activity was not explained by source of protein. Studies on mice cohorts generally supported most other findings.

Table 18: The main results from all non-rodent mammalian studies on effects of PR or AA-R on lifespan and all outcomes relevant to aging. All the identified studies explored effects of PR in humans.

Author and year	Study design	Effects on lifespan or miscellaneous outcomes
Blum et al. (1989)	Observational, cross-sectional. PR: healthy vegetarians, maximal 30g (5-6%), plant-based protein per day. CON: healthy medical staff, minimum 100g (16-20%), protein per day	*No association between protein intake and kidney function*
Fontana et al. (2008)	Observational, cross sectional. CR: 30% average, 23.5% energy from protein. PR: vegetarians, 9.6% energy from protein. CON: 15.9% energy from protein Randomized controlled trial: 20% CR versus CON	*PR but not CR was significantly associated with reduced serum IGF-1 and IGF-1: IGFBP-3 ratio. Both PR and CR significantly associated with reduces serum insulin and CRP*
Levine et al. (2014)	Observational, prospective. High protein group: more than 20% energy from protein. Medium protein group: 10-20 % energy from protein. PR (low protein group): less than 10% energy from protein. Age groups: 50-65 years and 66 years+	*PR significantly associated with lower all-cause mortality in the 50-65 years age group. In the 66+ age group, PR was associated with significantly increased all-cause mortality. Associations on all-cause mortality linked to intake of animal-based protein alone. PR significantly associated with protection against cancer-mortality in the 50-65 age group, but oppositely associated with increased mortality in those aged 66+. High IGF-1 activity linked to high protein intake, but in mice, IGF-1 activity was not explained by source of protein*

3.2.4 Summary and answer to thesis aim two

Out of the included studies on effects of PR in rodents in chapter 3.2.1, 75% reported overall positive effects on lifespan, but only 50% reported significant increases for median lifespan. From the included studies on both Meth-R and Trypt-R from chapter 3.2.2, 100% of the studies reported overall positive effects on lifespan, although none of these were significant for median lifespan. Overall, the findings from the systematic search indicate a strong trend

of increased lifespan with PR, Meth-R and Trypt-R in rodents, but sample size of uncovered studies is too low to make definite conclusions.

The results from chapter 3.2.3 show strong for health benefits of PR in humans, but somewhat surprisingly the one study with effects on lifespan indicates that PR is only beneficial before an age of 65 years and may have a negative contribution at older age. Although population studies like this are often statistically superior to establish strong associations, they cannot confirm the precise cause and effect relationship to the same extent as prospective randomized controlled trials, and these results need to be regarded in this context.

From the summary above, thesis aim number two can be answered:

- Can PR (including AA-R) provide increased lifespan or improved health at old age in mammals and especially humans?
  - In rodents, PR, including AA-R, seem to provide increased lifespan and improved health at old age. For humans, PR might also provide these positive effects on lifespan and health at old age, but the PR regimen may have a negative contribution after a certain age, and should perhaps not be maintained throughout life.

3.3 Discussion and comparison of CR and PR

3.3.1 Mechanisms of CR and PR

Several of the studies examined in this thesis provide valuable insight into the effects and possible mechanisms of CR and PR in mammals. As described in chapter 1.3 and 1.4 the mechanisms and pathways expected to play a major part in the effects of CR and PR are widely complex. As the nutrient sensing pathways provide a link between the interventions in question and the observed effects on health and lifespan these have been given a special focus. As expected the GH/IGF-1, mTOR, Sirtuin and AMPK signaling pathways seem to be the main pathways mediating these effects. However, effects on fundamental mechanisms of aging should also be expected to be influenced by other independent signaling pathways. Still, the fact that these nutrient sensing pathways act as cellular "switches" to control a wide range of cellular functions, and are expected to be the major pathways regulating longevity, makes them ideal targets to be monitored in aging-related interventions. Table 19 provides a detailed insight into the effects of PR and CR on the previously mentioned signaling pathways. These findings are based on the studies identified in chapter 3.1 and 3.2.

