Universidade de Aveiro Departamento de Ciências Médicas

2016

Stephany Marques Francisco

Protein Aggregation and Proteostasis in Aging Mice

Agregação proteica e proteostase no envelhecimento

Universidade de Aveiro Departamento de Ciências Médicas

2016

Stephany Marques Francisco

Protein Aggregation and Proteostasis in Aging Mice

Agregação proteica e proteostase no envelhecimento

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Doutora Ana Raquel Santos Calhôa Mano Soares, investigadora de Pós-Doutoramento do Departamento de Ciências Médicas da Universidade de Aveiro e co-orientação do Doutor Manuel António da Silva Santos, Professor associado do Departamento de Ciências Médicas da Universidade de Aveiro.

O júri

Presidente	Doutora Ana Gabriela da Silva Cavaleiro Henriques Professora Auxiliar Convidada, Universidade de Aveiro
Vogal	Doutora Marisa Susete Reverendo Simões Investigadora de Pós-Doutoramento, Centre D´immunologie de Marseille-Luminy
Vogal	Doutora Ana Raquel Santos Calhôa Mano Soares Investigadora de Pós-Doutoramento, Universidade de Aveiro

acknowledgements

First and foremost, I would like to thank my advisor, Dr. Ana Raquel Soares, for giving me the opportunity to work in her research project. I am grateful for all the support and guidance during my time here. Now I can say I am a good scientist under your leadership.

Many thanks to Dr. Manuel Santos for his guidance throughout this project and Master´s program. I would also like to thank my lab mates, in particular Sofia Domingues, Diogo Tomé, Marisa Pereira, Sofia Varanda and Mafalda Santos, and the rest of the RNA Biology Lab Group for all their help and support.

A special thanks to my family: my parents, my brother Rui and sister-in-law Katty, and baby Sofia for all their love and unconditional support during this project. A very special thanks to Tiago for being patient with me during this project and degree. To my friends and colleagues that I have made in this master´s program, that I hold near and dear to me especially Raquel Fernandes, Tânia Marante, Ana Luísa Graça, we have managed to surpass an important hurdle in our academic lives and we did it together. And to the rest of the classmates and teachers who have helped me during this journey, a big thank you to all!

Keywords Aging Mice, protein aggregation, insoluble proteins, ubiquitination, heat shock response, carbonylation.

Abstract Normal aging is the process of becoming older due to various biological and physiological changes accumulating over time. Many studies have focused on understanding the processes involved in age-related pathologies, such as protein aggregation in Alzheimer´s disease, Huntington´s disease among numerous others. However, the events that occur at a protein level in normal aging have not been explored as extensively. Most of what is known about normal aging has been found the nematode *Caenorhabditis elegans* (*C.elegans*), yeast and in mice. In this study, the role of protein misfolding and aggregation in aging as well as the deficiencies in the cellular pathways that maintain proteostasis were investigated. This was done through analysis by SDS-PAGE of insoluble proteins present in mouse samples of different ages (1 month old (mo), 3 mo, 6 mo and 12 mo). Then by Western Blot analysis, we analyzed the expression of ubiquitin and chaperone proteins such as Hsp70, Hsp90, and BiP in the same mouse tissues. Carbonyl content was also measured. Results showed a significant difference between older mice and younger mice in the diencephalon in terms of relative amounts of insoluble protein and ubiquitinated proteins. These results provide more insight into the age that the proteostasis begins to decline in normal aging in mice. The difference seen among these tissues shows that mouse tissues age at different rates. In particular, certain brain regions such as the diencephalon age more quickly than other tissues. These results provide further knowledge about normal aging and which tissues are more vulnerable to age-related changes.

Palavras chave envelhecimento, agregação proteica, proteínas insolúveis, ubiquitinação, resposta de choque térmico, carbonilação

Resumo

O envelhecimento é o processo resultante das mudanças biológicas e fisiológicas que se acumulam ao longo do tempo. Muitos estudos se têm focado na compreensão dos processos envolvidos em patologias relacionadas com a idade, tais como a agregação de proteínas na doença de Alzheimer, a doença de Huntington, entre outras. No entanto, os eventos que ocorrem ao nível proteico no envelhecimento normal ainda não foram explorados extensivamente. A maioria do que está escrito sobre o envelhecimento normal foi estudado no nematode *Caenorhabditis elegans (C.elegans)*, em leveduras e em ratos. Neste estudo, foi investigada a formação de agregados proteicos durante o envelhecimento assim como as deficiências nos processos celulares da proteostase em ratinhos saudáveis. Isto foi feito através da análise por SDS-PAGE de proteínas insolúveis presentes em amostras de ratinhos de diferentes idades (1 mês, 3 meses, 6 meses e 12 meses de idade). Em seguida, por análise de Western Blot, analisamos a expressão de ubiquitina e proteínas chaperonas tais como Hsp70, Hsp90 e BiP nos mesmos tecidos de ratinho. Também foi medido o teor de carbonilação nos tecidos. Os resultados mostraram uma diferença significativa entre ratinhos com 12 meses de idade e ratinhos de 1 mês de idade no diencéfalo em termos de quantidades relativas de proteína insolúvel e proteínas ubiquitinadas. Estes resultados proporcionam uma maior compreensão da idade em que o declínio da proteostase começa durante o envelhecimento normal em ratinhos. A diferença observada entre os tecidos revela que os tecidos de ratinho envelhecem a taxas diferentes. Em particular, o diencéfalo envelhece mais rapidamente do que os outros tecidos. Estes resultados fornecem mais conhecimento sobre o envelhecimento normal e quais os tecidos mais vulneráveis às mudanças relacionadas com a idade.

Table of Contents

Table of Figures

List of abbreviations

APS	**Ammonium persulfate**
ATF4	**activating transcription factor 4**
ATF6α	**activating transcription factor 6 alpha**
ATP	**adenosine triphosphate**
BCA	**Bichionchic Acid Assay**
BiP	**Binding immunoglobulin protein**
CHOP	**CCAAT-enhancer-binding protein homologous protein**
CMA	**chaperone-mediated autophagy**
eIF2α	**eukaryotic translation initiation factor 2 alpha**
ER	**endoplasmic reticulum**
ERAD	**Endoplasmic-reticulum-associated degradation**
GADD34	**Growth arrest and DNA damage-inducible protein 34**
HSF-1	**Heat shock factor protein 1**
HSP	**Heat shock protein**
HSR	**Heat shock response**
ILS	**Insulin-like Signalling**
IGF-1	**Insulin Growth Factor 1**
IRE1α	**inositol-requiring protein 1 alpha**
PDI	**protein disulfide isomerase**
PERK	**PRKR-like ER kinase**
ROS	**Reactive oxygen species**
SDS	**Socedyl disulfide**
SDS-PAGE	**Socedyl disulfide polyacrylamide gel electrophoresis**
TEMED	**Tetramethylethylenediamine**
TOR	**target of rapamycin**
UPR	**unfolded protein response**
UPS	**ubiquitin proteasome system**
XBP1	**X-box binding protein 1**

Chapter I: Introduction

Normal aging is the process of becoming older due to various biological and physiological changes accumulating over time. Many studies have focused on understanding the processes involved in age-related pathologies, such as protein aggregation in Alzheimer´s disease, Huntington´s disease among numerous others [1]. However, the events that occur at a protein level in normal aging have not been explored as extensively. Most of what is known about normal aging has been found the nematode *Caenorhabditis elegans* (*C.elegans*), yeast and in mice [1]. Literature suggests that protein homeostasis, the process of maintaining normal protein expression in the body, is disrupted in aging [1]. The integrity of the proteome is important in maintaining efficient cellular functioning and biological processes with the goal of optimizing the health and longevity of the organism [2]. In this thesis, the current knowledge of the role of protein misfolding and aggregation in aging as well as the deficiencies in the cellular proteostasis pathways is explored. The goal of this work is to acquire a better understanding of the mechanisms involved in protein misfolding and aggregation and at what age these pathways are affected. This knowledge can provide insights into potential target mechanisms for treatments of age-related pathologies and also putative reasons for why aging itself occurs.

1.1 The Role of Proteostasis in Aging

Many studies have discussed that the cell´s capacity to maintain a well-functioning proteome decreases with age [2]. Cells possess machinery that can monitor and maintain the proteome. In order to maintain protein homeostasis, cells have processes that can refold or degrade misfolded proteins [3]. Cellular proteostasis mechanisms may be hindered due to metabolic stress, heat shock, and oxidative stress [2,3]. There are several forms of metabolic stresses that may overwhelm the processes involved in maintaining protein homeostasis [3]. The most common are: mitochondrial dysregulation, reactive oxygen species (ROS) and imbalance of nutrients [3]. The accumulation of deleterious mutations in cells over time overloads cellular mechanisms that maintain protein homeostasis [3]. As a result, the efficiency of these processes is hindered, resulting in an increase of damaged proteins, most of which have undergone oxidation [3]. This is followed by protein aggregation that is widespread throughout the organism, toxicity, and eventually cell death [3]. However, an unanswered question arises, is protein misfolding a cause of aging or a consequence due to the gradual decline of proteostasis? There are several quality control processes that prevent protein aggregation. These quality control processes can be found as cytoplasmic quality control, or as organelle-specific in the nucleus, endoplasmic reticulum, and mitochondria [4,5]. Protective cellular mechanisms such as the unfolded protein response (UPR), the ubiquitin proteasome system (UPS), the heat shock response (HSR), and autophagy will be further

discussed and explored in this thesis. Figure 1 displays several cellular mechanisms involved in maintaining proteostasis.