From table 19, it can be seen that CR shows consistent[6] and strong effects on downregulation of the GH/IGF-1 pathway. Several studies show reduced GH/IGF-1 or increased IGFBP leading to lower GH/IGF-1 to IGFBP ratio and lower activation of this pathway. In addition, several downstream mediators such as PI3K, PKB/Akt, AC, and PKA are downregulated, as well as increased activity of the FOXO transcription factor expected to mediate pro-longevity effects. One out of many possible pro-longevity effects of increased FOXO activity is increased expression of the telomere-protective shelterin protein POT1a (Ye et al. 2014). FOXO activity is also strongly linked to several of the fundamental mechanisms of aging such as autophagy, oxidative stress resistance and stem cell function (Martins et al. 2016).

[6] Specifically, increased PI3K expression as shown in table 19 do not fit with the pattern described above. Nijland et al. (2007) reported this finding from a study of maternal CR baboon fetuses. Although findings were compared to fetuses from normal caloric mothers, it could possibly influence several pathways different from CR initiated after the pregnancy or neonatal period.

Table 19: A detailed overview of the effects on signaling pathways of CR and PR identified in this thesis. Due to the complex nature of these pathways, some details have been excluded for simplicity and increased clarification.

	Pathways influenced by CR	Pathways influenced by PR
Rodents	GH/IGF-1 pathway signaling: • Reduced GH and IGF-1 (Breese et al. 1991) and increased IGFBP (Sun et al. 2009) • Reduced PI3K, PKB/Akt, AC, PKA, and increased FOXO (Cheng et al. 2014; Sun et al. 2009) • Increased FOXO expression and activity (Cheng et al. 2014; Sun et al. 2009) mTOR pathway signaling: • Decreased mTOR and S6K activity (Cheng et al. 2014) • Reduced 4E-BP phosphorylation (maintaining eIF-4E inhibition) (Sun et al. 2009) Sirtuin signaling: • Increased Sirt1 (Sun et al. 2009)	GH/IGF-1 pathway signaling: • Reduced IGF-1 and increased IGFBP-1 (Levine et al. 2014) Meth-R only: • Reduced IGF-1 and IGFBP (Sun et al. 2009) • Reduced PKB/Akt activity (Sun et al. 2009) mTOR pathway signaling: • Lower BCAAs and lower mTOR activity (Solon-Biet et al. 2014)
Non-humans primates	GH/IGF-1 pathway signaling: • Reduced IGF-1, and increased IGFBP (Li et al. 2009) • Upregulated PI3k gene expression (Nijland et al. 2007) • Reduced PKB/Akt activity (Li et al. 2009) mTOR pathway signaling: • Reduced mTOR activation, mTOR normally activated by hormones, growth factors, AAs and PKB/Akt (Nijland et al. 2007) • Downregulated S6K and eIF-4E gene expression (Nijland et al. 2007) Sirtuin signaling: • increased SIRT1 activity (Qin et al. 2006)	No data identified
Humans	GH/IGF-1 pathway signaling: • Increased IGFBP-1, reduced IGF-1: IGFBP-1 ratio, no effects on IGF-1 or IGF-1: IGFBP-3 ratio (Fontana et al. 2016) • Downregulated PI3K and PKB/Akt expression (Mercken et al. 2013) • Increased FOXO expression (Mercken et al. 2013) Sirtuin signaling • Increased SIRT2, SIRT4, SIRT5 expression (Mercken et al. 2013) • Upregulated PGC-1α expression (Mercken et al. 2013) AMPK signaling: • Increased AMPK expression (Mercken et al. 2013) • Upregulated PGC-1α expression (Mercken et al. 2013)	GH/IGF-1 pathway signaling: • Reduced serum IGF-1 (Fontana et al. 2008; Levine et al. 2014) • Reduced IGF-1: IGFBP-3 (Fontana et al. 2008)

PR, similar to CR, also seems to downregulate the GH/IGF-1 pathway, but the only evidence uncovered here are lower IGF-1 and increased IGFBP levels, leading to lowered upstream activation of the pathway. It can be expected that this downregulation of GH/IGF-1 signaling by PR is quite strong, but not to the same extent as for CR.

CR seems to strongly downregulate the activity of the mTOR pathway. Lower mTOR activation does also appear to be the case for PR. As high AA levels are consistently highlighted as one of the strongest activators of this pathway, this effect can be expected to be comparably strong (and cumulative) for both CR and PR.