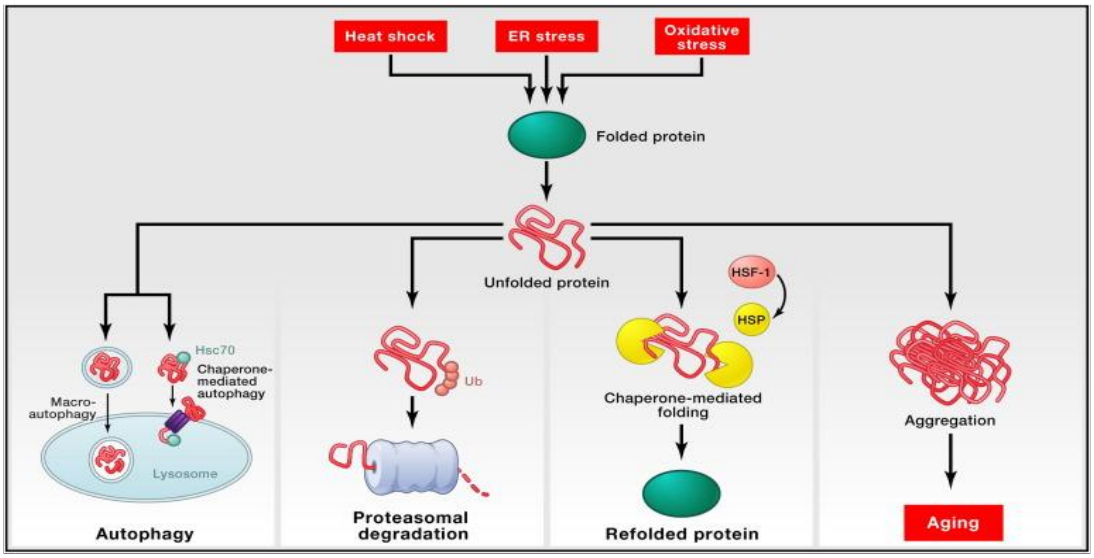


Figure 1. Cellular pathways that are involved in proteostasis. Following exposure to various stresses such as heat shock, ER stress, and oxidative stress, proteins undergo changes to their functional conformations. Cellular responses such as autophagy, proteasomal degradation via ubiquitination, and chaperone-mediated folding are able to assist in refolding or degradation of misfolded proteins Adapted from: López-Otín C., Blasco MA., Partridge L., Serrano M., and Kroemer G. The Hallmarks of Aging. Cell. 2013; 153:1194–217.

1.2 The Heat Shock Response

Exposure to elevated temperatures stimulates the production of several proteins called heat shock proteins (HSPs) in almost all organisms [6]. These proteins play an important role in maintaining homeostasis [6]. Among the most common heat shock proteins are Hsp10, Hsp27, Hsp60, Hsp70, Hsp90, and Hsp110 [6,7]. In order to understand how heat shock proteins are involved in aging, the function of each type of HSPs needs to be explored. In particular, a class of HSPs called molecular chaperones play an important role in proteostasis [7,8,9]. Molecular chaperones including Hsp70 and Hsp90 aid in stabilizing, refolding of unfolded or misfolded proteins in order for these proteins to acquire the correct functional conformation [9,10, 11]. Molecular chaperones are particularly important in aging because they are able to bind to misfolded proteins that have a tendency to aggregate due to these proteins having exposed hydrophobic domains [10, 11]. For instance, in Huntington´s disease, Hsp70 has been shown to directly bind to mutant huntingtin aggregates, preventing further aggregation [12].

Figure 2 shows the process of chaperone-assisted refolding of unfolded or misfolded proteins [13]. The process begins with the unfolded protein being recognized by molecular chaperones Hsp70 and Hsp40 [11]. Hsp70 contains a subdomain which recognizes and binds to exposed hydrophobic segments present on unfolded proteins, initiating the refolding process of these proteins [11]. The refolding of proteins via molecular chaperones is ATP-dependent [11,13]. Hsp70 and Hsp90 are able to form complexes with other proteins called co-chaperones that assist in the refolding process [11,13]. During the process of refolding, misfolded or unfolded proteins are transferred from Hsp70 to Hsp90 via a co-chaperone called Hsp70-Hsp90 Organizing Protein (HOP)[14]. Hsp90 is abundantly expressed in eukaryotes and is responsible for stabilizing and completing the maturation of proteins in the refolding process [11,15]. When Hsp90 is bound to ATP, the complex is in a closed conformation and allows for stable binding of the protein being folded [11,15,16]. Following ATP hydrolysis, Hsp90 switches to an open conformation and releases the newly folded protein [11,15,16]. During aging, HSP expression decreases [8]. For instance, studies have shown that Hsp40 and Hsp70 expression significantly decreases in cardiac tissues of aged rats [17].

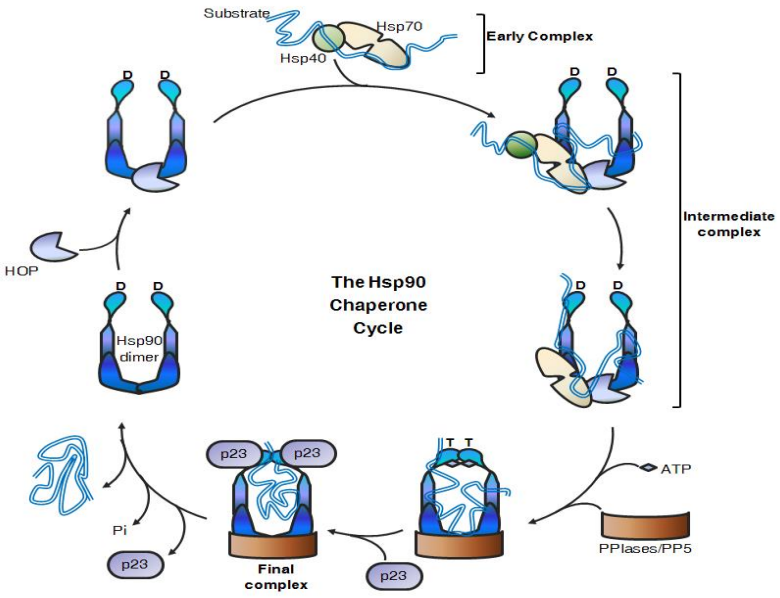


Figure 2. Chaperone-assisted protein folding. Molecular chaperones, Hsp40 and Hsp70 (early complex) recognize the substrate (unfolded protein), then co-chaperone HOP allows for the substrate to be transferred to Hsp90, which stabilizes the substrate, and finally co-chaperone p23 is recruited to be part of the final complex, where it helps maintain a closed conformation of Hsp90 in order to complete the refolding process. The correctly folded substrate is released after ATP hydrolysis which opens up the Hsp90 complex. Adapted from: HSPIR Hsp90. [Internet] HSPIR, 20 Jan. 2014. Web. 06 Dec. 2016 Available from: http://pdslab.biochem.iisc.ernet.in/hspir/hsp90.php

1.2.1 Initiation of the Heat Shock Response via Heat Shock Factor 1

Heat shock factor 1 (HSF-1) is a transcriptional factor that activates the transcription and production of heat shock proteins [7]. Figure 3 shows how the heat shock response is activated via HSF-1. In the absence of stress, HSF-1 is repressed by a multi-chaperone complex with Hsp40, Hsp70 and Hsp90 [7]. In the inactive state, HSF-1 is a monomer and is unable to bind to heat shock elements present in the promoter regions of HSP genes [7,8,10]. However, when there is stress, HSF-1 dissociates from the complex and becomes active as a trimer through hyperphosphorylation [7,9,10]. After these modifications, HSF-1 binds to heat shock elements and activates transcription of HSP genes leading to more HSPs being present in cells. In *c. elegans*, the inhibition of HSF-1 leads to a decreased lifespan whereas overexpression of HSF-1 extends lifespan [7,10].

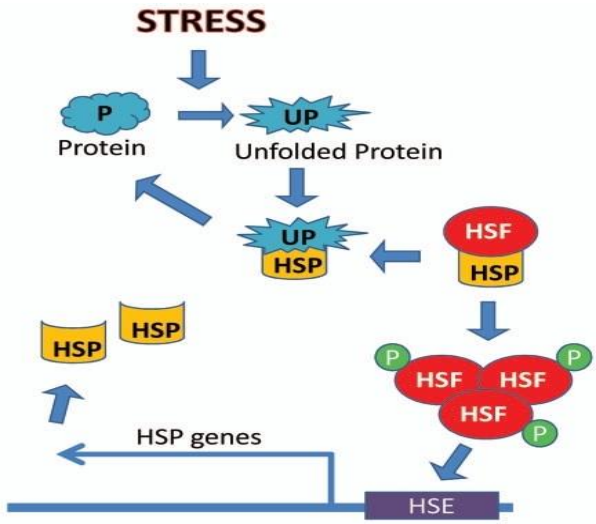


Figure 3. Activation of Heat Shock Response via Heat Shock Factor 1. In conditions of stress, HSF-1 dissociates from the complex with Hsps and becomes active as a trimer through several phosphorylation reactions. After these modifications, HSF-1 binds to heat shock elements, activating transcription of HSP genes leading to more HSPs being available in cells. Adapted from: Krivoruchko A, Storey KB. Forever young: mechanisms of natural anoxia tolerance and potential links to longevity. Oxidation Medical Cellular Longevity 2010 (May-Jun).

1.2 The Unfolded Protein Response

Aging impairs the unfolded protein response (UPR), a pathway that prevents the misfolding of proteins present in the lumen of the endoplasmic reticulum (ER) by recruiting chaperones [4]. During aging, there is a natural and gradual decline in UPR function [4,5,6]. The levels of expression of some crucial components of the UPR, such as molecular chaperone immunoglobulin heavy-chain binding protein (BiP), protein kinase RNA-like endoplasmic reticulum (PERK) kinase, and eukaryotic translation initiation factor 2 alpha (eIF2α) decrease during aging [4,5]. Chronic stress of the ER leads to dysfunctional activation of the UPR response [5].

When there is stress in the endoplasmic reticulum (ER), unfolded and misfolded proteins bind and sequester BiP, activating the UPR [6]. The UPR consists of three parallel signaling branches: the PERK- eIF2α pathway, the inositol-requiring protein 1α (IRE1α)–X-box binding protein 1 (XBP1) pathway and activating transcription factor 6 (ATF6) pathway [18]. Figure 4 shows the three signaling cascades of the UPR.