Also highly interesting, CR seems to increase the activity of AMPK signaling and the activity of specific sirtuins with increased PGC-1α activity (and mitochondrial biogenesis) as one of the downstream effects. Recently, activation of AMPK has also been linked to increased mitophagy and improved muscle function in rodents (Ryu et al. 2016). Collectively, improved regulation of mitochondrial function could be one of the major downstream effects of increased sirtuin and AMPK signaling. Still, sirtuin and AMPK signaling activated with CR are unlikely to contribute to pro-longevity effects in the same extent as downregulated GH/IGF-1 and mTOR signaling. No studies examined in this thesis have uncovered any effects of PR on sirtuin or AMPK signaling, and this could likely explain some of the stronger observed effects with CR compared to PR as shown in the next subchapter.

The expected major overall effects of CR and PR on these pathways are shown in table 20. No other major pathways expected to regulate longevity to the same extent as these four pathways have been identified in the work with this thesis.

Table 20: The effects of CR and PR on the four major nutrient sensing pathways.

Signaling pathway	CR	PR
GH/IGF-1 signaling	↓ ↓ ↓	↓ ↓
mTOR signaling	↓ ↓	↓ ↓
Sirtuin signaling	↑	
AMPK signaling	↑	

From table 20 it seems that CR and PR work through the downregulation of the two shared pathways of GH/IGF-1 and mTOR signaling. In addition, upregulation of the two additional signaling pathways, sirtuin and AMPK signaling seems to contribute to the effects of CR, and these are most likely not part of the mechanisms of PR.

As described by Mattison et al. (2012) it is expected that an intervention able to increase both average/median and max lifespan is likely to mediate this effect by influencing some of the fundamental mechanisms of aging described in chapter 1.3. In many studies identified in this thesis, CR and PR increase both median and max lifespan. In fact, the tendency is that the magnitude of increases on all parameters of max lifespan such as 25% max, 10% max or absolute max lifespan is stronger and more consistent than the effects observed on median lifespan. The results from the studies identified in this thesis and the findings on mechanisms described here, therefore strongly support the idea that both CR and PR work through some of the fundamental mechanisms of aging. Next, figure 6 shows an overly simplified depiction to how CR and PR are expected to regulate lifespan and longevity. Knowledge about the precise mechanisms in many of the steps illustrated in figure 6 are currently limited.

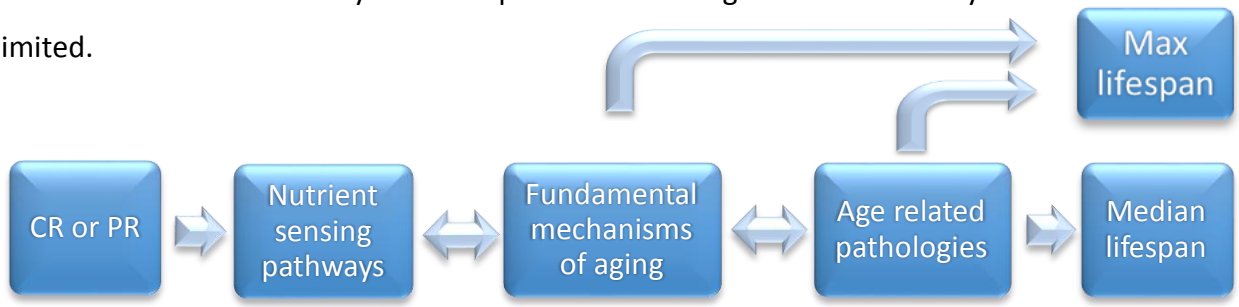


Figure 6: A depiction of how CR or PR is expected to influence lifespan and aging.

3.3.2 Comparison, summary and answer to thesis aim three

Chapter 3.3 aims to explore if PR can provide the same effects as seen with CR. An aim of this chapter was also to explore if the concomitant PR that often follows with CR studies could cause the effects reported by CR. Some studies identified here provides valuable comparative insight, with PR and CR tested in the same setting. As shown in table 21, out of the included studies of PR in rodents, six of these compared the separate effects of CR and PR. Five out of these six studies showed stronger effects by CR compared to PR, with only one study indicating the opposite conclusion.