In the absence of stress, PERK is an ER transmembrane protein and is bound to BiP [18]. When there is stress, such as the accumulation of misfolded proteins in the lumen of the ER, BiP is released from PERK and PERK undergoes autophosphorylation [18]. PERK blocks protein translation by phosphorylating eukaryotic translation initiation factor 2α (eIF2). Phosphorylated eIF2α leads to the eventual shutdown of protein synthesis [18]. Alzheimer´s, Parkinson´s and prion diseases show increased phosphorylated eIF2α (eIFα-P) levels [19]. When there are rising levels of unfolded proteins detected by BiP in the ER, auto-phosphorylation of PERK occurs [19]. PERK-P phosphorylates eIF2α, blocking the initiation step of protein translation [19]. Then, eIF2α-P induces ATF4 and CCAAT/enhancer-binding protein homologous protein (CHOP) expression, leading to caspase-12 cleavage, and expression of GADD34, which is a stress-induced eIF2α-P-specific phosphatase and an effector of a negative feedback loop that terminates UPR signaling [19]. When GADD34 is overexpressed, there is a decrease in eIFα-P levels, and protein translation begins again [19]. The activation of the UPR increases protein folding, transport and endoplasmic-reticulum-associated protein degradation (ERAD), while decreasing protein synthesis [18,19]. If protein misfolding is not resolved, cells enter apoptosis [18-20].

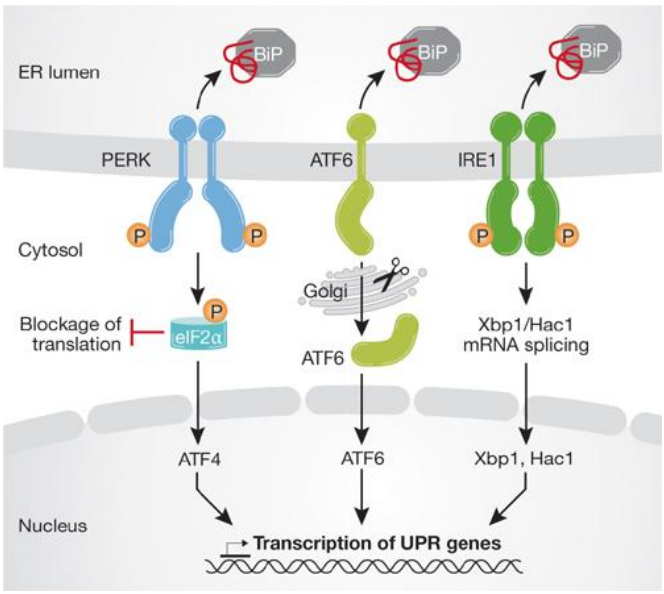


Figure 4. The three pathways of the UPR. The unfolded protein response consists of three signaling cascades: the IRE1-XBP pathway, the PERK- eIF2α pathway, and the ATF6 pathway. Adapted from: Cyr DM, and Hebert DN. Protein Quality Control-linking the unfolded protein response to disease. EMBO Reports. 2009; 10 (11): 1183-1279.

In the IRE1-XBP1 pathway, the inactive form of IRE1 is bound to protein chaperone BiP [20,21]. When there is accumulation of misfolded proteins in ER lumen, IRE1 is released from BiP, dimerizes and undergoes autophosphorylation [20,21]. Phosphorylated IRE1 removes an intron in the *XBP1* mRNA, thereby activating a spliced form of XBP1 which is able to activate UPR target genes that code for chaperones and ERAD proteins [20,21].

The last branch of the UPR is the ATF6 response. ATF6 is found in the ER membrane bound to BiP when inactive [20,22]. When there is stress, ATF6 is released from BiP and is translocated to the Golgi complex [20,22]. ATF6 is cleaved into ATFα and ATFβ which are then translocated to the nucleus where they bind to ER stress response elements and activate transcription of UPR target genes [20,22].

1.3 Oxidative Stress and Protein Carbonylation

In recent studies, protein carbonylation has been suggested as a biomarker of aging [23]. Protein carbonylation is an irreversible process where amino acid side chains are modified into carbonyl derivatives (either aldehydes or ketones) driven by reactive oxygen species (ROS) [23]. Protein carbonylation is an oxidative process and carbonyls can be incorporated into proteins in various ways [23]. One way is through the direct oxidation of amino acid side chains specifically modifying proline,

arginine, lysine and threonine residues into carbonyl derivatives [23]. Some proteins have more susceptibility of becoming carbonylated than others for instance, human brain copper-zinc superoxide dismutases (SOD1) are common targets of oxidation found in patients with Alzheimer´s and Parkinson´s Disease [24]. Protein carbonylation is also common in other age-related diseases such as diabetes [24]. Figure 5 shows that protein carbonylation increases with age across several species including worms, flies, rats and humans.

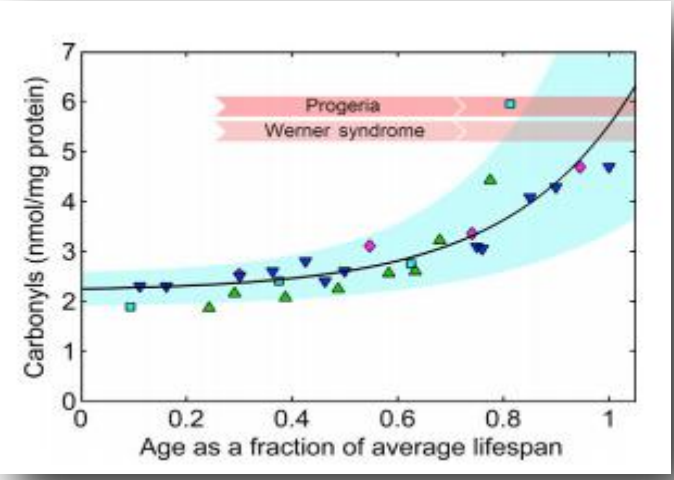


Figure 5. Protein carbonylation rates in several species during aging. Carbonyl content with age in worms (purple diamonds), flies (green triangles), rats (light blue squares) and humans (blue downward triangles). The horizontal pink bands correspond to the maximum carbonyl levels measured toward the end of life in people with premature aging diseases progeria and Werner syndrome. Adapted from: Graff AMR De, Hazoglou MJ, Dill KA, Graff AMR De, Hazoglou MJ, Dill KA. Highly Charged Proteins: The Achilles Heel of Aging Theory Highly Charged Proteins: The Achilles' Heel of Aging Proteomes. Structural Design [Internet]. Elsevier Ltd. 1-8.

The endoplasmic reticulum plays a crucial role in aging, this is due to the ER being a main target for endogenously generated oxygen species (ROS). During aging, chaperones located in the ER that partake in protein folding are impaired via oxidation [5,25]. The affected chaperones include: BiP, protein disulfide isomerase (PDI), and calnexin [25]. In particular, molecular chaperone BiP is integral in the UPR response, interacting with enzymes IRE1, PERK, and ATF6, in normal conditions [25]. However, under stress, BiP is unable to interact with other proteins resulting in activation of the UPR response. On the other hand, PDI is involved in oxidative folding of proteins in the ER [25]. A study by Nuss and

colleagues found that the enzymatic activity of chaperones BiP and PDI were significantly reduced with age in mouse livers [25]. The authors suggested two ways that chronic oxidative stress is a cause for the accumulation of misfolded proteins. First off, the direct oxidation via ROS of PDI and BiP and second, the decline of chaperones being able to properly fold nascent proteins [25]. The results confirm that abnormal proteins accumulate and form aggregates due to chaperones BiP and PDI undergoing oxidative modifications, in this case carbonylation, resulting in the decline of activity to correctly fold proteins and degrade proteins. Also, the decrease in the efficiency of the chaperones may be due to the structural changes that arise as a result of being carbonylated [25].

1.4 The Ubiquitin Proteasome System in Aging

The role of the ubiquitin proteasome (UPS) system is to degrade damaged and abnormal proteins [26]. The UPS system is a cytoplasmic cellular control network which consists of damaged proteins being tagged by ubiquitin and then degraded by the proteasome [26]. The proteasome itself consists of a barrel-shaped complex with multiple subunits, specifically a 20S catalytic core particle and two 19S regulatory particles [26]. In aging, the activity of the proteasome declines, which incapacitates cells from efficiently removing damaged proteins [26]. Protein chaperones such as HSP70 are able to initiate the ubiquitin-proteasome cascade for protein degradation [11, 26]. The link between chaperones, the UPS and aging has been observed in mice [27]. Hsp70 is able to activate Carboxyl-terminus of Hsp70 Interacting Protein (CHIP) which is a ubiquitin ligase that is part of the UPS. When CHIP levels are depleted in mice, there is a decrease in lifespan and aging phenotypes arise [27, 28]. Figure 6 explains in detail the signaling cascade of the UPS pathway. Ubiquitination of proteins involves three sequential reactions by three enzymes called ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3) [28].