Although both CR and PR show great promise in increasing lifespan and health for humans, findings in this thesis indicate that CR is likely to mediate a stronger effect than PR. These

findings are in line with those of Speakman et al. (2016) in the previously mentioned meta-analysis on rodents, who also reported much smaller impact from PR with 15% increased lifespan compared to 30% by CR. In contrast, a review by Le Couteur et al. (2016a) concluded that diets low in protein (PR) strongly increased lifespan in insects and mice, while total caloric intake (CR) had minimal effect. Le Couteur et al. (2016a) also concluded that this was indirectly supported by observational studies in humans. Findings in this thesis support that PR is likely to improve health and lifespan but the effects of CR in mammals identified here contrast the conclusions by Le Couteur et al. (2016a), although it could be argued that few observational studies of PR in humans were identified and included in this thesis. Importantly, effects on lifespan requires long follow up time in experimental settings, and this relationship is hard to establish in non-human primates and humans, while effects on other health outcomes show quite consistent positive effects for both CR and PR as these are easier to establish with shorter follow up.

Table 21: An overview of included studies comparing the effects of both individual CR and PR.

	Author (year)	Overall effects of PR on lifespan	Overall effects of CR on lifespan	Strongest effects on lifespan by CR or PR?
Mice	Fernandes et al. (1976)	*Positive*	*Positive*	*CR*
	Solon-Biet et al. (2014); Solon-Biet et al. (2015)	*Positive*	*No effect*	*PR*
Rats	Anantharaman (1983)	*No effect*	*Positive*	*CR*
	Davis et al. (1983)	*Negative*	*Positive*	*CR*
	Yu et al. (1985)	*Positive*	*Positive*	*CR*
	Horakova et al. (1988)	*Positive*	*Positive*	*CR*

Based on the identified studies in chapter 3.1 - 3.2 and the results summarized below, a comparison between the effects of CR and PR can be made as seen in table 22. There is strong evidence that CR can lead to increased lifespan in rodents. Well designed prospective studies show a positive trend on effects of CR in non-human primates, but the results are not consistent. Based on these findings in other mammals and short-term CR studies in humans, it is likely that CR might provide both improved health and increased lifespan for humans, although more studies are needed to establish this relationship precisely. PR in rodents as well as selective AA-R with tryptophan or methionine show positive trends of increased lifespans, although no definite conclusions can be drawn. PR in humans shows a

positive trend on both health and lifespan but only up to 65 years of age. Based on these findings on PR uncovered here, it is likely that PR might provide improved health and increased lifespan for humans, although the evidence is weaker than for CR.

Table 22: A comparison of the effects on lifespan by CR and PR identified in chapter 3.1 and 3.2.

Increased lifespan by CR or PR				
Species	CR	Quality of evidence	PR	Quality of evidence
Rodents	*Yes*	*Very strong: Two independent meta-analyses*	*Inconclusive but positive trend*	*Strong: 8 (14 including AA-R) prospective interventional studies with CON groups*
Non-human primates	*Inconclusive, but positive trend*	*Strong: Two prospective lifelong controlled trials*	*No data identified*	
Humans	*No data identified*		*Inconclusive, but positive trend (until 65 years of age)*	*Low: One observational, high age onset prospective study*

Whether the concomitant PR in CR studies could cause most of the observed effects, findings in this thesis do not indicate that to be the case. However, studies of CR that often also have a concomitant PR are likely to provide stronger effects compared to studies of CR where the protein intake is corrected to maintain a normal intake. Based on the mechanisms previously described, the effects of PR and CR should show a small degree of additive effects.

All together, the comparisons between CR and PR provides the ability to answer thesis aim number three:

- Can PR provide the same effects as seen with CR, and if so can the concomitant PR that often follows with CR studies cause the effects reported by CR?
  - PR seems to provide many of the same effects as seen with CR, but these effects appear to have a lower impact. Both CR and PR can lead to downregulated GH/IGF-1 and mTOR signaling, while only CR appears to give an activation of sirtuin and AMPK signaling. PR can possibly be the cause, or contributor to some, but not all the effects seen with CR.

3.3.3 Discussion of related topics

Influence of telomere attrition

When studying the aging process, conclusions drawn from laboratory model organisms might offer additional problems because of the differences in lifespan compared to humans (Blackburn et al. 2015). Some of the mechanisms expected to play a contributory role in the human aging process, such as telomere maintenance might not be relevant over the much shorter lifespan of many commonly used model organisms. For instance, rodents generally have high levels of telomerase, the telomeres in mouse chromosomes are about five times longer than in humans, and they typically die with intact telomeres. Most other short-lived laboratory animals also appear to have minimal telomere shortening during their lifetime as well.