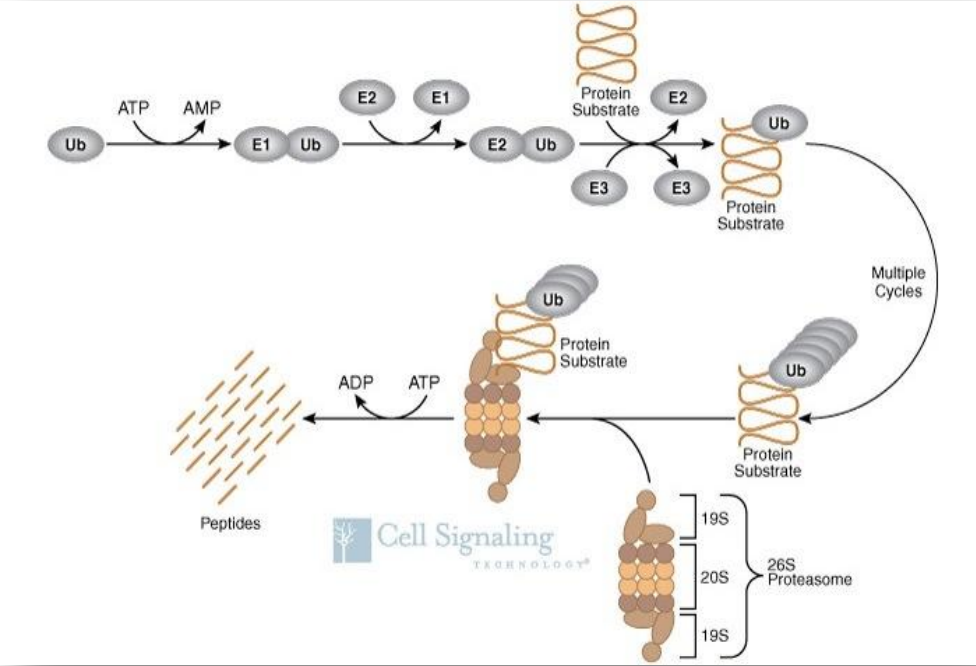


Figure 6. Steps of the ubiquitin-proteasome pathway. Ubiquitin activating enzyme (E1) binds to a ubiquitin molecule with the use of one ATP in order to create the bond between them which activates ubiquitin. Then, the ubiquitin-conjugating enzyme (E2) takes over the active ubiquitin from E1. The active ubiquitin is transferred by E2 to a protein substrate bound to the ubiquitin ligase (E3). Following this, there is the extension of the ubiquitin chain. The ubiquitinated protein undergoes binding with either 1, 2, 3 or 4 ubiquitin molecules and then is sent to the 26 S proteasome to be degraded. Deubiquitinating enzymes release the ubiquitin molecules. Adapted from: Ubiquitin and Ubiquitin-Like Proteins [Internet]. Cell Signal. 2016 [cited 6 December 2016]. Available from: https://www.cellsignal.com/common/content/content.jsp?id=science-pathways-ubiquitin.

In order to evaluate how different components of the proteasome affect aging, several studies have manipulated genes of ATPases and other components of the proteasome that are crucial in maintaining the integrity of the proteasome. The results of these studies are summarized in **Table 1** [5].

Subunit deficiency/overexpression	Function	Phenotype	Model
Rpt2 inactivation	ATPase	Ubiquitin and α-synuclein positive Lewy like intraneural inclusion in neurons and neurodegeneration	*Mus musculus*
Rpt3 inactivation	ATPase	TDP43, FUS accumulation, basophilic inclusion bodies in neurons, locomotor impairment, loss of neurons	*Mus musculus*
β5t deletion	Chymotrypsin-like proteolytic activity	Shortening of lifespan, accumulation of polyubiquitinated and oxidized proteins, aggravated age-related metabolic disorder	*Mus musculus*
Rpn11 overexpression	Deubiquitination of the proteasome substrate	Extension of lifespan, suppression of polyQ induced toxicity	*Drosophila melanogaster*
Rpn6 overexpression	Stabilizing the interaction between CP and RP	Extension of lifespan under mild stress condition	*Caenorhabditis elegans*

Table 1. The Effect of Genetic Modulation of Proteasome in Different Models on Aging and Disease. This table highlights the effect of genetic modification in subunits of the proteasome and how these alterations affect aging. In transgenic mice, a shortened lifespan was observed when chymotrypsin-like proteasome activity decreases through β5t deletion. As a result, the transgenic mice had an accumulation of damaged and oxidized proteins and developed age-related metabolic disorders. Also, the normal expression of non-ATPase 19S subunit Rpn11 was shown to maintain the proper functioning of the proteasome and suppress toxicity in *Drosophila melanogaster*. Most importantly, when Rpn11 is overexpressed in adulthood as seen in Table 1 row 5, the mean lifespan in *Drosphila* is extended. Similar extension of lifespan was observed when Rpn6 subunit expression was increased in a mutant form of *C. elegans*. Adapted from: Contreras KC., Mukherjee A., and Soto C. Role of Protein Misfolding and Proteostasis Deficiency in Protein Misfolding Diseases and Aging. International Journal of Cell Biology 2013:1-10.

1.5 Recent Findings in *C. elegans*

A recent study by Labbadia and Morimoto has changed the way researchers understand the changes in proteostasis during aging. The authors propose that in *C. elegans*, the decline in proteasomal activity is not gradual as originally thought [29]. The hindrance of proteostasis is actually due to a sudden early life event that initiates several downstream cascades and processes [29]. Figure 7 shows the age and reproductive stage at which alterations in proteosomal responses occurs in *c.elegans*.

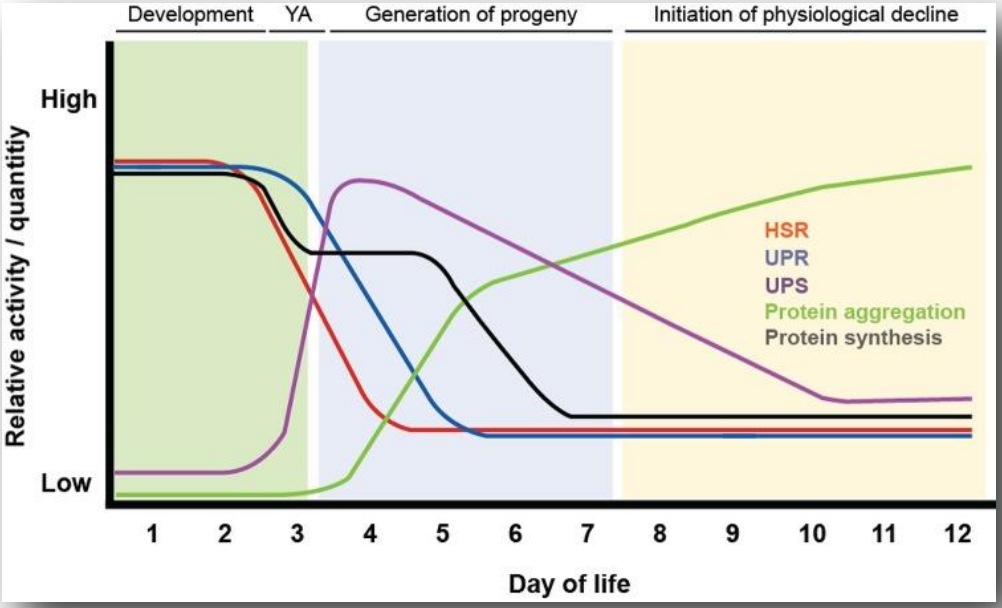


Figure 7. **Changes in proteostasis, reproduction and aging in *Caenorhabditis elegans***. There are three distinct stages in the lifespan of *C. elegans*: the developmental stages - larval stages to young adults (pale green region), maturation into reproductively active adults (pale blue region), and post-reproductive adults (pale red region). These life stages correlate with changes in the heat shock response (HSR), (red line), unfolded protein response (UPR) (blue line), ubiquitin-proteasome system (UPS) (purple line), protein synthesis (black line), and onset of protein aggregation (green line) early in life (colored lines). All three responses decline after reproductive age (pale blue region) is reached. Protein aggregation begins increasing at age 4 and continues increasing in the post-reproductive stage. Adapted from: Labbadia J, Morimoto RI. Proteostasis and Longevity: when does aging really begin? Prime Reports. 2015;6(1):435–64.

The authors provide further evidence that aggregation of proteins in neurons and muscle cells was observed by day three and became widespread by day seven, before cessation of reproduction which occurs around day seven [29]. In another study, where UPS activity was monitored via green fluorescent protein tag [30]. The results showed that after only two days there was a decline in fluorescence in all somatic tissues [30]. Nevertheless, the function of having changes in proteasomal activity in early stages of adulthood should be further investigated. One suggestion is to monitor protein degradation pathways in earlier stages of life and record the changes in the proteasomal network [30]. Further studies will provide insight to see if increased proteosomal activity is beneficial as a protective measure against accelerated aging and at the same time ensures lengthened longevity.

1.6 Autophagy, Metabolic Signalling Pathways, and Longevity

Autophagy is the method used by cells for removal of large protein aggregates. There are three forms of autophagy: chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy [31]. The types vary depending on the targets each recognize and the manner in which targets are directed to lysosomes for degradation [31]. Declines in proteostasis may arise when there are errors in any of the three types of autophagy [26,31]. Higher levels of activity of the autophagy pathway in early adulthood has also been implicated as a protective measure for tissues from proteostasis collapse [26]. Target of Rapamycin protein (TOR) regulates autophagy [31]. When activated, TOR triggers anabolic processes which increase protein synthesis. TORC1 which is a TOR complex is inhibited by Rapamycin (a compound) [5,31]. Rapamycin binds to FKBP12, forming a complex that binds and inhibits TOR, in order to activate autophagy [31]. Studies in *C. elegans* have revealed that Rapamycin treatment and genetic knockdown of TORC1 signaling increased resistance to stress and autophagy and increased health and lifespan [31]. Interestingly, the mechanisms that promote longevity via autophagy are highly interconnected with the activity of components in metabolic signaling pathways [26,31].

Aging can be regulated via three metabolic signaling pathways, these being dietary restriction, insulin/IGF-1 signaling, and reduced mitochondrial function. Restrictions on food intake has been shown to lengthen lifespan [32]. In worms, mice, and flies, dietary restriction increases lifespan via downregulation of TOR activity [32]. Figure 8, shows the pathways triggered by dietary restriction [32]. The TOR pathway, shown in purple is responsible for regulating autophagy and translation. In order to lengthen the lifespans of all three species via TOR inhibition autophagy is required [32]. Autophagy inactivates S6 Kinase, resulting in a longer lifespan [32].

The insulin-like signaling (ILS) pathway is known to regulate metabolism and is involved in the modulation of the adult lifespan of *C.elegans* [32,33]. Downregulating the insulin-like signaling (ILS) pathway increases longevity and resistance to stress, while suppressing the aggregation of polyglutamine expansion proteins and Aβ toxicity. Heat shock factor protein 1 (HSF-1) is a stress response factor in the ILS pathway [32]. A study done by Ben Zvl and colleagues (2009) showed that enhancing HSF-1 and/or DAF-16, a forkhead transcription factor, can suppress protein misfolding in *C.elegans* [34]. Therefore, augmenting HSF-1 and DAF-16 activity restores the integrity of the proteome [34]. Increased activity of both HSF-1 and DAF-16 in development and early adulthood may prevent the proteosomal collapse that is commonly seen in aging [34]. Specifically, the reduction of ILS signaling results in the activation of DAF-16 which increases lifespan [34]. At the same time, the down-regulation of HSF-1 results in a shorter lifespan as well as accelerated aging in *C. elegans* whereas HSF-

1 overexpression increases lifespan [2,34]. The results of this study establish a link between lifespan and metabolic control.