If telomeres are shortened at the same constant rate in most species, possible effects of interventions on telomere shortening could more easily be revealed in longer-lived species such as humans or long-lived primates. This might be of importance in many model organisms where telomere shortening is unlikely to contribute to the aging process, when on the contrary, telomere shortening is believed to have an important impact on the aging process in humans (Alberts et al. 2015; Blackburn et al. 2015). However the report by Smith et al. (2011) from the NIA CR study on rhesus monkeys showed no effects on telomere shortening. But it should also be taken into account that the main results from the NIA study found only small effects of CR on their main parameters, perhaps due to unexpected long lifespan and good health of CONs. Seen in this context, a significant effect of CR on telomere shortening from the NIA study should not be expected either. However, it would have been interesting if the effects on telomere length by CR were reported in the WNPRC study where CR was found to have significant positive effects on lifespan/HR for death.

Source of protein

Some of the effects of PR might be highly dependent on source of protein as indicated by Levine et al. (2014), who showed that protein from animal-based sources explained much of the increased risk of diseases associated with high protein intake. Another study of CR on mice by Shimokawa et al. (1993) showed that replacing the casein-based protein source with a soy-based protein source markedly decreased the progression of neuropathy with increased age. A switch from casein-based protein source to a lactalbumin-based protein

source gave no difference on this specific parameter. If the pro-aging effects associated with high protein intake are mediated in part by animal-based protein alone, it could be expected that results of both CR and PR studies would vary greatly based on the choice of protein source, with studies based on plant-based protein sources getting smaller benefits of both interventions. This could also explain some of the opposing results from the NIA and WNPRC primate CR studies. If protein intake from animal-based sources alone could explain the pro-aging effects associated with high protein intake this also raises the possibility that other bioactive molecules in addition to AAs could play a major role in mediating this effect. Protein from animal-based sources contains more leucine compared to protein from plant-based sources and this could likely explain some of the pro-aging effects by increased activation of the mTOR pathway (van Vliet et al. 2015).

Protein supplementation and physical exercise

Another interesting aspect is the wide use of protein and BCAA supplements used in sports for its purported ability to aid muscle growth and recovery. According to the findings in this thesis, it is likely that high protein and BCAA intake can increase muscle growth and recovery from muscle injury by maintaining high activity of the GH/IGF-1 and mTOR signaling pathways. This effect would thus likely lead to negative long-term effects on lifespan and health. As physical exercise is associated with improved health, it is highly interesting that several of the nutrient sensing pathways associated with aging, are also strongly implicated as some of the molecular mechanisms in the response to exercise (Hoppeler et al. 2011). Low intensity, endurance type exercise has been described to be a strong activator of AMPK signaling and the downstream PGC-1α, while high-load, strength type exercise, induce muscle growth in part through increased activation of mTOR and GH/IGF-1 signaling (Hoppeler et al. 2011). The latter implies that strength type exercise would have a negative long-term impact on lifespan and health. This might partially be true, but it seems likely that any endurance type component of an exercise regime would activate AMPK and lead to overall positive benefits, possibly due to the ability of activated AMPK to inhibit mTOR signaling (Alers et al. 2012; Nijland et al. 2007).

Can CR be combined with exercise?

A relevant question is whether CR can be combined with the benefits of physical exercise. Essentially, this depends on whether the nutrient sensing pathways are responding to the

amounts of nutrients or energy availability only. It seems as if the regulation of GH/IGF-1 and mTOR pathways to a huge extent respond to the amount of macronutrient, and especially carbohydrates/glucose, AAs and insulin levels in addition to growth factors. Downregulation of these pathways are unlikely to be achieved in the same extent for individuals combining physical exercise with CR, compared with the lower calorie intake of CR alone. However, as AMPK responds to intracellular energy levels, and is known to be activated by endurance type exercise, this activation can likely be achieved with combined exercise and CR. This activation of AMPK can possibly inhibit mTOR and indirectly downregulate this pathway. Low energy availability might also be able to activate sirtuins (Jiang et al. 2013). In addition, if post-exercise cellular energy levels are only restored at a slow rate following several hours, this is likely to provide stronger beneficial effects on these two pathways. In fact, a new area of focus in sports nutrition is the potential applications for training in a low glycemic state, possibly combined with slow post-exercise carbohydrate-refueling, to more strongly activate AMPK and related signaling pathways in order to induce stronger adaptations (Bartlett et al. 2015).