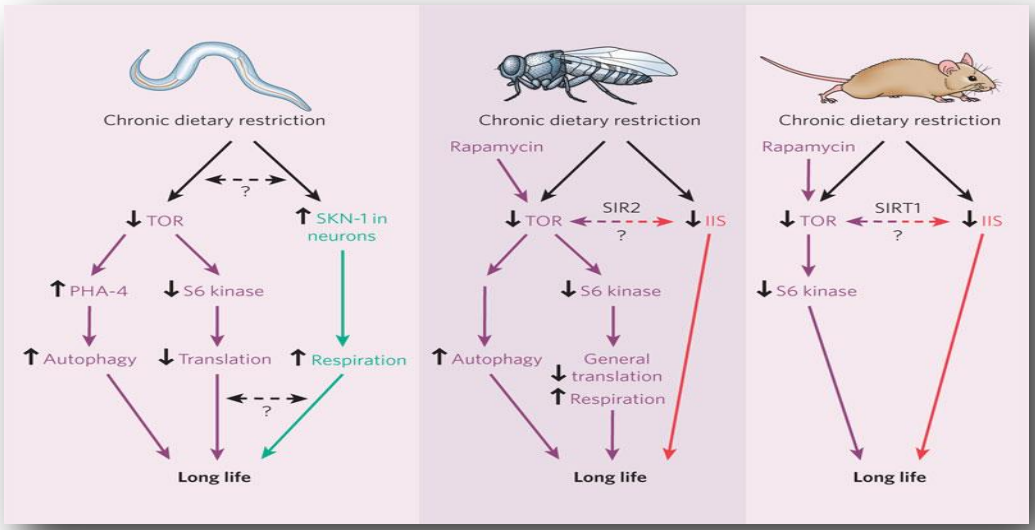


Figure 8. Pathways of Chronic Dietary Restriction includes the TOR pathway in purple and ILS in red. Adapted from Contreras KC., Mukherjee A., and Soto C. Role of Protein Misfolding and Proteostasis Deficiency in Protein Misfolding Diseases and Aging. International Journal of Cell Biology 2013:1-10.

Another pathway involving the reduction of the mitochondrial electron transport chain (ETC) is linked to promoting longevity [2, 35]. Originally shown in *C. elegans*, reducing expression of mitochondrial genes such as ETC complexes I, II, III, IV, and V via RNAi lengthened lifespan. However, this mechanism´s pathway is still unknown [34,35].

1.7 Human Aging and Protein Aggregation

In recent years, several reports have argued that humans with lower insulin-like signaling activity live longer [35]. When mutations arise in the insulin-like growth factor receptor (IGF-1), the activity of the IGF-1 signaling pathway decreases [36,37]. These mutations were found more abundantly in Jewish Ashkenazi centenarians than control groups [37]. Furthermore, mutations in the FOXO3a (DAF-16 orthologue) were more commonly found amongst two centenarian groups, that were German and Japanese-Hawaiian [36,37]. These findings prove that ILS signaling is closely linked to longevity not only in other species but in humans as well.

When analyzing the aging phenotype, one of the first changes to occur is the gradual loss of vision [38]. By using immunohistochemistry, Leger and colleagues (2011) have been able to measure the location and aggregation of several proteins in the human retina [38]. These proteins include tau, βA4-amyloid, α-synuclein, and ubiquitin [38]. Results have shown that in the cytoplasm of ganglionic cells in the retina, there was an increase of tau protein aggregates as the age of the patients increased [38]. However, there were no β-amyloid aggregates found. Both α-synuclein, and ubiquitin were found together in the inner nuclear layer and these aggregates were significantly higher in older patients [38]. Thus, the aging human retina contains protein aggregates of tau, α-synuclein, and ubiquitin [38].

In terms of protein aggregation and expression levels of human genes, via DNA microarray techniques, experimental *in vitro* rates of protein aggregation have been found to be negatively correlated with *in vivo* levels of corresponding gene expression [39]. Human proteins have resistance against aggregation however, in the event of a mutation or environmental damage, there are no measures that can deal with the resulting altered rates of aggregation [39]. This provides further knowledge about the nature of proteins and their ability to aggregate into forms that may be detrimental to the functioning of cellular mechanisms such as β-amyloid and tau aggregates in Alzheimer´s disease and α-synuclein in Parkinson´s disease [39].

1.8 Mice as a Model for Aging Research

Mice as opposed to other species share approximately 99% of their genetic material with humans [40]. For aging and longevity studies, mice are the most ideal mammalian models due to being easy to handle as well as having a short generation time [40]. Mice also have relatively short lifespans of approximately 24 months on average [40].

Recent aging mice studies have analyzed the presence of protein aggregation via extraction of detergent-insoluble proteins from mouse tissues followed by gel electrophoresis [41]. Protein aggregates have been shown to be insoluble in buffers that contain detergent and can be separated by SDS-PAGE [29,41]. A very recent study by Ayyadevara and colleagues analyzed the proteomic profile of aging and hypertensive mouse hearts by isolating detergent-insoluble proteins and running the samples with two dimensional gels [41]. Based on these recent aging mouse studies, we decided to analyze the presence of detergent-insoluble protein aggregates in various mouse tissues in this thesis.

1.9 Objectives

The aim of this thesis is to investigate how aging affects cellular mechanisms that maintain proteostasis and to determine at what age and in which tissues protein aggregates appear as a result of aging. In order to see this, several mouse tissues including the brain, heart, lungs, kidneys, liver were homogenized and protein extraction was performed. Protein extraction was performed in order to isolate insoluble proteins and compare the amounts of insoluble proteins between mice of different ages. Another way to observe the efficiency of the cellular mechanisms involved in the degradation of misfolded proteins was to analyze if proteins are being ubiquitinated in these tissues and if these levels increase in older mice. In addition, expression of proteins involved in heat shock response in tissues was also seen to determine if this pathway is also hindered in older mice. Also, protein carbonyl content was measured to observe if in fact, with age there are more proteins that have undergone modifications through oxidative processes.

Therefore, the biological questions addressed in this thesis include:

1. Are there protein aggregates present in mouse tissues? If so, at what age do these protein aggregates begin to appear and do they accumulate with age?
2. Are there declines in the efficacy of protein control systems such as UPS and HSR during aging in mice?
3. If there are declines in cellular mechanism function, at what age do these mechanisms start becoming inefficient?
4. Are there more proteins that have become undergone alterations or modifications such as carbonylation in older mice in comparison to younger mice?

Chapter II: Methods and Materials

Compositions of all solutions are included in Appendix 1.

2.1 Mouse Samples

Tissues extracted from C57BL/6 Mice (1,3, 6, and 12 months old), namely the diencephalon, cortex, cerebellum, heart, lungs, kidneys, and liver. The mice were provided by the Life and Health Sciences Research Institute at the University of Minho in Braga, Portugal.

2.2 Protein Extraction

Approximately 30 mg of mouse tissue was added to tubes with 320 µl of ELB buffer (Triton X-100, pH7 1M HEPES, 5M NaCl, 100.0 mM Na_3VO_4, 1M DTT, 1M NaF, 0.5M EDTA, 100.0 mM EGTA, 40 mM PMSF, 50x Roche Inhibitor Cocktail) and then manually homogenized. Next, the homogenized tissues were sonicated using a sonicator (IKA Laboratechnik U200Scontrol) at 50% for 15 seconds. Samples were then centrifuged at 200g for 20 minutes at 4°C. Afterwards, the supernatant was removed and kept as the total protein solution. Total protein concentrations in the samples were then quantified using a bicinchoic acid (BCA) protein assay kit (Pierce).

2.3 Protein Quantification

Total protein quantification was performed using a bicinchoic acid (BCA) protein assay kit (Pierce). BCA relies on how proteins are able to form complexes with Cu^{2+} in an alkaline environment, followed by the reduction of Cu^{2+} to Cu^{+} also known as a Biuret reaction [42]. When reduced Cu^{+} ions are present in samples, a purple color appears which indicates the chelation of BCA molecules with one Cu^{+} ion [42]. The BCA- Cu^{+} complexes have an absorbance maximum at 562 nm [42]. Absorbance can be read by a spectrophotometer [42]. Protein concentrations of each sample are determined based on the standard curve [42]. Standards contain bovine serum albumin that have a known concentration and are assayed alongside the samples that have unknown concentrations [42].

10 µl of each sample and standards were pipetted into microplate wells. Samples and standards were incubated with 100 µl of working reagent which consists of 50 parts of reagent A to 1 part of reagent B. All samples were incubated at 37ºC for 30 minutes, then cooled and measured at 562 nm absorbance with a spectrophotometer.

2.4 Insoluble Protein Extraction

Protein aggregates have been shown to be detergent- insoluble in buffers with detergents such as Triton X-100 [29]. Following protein quantification, 300µg- 400µg of protein from each sample

solution was collected and centrifuged at 16,000 g for 20 min at 4°C. After removing the supernatants, the remaining insoluble pellets were washed with 80 µl ELB and 20µl of Triton X-100, sonicated (two times at level 4 and duty cycle 50%), and centrifuged once again at 16,000 g for 20 min at 4°C. Afterwards, the supernatants were discarded and the remaining pellets were washed in 50µl of ELB and then sonicated (twice at level 2 and duty cycle 50%). After sonication, 10 µl of 6x SDS loading buffer was added to each sample and then boiled for 5 minutes at 95°C in a thermomixer. Samples were then loaded and separated by SDS-PAGE (10%) and stained by Coomasie Brilliant Blue R (Sigma) (Appendix 1). Experiments were performed with at least four replicates each time.

2.5 SDS-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separates protein samples based on molecular weight and charge [43,44]. SDS-PAGE consists of proteins migrating through a polyacrylamide gel under an electrical field [43,44]. Sodium dodecyl sulfate (SDS) is a detergent that disrupts tertiary structures of proteins, making them linear proteins [43,44]. SDS coats all proteins with a negative charge, allowing them to migrate from the negatively charged cathode toward the positively charged anode [43,44]. The proteins migrate toward the positive anode at different rates based on their molecular weight [43,44]. Polyacrylamide gels are used due to these being chemically inert [43,44]. The polyacrylamide gels consist of a stacking and running gel [43,44]. Proteins with a higher molecular weight migrate slower than proteins with lower molecular weight throughout the gel [43,44]. 10µg of total protein and 30µg of insoluble fraction samples underwent SDS-PAGE (See Appendix 1).

2.6 Western Blot

Western Blot analysis detects specific proteins in samples that consist of a mixture of proteins [45]. First, proteins are separated by size via electrophoresis, followed by transfer to a solid membrane and finally, incubation with antibodies specific to the proteins of interest [45]. In order to analyze the expression of heat shock proteins and ubiquitinated proteins, SDS-PAGE (Appendix 1) was done with 30-50µg of protein in each well and then the gels were transferred onto nitrocellulose membranes (BioRad) using a Trans-Blot Turbo Transfer System (BioRad). Membranes were then blocked during 1 hour in Trisbuffered saline with 0.1% Tween20 (TBS-T) and 5% Bovine Serum Albumin (BSA) from NYZTech and then incubated overnight with the primary antibody in TBS-T with 5% BSA. The primary antibodies used were: anti-Hsp70 (Enzo, 1:1000) anti-Hsp90 (StressMarq Biosciences,1:1000), anti-HSF1 (Enzo, 1:1000), anti-BiP (StressMarq, 1:1000) and anti-ubiquitin (Covance, 1:1000).

Afterwards, membranes were washed 3x with TBS-T and then incubated with a secondary antibody (either anti-mouse IRDye® 800CW LI-COR, 1:10000 or anti-rabbit IRDye® 680LT LI-COR 1:10000) in the dark for at least 1 hour. After incubation, membranes were washed an additional 2 times for 5 minutes with TBS-T and once with TBS. Then, the membranes were scanned using an Odyssey® IR scanner. The intensities of the bands were measured using ImageJ.

	Origin	Secondary Antibody
Anti-HSF1	Rabbit	Anti-Rabbit IRDye® 680LT LI-COR
Anti-HSP70	Rabbit	Anti-Rabbit IRDye® 680LT LI-COR
Anti-BiP	Rabbit	Anti-Rabbit IRDye® 680LT LI-COR
Anti-HSP90	Mouse	Anti-Mouse IRDye® 800CW LI-COR
Anti-Ubiquitin (1:1000)	Mouse	Anti-Mouse IRDye® 800CW LI-COR

Table 2. Antibodies used for Western Blot Analysis

2.7 Protein Carbonylation

The compound 2,4-dinitrophenylhydrazine (DNPH) reacts with protein carbonyls present in protein samples forming DNP-protein precipitates or hydrazones which are detected using a spectrophotometer [23,24]. Carbonyl groups were determined by first dissolving 10mM of 2,4-dinitrophenylhydrazine (DNPH) in 2M hydrochloric acid (HCl). Then 500µl of the solution was added to 100µl of protein sample (containing 500µg-2mg of total protein). A blank was prepared by adding 500µl of HCl 2M without DNPH and 100µl of milliQ H_2O. Samples were incubated for 1 hour at 20ºC in darkness. After incubating, 500µl of 20% trichloric acid (TCA) were added to all the samples then incubated for 15 minutes at 4ºC. Then samples were centrifuged at 10 000g during 15 minutes at 4ºC. Then supernatant was discarded and remaining pellets were washed with 500µl of ice cold acetone and placed at -20ºC for 5 minutes up to 1 hour. The samples were then centrifuged during 2 minutes at 10 000g at 4ºC. The supernatant was discarded and pellets were dissolved in 1ml of 6M guanidine and HCl and incubated at 37ºC during 30 minutes. In order to quantify the absorbance of each sample, 1ml of each sample was placed in a cuvette and read by a spectrophotometer (Beckman DU®530) of each sample was quantified at 370nm. The following formula was used to determine the relative amount of protein carbonyl present in each sample.

Concentration of Protein Carbonyl = Concentration at absorbance at 370nm / Concentration of total protein

2.8 Statistical Analyses

All data was analyzed using GraphPad Prism 5. Statistical significance for insoluble protein data, western blot analysis and protein carbonylation data was analyzed using the Mann-Whitney U test. The Mann-Whitney U Test is a non-parametric test that does not assume that the difference between samples follows a normal distribution and is an alternative to the student´s independent sample t-test [46]. This test is used to compare two population means that come from the same population as well as determine if the two means are equal or not [46].

Chapter III: Results

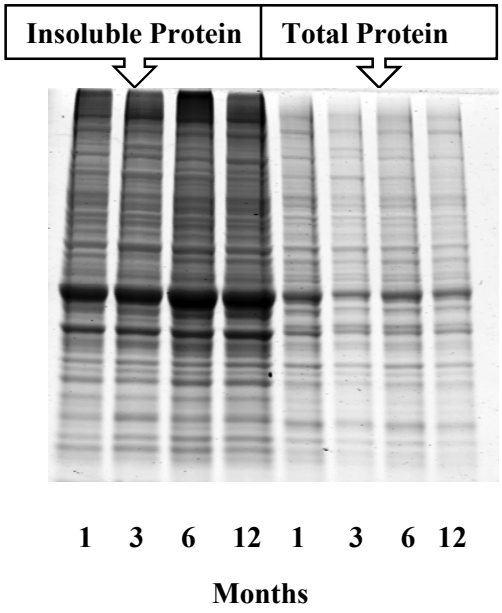
3.1. Analysis of Insoluble Protein in Mouse Tissues

After protein extraction and quantification through BCA, insoluble protein extraction was completed to isolate the insoluble proteins present in the mouse tissues. Protein aggregates have been shown to be insoluble in buffers with detergent and can be separated by SDS-PAGE [29]. SDS-PAGE was performed using samples from the mice aged 1 month, 3 months, 6 months, and 12 months (mo.). All images were taken with an Odyssey® IR scanner. All of the images in this section consist of the first four lanes being the insoluble protein extracts from 1 mo., 3mo., 6 mo., and 12 mo. mice and the last four lanes with the total protein of each sample which were ran alongside in order to compare the intensities of each lane. The intensities of the extracts were measured using ImageJ. The relative amount of insoluble protein for each age group was calculated by measuring the intensities of the insoluble protein extracts and dividing these values by the intensities of the total protein extracts for each corresponding sample. The bar graphs show the mean values and standard deviation of the relative amounts of insoluble protein for each mouse age group. The relative amounts are in all arbitrary units (au). In order to test for significance, a non-parametric t-test called the Mann-Whitney U Test was used.

3.1.1 Increase of Insoluble Protein in the Diencephalon of Aging Mice

SDS-PAGE was performed using insoluble and total protein samples from the diencephalon of mice aged 1 mo., 3 mo., 6 mo., and 12 mo. (Figure 9 a). The relative amount of insoluble protein in the diencephalon for each age group which consists of the intensities of the insoluble protein extracts divided by the intensities of the total protein extracts is shown in Figure 9 b). In the diencephalon, the amount of insoluble protein was significantly greater in 12 mo. mice than in 1 mo. mice.

a)



b)

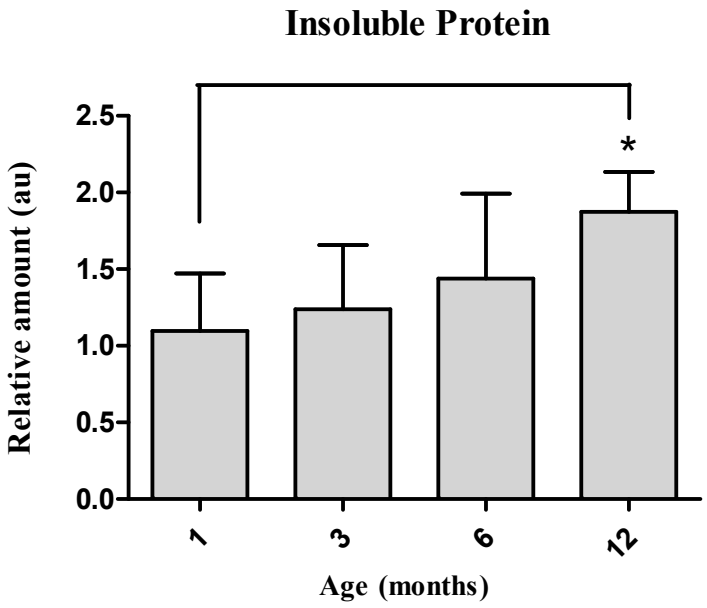


Figure 9 a) Insoluble Protein in the Diencephalon. Image of Protein Gel of Insoluble Protein and Total Protein of Diencephalon samples from 1, 3, 6, and 12 mo. mice. **Figure 9 b) Graph of relative amount of insoluble proteins in the diencephalon samples aged 1, 3, 6, and 12 mo.** The amount of insoluble protein increases as the age of the mice increases. There was a significant difference between the average insoluble protein in 1 mo. mice and 12 mo. mice. (Mann-Whitney U Test, * p= 0.0286, n= 4).

3.1.2 Insoluble Protein in the Cortex

Insoluble and total protein extracts of cortex samples from mice aged 1 mo., 3 mo., 6 mo., and 12 mo. were separated by SDS-PAGE (Figure 10 a). In this gel, there was an increase of insoluble protein as the age of the mice increased however, after statistical analyses, the increase was not significant (Figure 10 b).