Homocysteine

Homocysteine metabolism has also been implicated in several age related diseases such as CVD and neurodegenerative disorders/cognitive decline (McCully 2015). Cysteine restriction was not included in the search query used in chapter 3.2 because early attempts to construct the systematic search resulted in no additional records. It seems likely that cysteine intake alone only has a minor role in this context and that dietary methionine along with folate/B_6 and cobalamin/B_{12} might have bigger impacts. Effects on homocysteine metabolism mediated by methionine could likely be one of several possible mechanisms for the pro-longevity effects seen with Meth-R.

Evolutionary aspects of changing nutrient availability

As illustrated in figure 7, activity of the GH/IGF-1 and mTOR pathways is likely to be involved in the trade of regulation between longevity and protection against age-related pathologies versus growth, reproductive function, wound healing and acute immune function (Grandison et al. 2009). From an evolutionary point of view, it is easy to understand why the latter functions clearly have been selected for and prioritized more than increased lifespan and protection against age related pathologies. It also shows that the pro-aging pathways

associated with CR and PR regulate many vital functions, and that these pathways need to maintain some activity to even reach an age where the typical age related pathologies could be lethal. After all, without modern medicine and protection from predation and starvation, individual members of a species rarely live longer than their reproductive period, meaning that selective pressure would select for high activity in these pathways because of their strong effect on growth and reproduction, leading to increased fitness (Goodell & Rando 2015). This is also supported by the findings of Solon-Biet et al. (2015) who found that diets with a high protein and low carbohydrate ratio lead to the greatest reproductive function while the opposite diets maximized longevity.

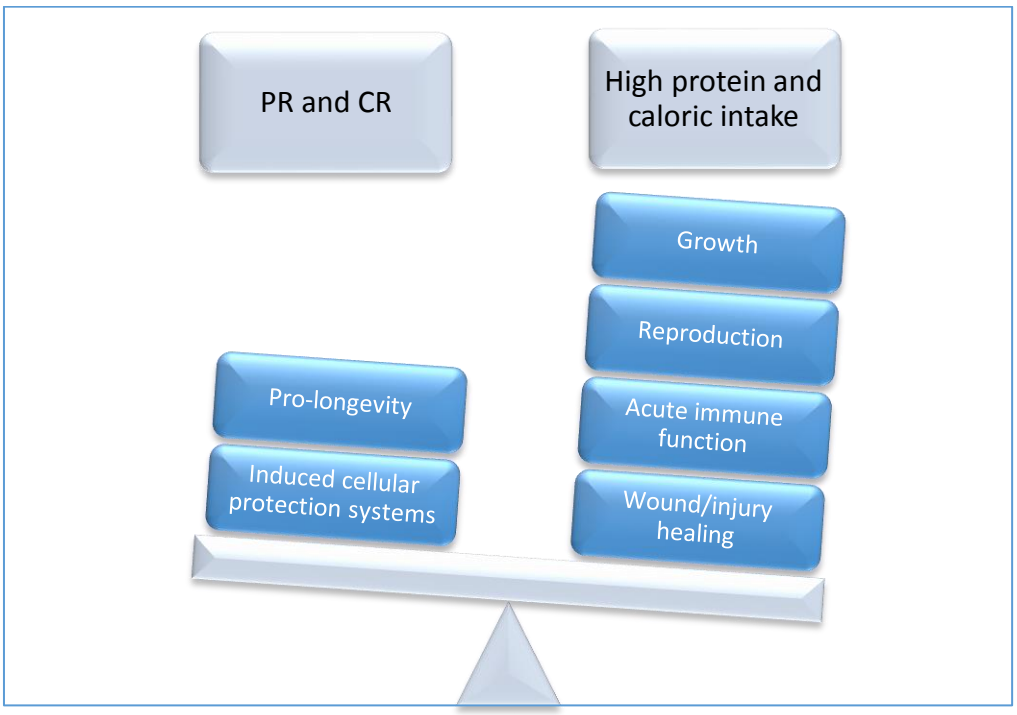


Figure 7: Intake of total calories and protein stimulate activity of the GH/IGF-1 and mTOR pathways regulating the balance between longevity and vital functions such as growth, reproduction, acute immune function and wound healing.