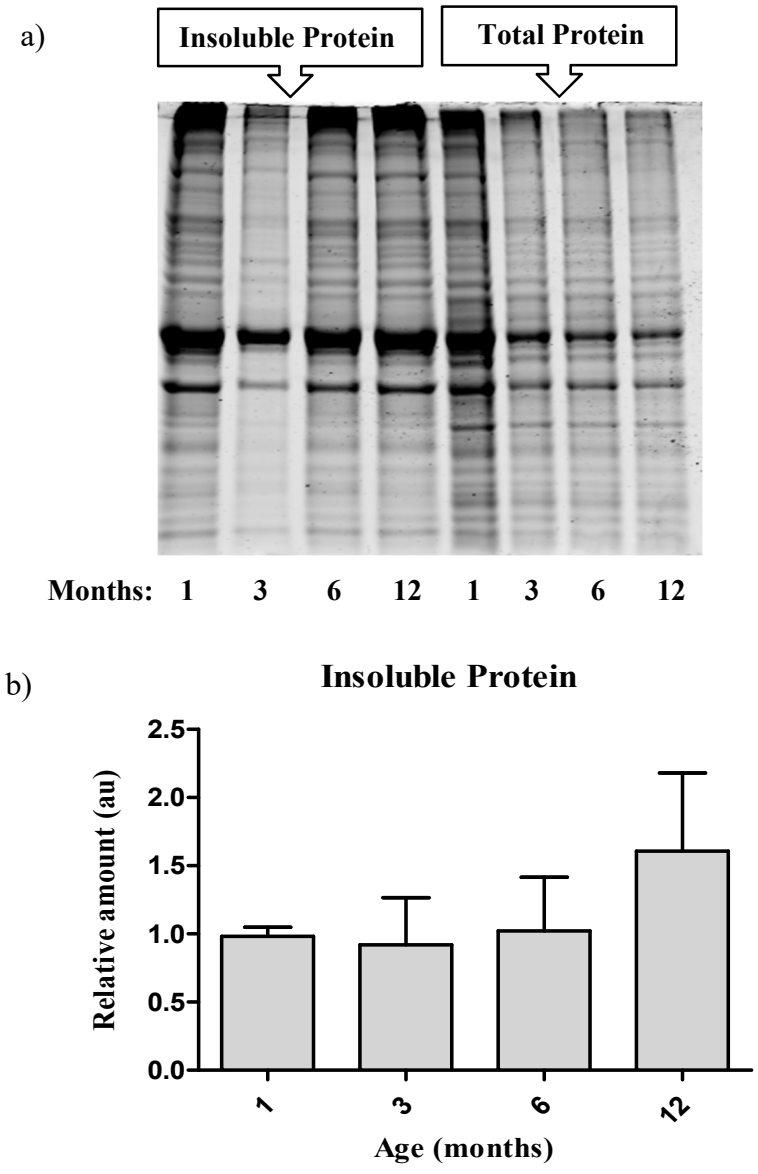
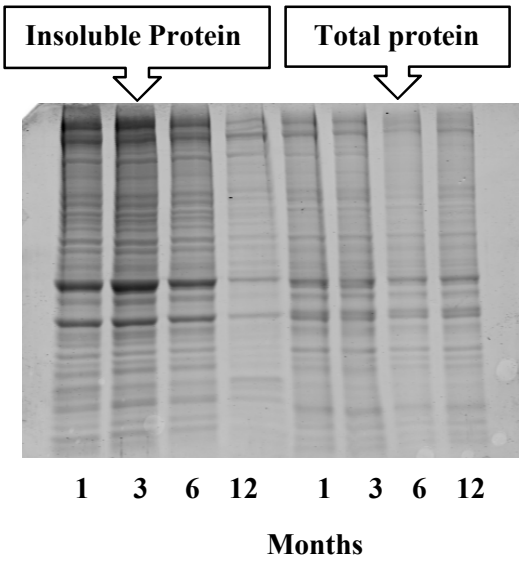


Figure 10 a) Insoluble Protein in the Cortex. Image of insoluble protein fractions of the cortex of mice aged 1, 3, 6, and 12 mo. **b) Graph of the Relative Amount of Insoluble Protein in the Cortex.** Graph of the relative amounts of insoluble protein present in the cortex of the mice. The difference in the amounts of insoluble protein between age groups was not significant (Mann Whitney U Test, $p>0.05$, $n=4$).

3.1.3 Insoluble Protein in the Cerebellum

An insoluble protein gel of the cerebellum of four mice groups aged 1 mo., 3 mo., 6 mo., and 12 mo. are shown in (Figure 11a). In the gel, there is a slight decrease of insoluble protein in the 12 mo. mice while the total protein intensities were identical for all age groups. After statistical analyses, the difference between age groups was not significant (Figure 11b).

a)



b)

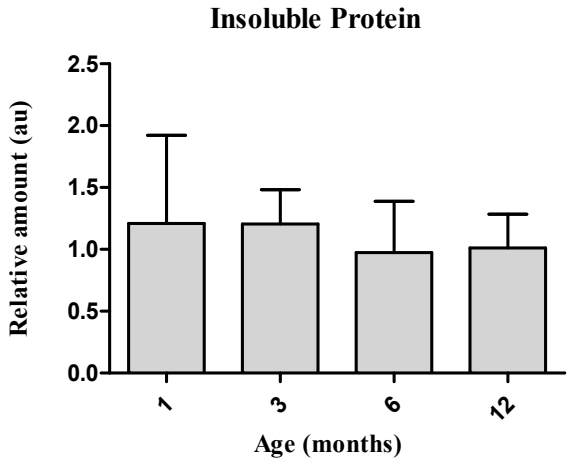
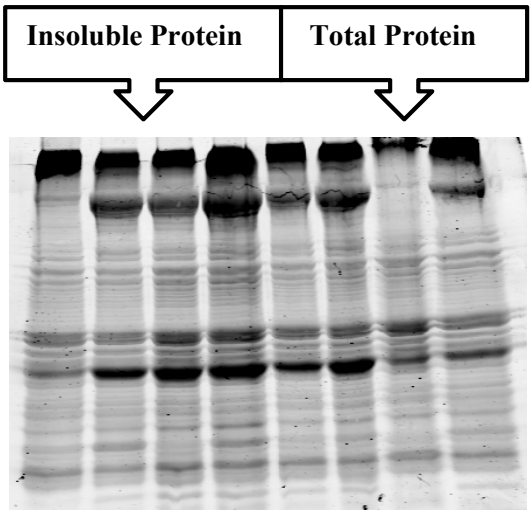


Figure 11 a) Insoluble protein in the cerebellum. Image of insoluble protein of the cerebellum of mice aged 1, 3, 6, and 12 mo. **b) Graph of the relative amounts of insoluble protein present in the cerebellum of mice.** There was no difference in the amounts of insoluble protein between age groups was not significant (Mann-Whitney U Test, $p>0.05$, $n=4$).

3.1.4 Heart

Insoluble protein extracts from the heart are shown in Figure 12 a). After quantifying the intensity of the extracts, the relative amount of insoluble protein was greater in older mice (12 mo.) than younger mice however, after statistical analyses, the increase was not significant (Figure 12 b).

a)



b)

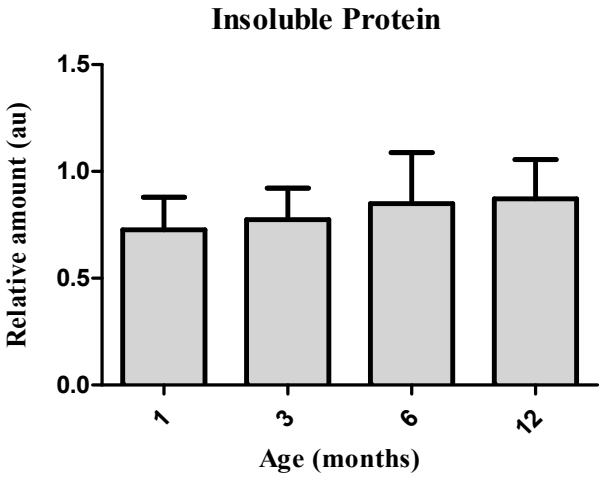


Figure 12 a) Insoluble Protein in the Heart. Image of insoluble protein fractions of the hearts of mice aged 1, 3, 6, and 12 mo. **b) Graph of Relative Amounts in the Heart.** The difference in the amounts of insoluble protein between age groups was not significant (Mann-Whitney U Test, $p>0.05$, $n=4$).

3.1.5 Lungs

Insoluble protein and total protein of lung samples from mice aged 1 mo., 3 mo., 6 mo., and 12 mo. is shown in Figure 13a. There appears to be almost no differences in the amount of insoluble proteins between age groups. After quantifying the intensity of the extracts, the relative amount of insoluble protein was greater in mice aged 12 mo. The relative amounts of insoluble protein for each mouse age group which reveal that after statistical analyses, there was no significant difference between mouse groups (Figure 13b).

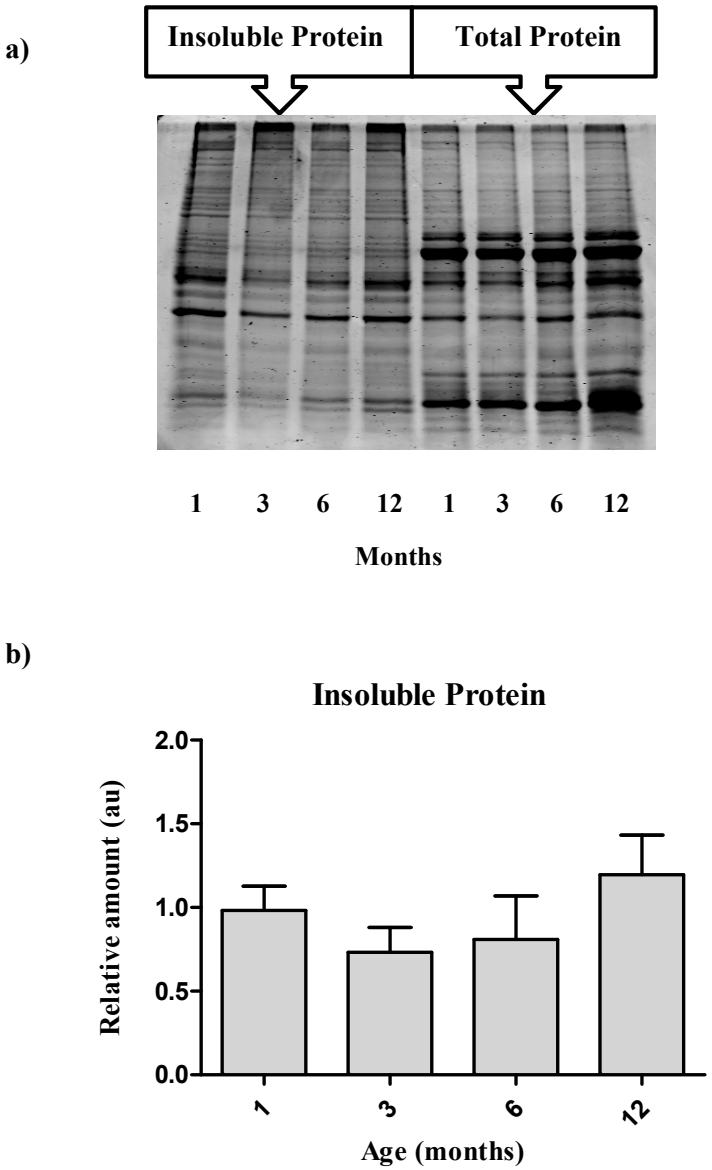


Figure 13 a) Insoluble protein in the lungs. Image of insoluble protein fractions of the lungs of mice aged 1, 3, 6, and 12 mo. **b) Graph of the relative amounts of insoluble protein present in the lungs of mice.** The difference in the amounts of insoluble protein between age groups was not significant (Mann-Whitney U Test, $p>0.05$, $n=4$).