For evolutionary survival, available recourses for an organism are used for either growth/survival or reproduction (Kirkwood 2005). As mentioned in chapter 1.4 if the nutrients (calories and protein) required for growth and reproduction are too low, the best adaptive mechanism for evolutionary survival is to induce cellular protection systems for survival until a later point where enough nutritional resources for growth and reproduction becomes available. This concept does to a huge extent explain why the GH/IGF-1 and mTOR pathways normally maintain high activity despite their detrimental pro-aging effects, and why pro-longevity effects are only induced in times of low energy or nutrient availability.

3.3.4 Future perspective

Although CR shows consistent increases in lifespan for rodents in experimental settings, these results might not be representative for rodents as a whole group. The high degree of variations in the magnitude of effects between rats and mice and across different strains of the same species, open up the question that genetic variation could play a major role in mediating the effects of CR through gene-diet interactions. This could likely be the case for PR as well. Research exploring the possible genetic mechanisms to this effect is warranted. Needed are also studies on CR and PR in rodent strains with a high degree of genetic diversity. The use of highly inbred strains with low genetic diversity for CR and PR studies might produce falsely positive results if strains known to give positive results tends to be chosen for these types of experiments.

It has been shown that in some experimental settings, CR can provide improved health and increased lifespans for non-human primates. One question that remains to be answered is why these effects are smaller or even absent in different settings. In-depth analysis of the possible variables leading to differential outcomes in the WNPRC and NIA prospective primate CR studies should be used to guide future research in the right direction.

Initiation of more prospective non-human primate studies on effects of CR or PR are urgently needed. These studies are costly and time consuming (usually 25 years or more), but gives some of the best possible insight into the complex effects and mechanisms relevant to humans. Ideally, for many mammalian species, the effects of both CR and PR alone should be examined with respect to natural based or synthetically based diets, sucrose content and protein sources, composition and content. Effects of these variables should preferably be explored in the same experimental environment. In addition, studies with cohorts of animals with different genetic origin would also provide valuable insight. Furthermore, effects of PR should also be examined both isolated and in combination with CR, to differentiate the effects of these interventions with each other.

It would also be of high interest to examine the effects of animal-based protein sources with one group receiving natural proteins from animal-based sources, and another group receiving the same protein content and composition, isolated from the same sources, but purified for other bioactive components. This would allow the exploration of the interesting

possibility that other bioactive molecules could be responsible for some of the detrimental effects associated with high protein diets.

Supported are also future research on effects of CR and PR in humans to be conducted as randomized controlled trials based on the design from the CALERIE research program. These interventions in humans need to be conducted with safety as a first priority and therefore only low degree CR or PR can be applicable in the nearest future. Long-term follow up on randomized controlled trials of CR or PR in humans are needed to possibly uncover effects on lifespan and pathologies.

In general, much research needs to be conducted on the fundamental mechanisms of aging. Understanding of how these mechanisms are influenced by the nutrient sensing pathways and other factors are strongly warranted. In addition, knowledge of how these mechanisms can interact or regulate different pathologies would be of great interest. This insight is needed to understand precisely how PR and CR provide effects on aging and lifespan, and it could help the identification of better biomarkers to measure interventional effects at an early stage. This insight would also accelerate the already progressing work with pharmacological interventions or CR mimetics[7] to delay aging and increase both lifespan and good health at old age (Testa et al. 2014).

In working with this thesis, observations have been made regarding diverse synonyms and abbreviations used for the different pathway mediators within the same species. As the network of signaling pathways are widely complex in nature, future research should be unified with the use of a single synonym that also could facilitate an easier comparison of conserved pathways between different species for increased simplicity and understanding.

There is also an urgent need for the unified use of a single unique terminology on the topic of PR expressed by Le Couteur et al. (2016a). The diverse terminologies on this topic existing today have a negative influence on the ability to construct a good systematic search with a high degree of relevant hits. A more unified terminology is also needed for the topic of CR, although only a few terminologies seem to be in use on this topic.

[7] CR mimetics refer to the development of pharmaceuticals or identification of natural substances that can activate the same mechanisms as CR. Although these are usually referred to as CR mimetics, development/identification of PR mimetics would also have a strong potential of use.

4. Conclusion

The evaluation of studies uncovered in this thesis, have provided answers to the three thesis aims:

Thesis aim number one asked if the positive effects and knowledge of CR on lifespan and health at old age in model organisms are relevant for mammals and especially humans. Findings support that much of the knowledge of CR from model organisms seem to be relevant also for mammals and humans. There is strong evidence that CR can increase lifespan in rodents and provide many of the same effects as seen in other model organisms. It is also likely that CR could provide many of these same effects in both non-human primates and humans.

Thesis aim number two asked if PR (including AA-R) could provide increased lifespan or improved health at old age in mammals and especially humans. Findings from the systematic search on effects of PR indicate that for rodents, PR, including AA-R seem to provide increased lifespan and improved health at old age. For humans, PR might also provide these positive effects on lifespan and health at old age, but the PR regimen may have a negative contribution after a certain age, and should perhaps not be maintained throughout life.

At last, thesis aim number three was to explore if PR can provide the same effects as seen with CR. This aim also questioned if the concomitant PR that often follows with CR studies could cause the effects reported by CR. Collectively, findings in this thesis indicate that PR seems to provide many of the same effects as seen with CR, but these effects appear to have a lower impact. Both CR and PR can lead to downregulated the GH/IGF-1 and mTOR signaling, while only CR appears to give an activation of sirtuin and AMPK signaling. Thus, PR can possibly be the cause, or contributor to some, but not all the effects seen with CR.

5. References

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6. Appendices

6.1 PubMed search on terminology, calorie restriction and energy restriction

PubMed searches were performed on June 3, 2016. These three search queries were set up to discover which of the terms “calorie/caloric restriction” or “energy restriction” that were most commonly used in relation to aging. Searches with either “caloric restriction”, “calorie restriction” or “energy restriction” in the title were combined with aging in the title or abstract. Below follows the search queries used, with the number of corresponding results to the right:

Search query: (caloric restriction[Title]) AND aging[Title/Abstract]) = 439 results

Search query: (calorie restriction[Title]) AND aging[Title/Abstract]) = 291 results

Search query: (energy restriction[Title]) AND aging[Title/Abstract] = 17 results

6.2 PubMed search query for systematic search on PR:

This systematic search was performed on June 30th 2016, with a total of 377 initial results. Below follows the exact search query that was used:

((((((((((ratio of macronutrients[Title/Abstract] OR protein restriction[Title/Abstract] OR low protein diet[Title/Abstract]) OR LPHC diet[Title/Abstract]) OR LPHC[Title/Abstract]) OR CPC diet[Title/Abstract]) OR low protein high carbohydrate[Title/Abstract]) OR low protein high carbohydrate diet[Title/Abstract]) OR macronutrient ratio[Title/Abstract]) OR protein carbohydrate ratio[Title/Abstract]) OR amino acid restriction[Title/Abstract]) OR methionine restriction[Title/Abstract]) OR "tryptophan restriction"[Title/Abstract]) OR "leucine restriction"[Title/Abstract] AND (((((aging[Title/Abstract] OR longevity[Title/Abstract]) OR lifespan[Title/Abstract]) OR life span[Title/Abstract]) OR life expectancy[Title/Abstract]) OR survival[Title/Abstract])

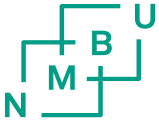
6.3 Calculations for the comparisons of the WNPRC and NIA diets

WNPRC diet composition:

	% of total weight	Energy per gram	Energy per 100g of total diet	Calculated % of total energy
Carbohydrate (including sucrose)	61%	17 kj / 4 kcal	1037 kj / 244 kcal	62%
Protein	15%	17 kj / 4 kcal	255 kj / 60 kcal	15%
Fat	10%	37 kj / 9 kcal	370 kj / 90 kcal	23%
			Total = 1662 kj / 394 kcal	
Sucrose	25-29%	17 kj / 4 kcal	425-493 kj / 100-116 kcal	25-29%

NIA study diet compositions:

	% of total weight	Energy per gram	Energy per 100g of total diet	Calculated % of total energy
Carbohydrate (including sucrose)	56.9%	17 kj / 4 kcal	967kj / 228 kcal	67%
Protein	17.3%	17 kj / 4 kcal	294kj / 69kcal	20%
Fat	5%	37 kj / 9 kcal	185kj / 45kcal	13%
			Total = 1446kj / 342kcal	
Sucrose	3.9%	17 kj / 4 kcal	66kj / 16kcal	5%



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