3.1.6 Kidneys

The insoluble protein gel of insoluble and total protein in the kidneys of mice aged 1 mo., 3 mo., 6 mo., and 12 mo is shown in Figure 14 a). After quantifying the intensity of the extracts, the relative amount of insoluble protein remained relatively the same between age groups. the relative amounts of insoluble protein for each mouse age group which reveals that after statistical analyses, the difference between age groups was not significant Figure 14 b) shows.

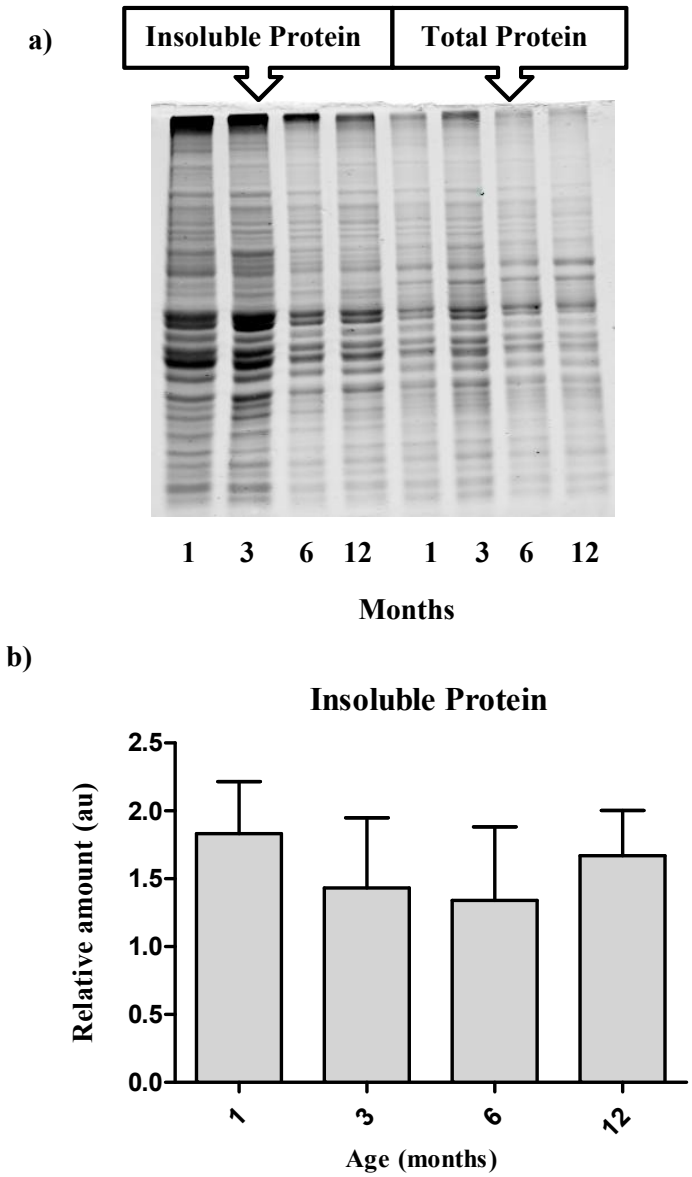


Figure 14 a) Insoluble protein in the kidneys. Image of insoluble protein fractions of the kidneys of mice aged 1, 3, 6, and 12 mo. **b) Graph of relative amounts in kidneys.** Graph of the relative amounts of insoluble protein present in the kidneys of mice. There was no significant difference between age groups (Mann-Whitney U Test, $p>0.05$, $n=4$).

3.1.7 Liver

The insoluble protein gel of insoluble and total protein in the liver of mice aged 1 mo., 3 mo., 6 mo., and 12 mo. is shown in (Figure 15 a). After quantifying the intensity of the extracts, the relative amount of insoluble protein remained relatively the same between age groups (Figure 14 b). After statistical analyses, the difference between age groups was not significant.

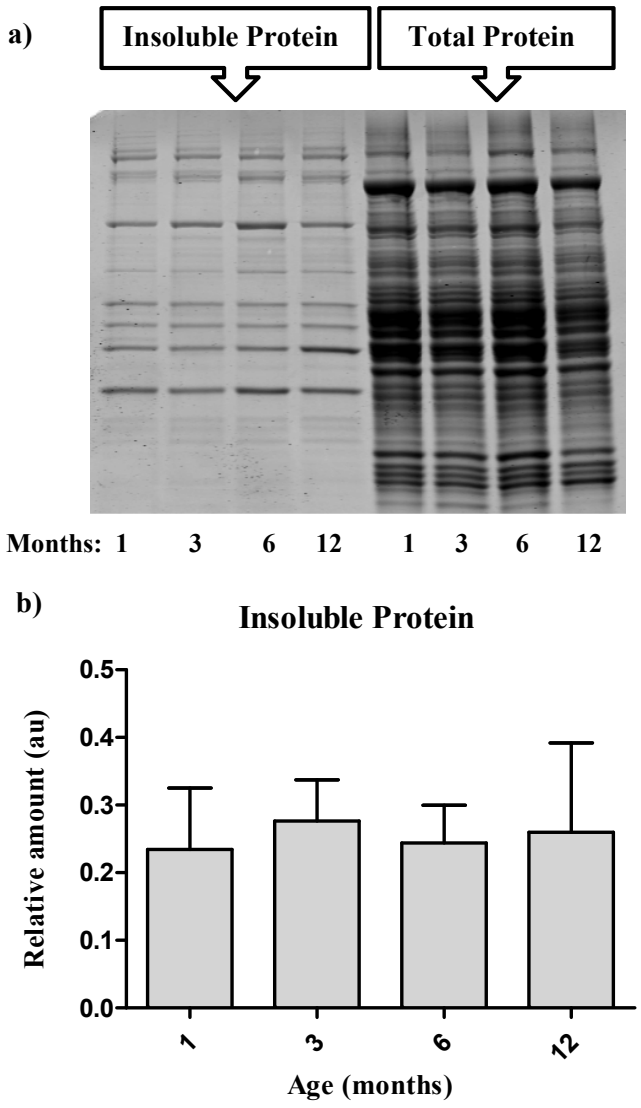


Figure 15 a) Insoluble protein in mouse liver. Image of insoluble protein fractions of the liver of mice aged 1, 3, 6, and 12 mo. **b) Graph of the relative amounts of insoluble protein in the liver of mice.** There was no significant difference between ages (Mann-Whitney U Test, $p>0.05$, $n=4$).

3.1.8 Summary of Results from SDS-PAGE

Tissue	Increase of Insoluble Protein with Age?	Were the differences significant?
Diencephalon	**Yes**	**Yes, *p=0.0286 between 1mo. and 12 mo. mice**
Cortex	**Yes**	**No**
Cerebellum	**No**	**No**
Heart	**Yes**	**No**
Lung	**Yes**	**No**
Kidney	**No**	**No**
Liver	**No**	**No**

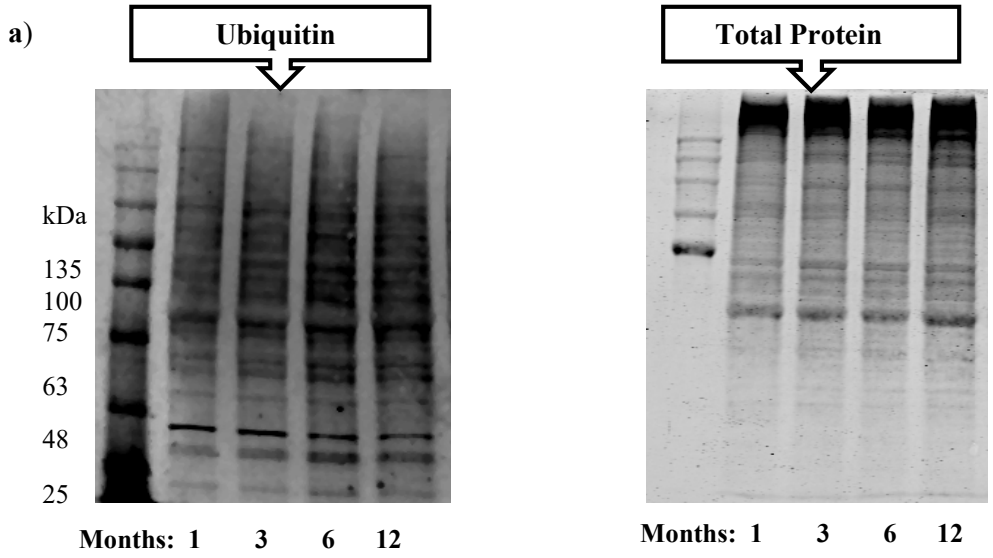
Table 3. Summary of SDS-PAGE results of relative insoluble protein amounts in each mouse tissue tested in this study.

3.2 Protein Ubiquitination

As a next step we investigated whether there were increased protein modifications, in this case, protein ubiquitination as the mice age. As stated in the introduction, when aging occurs, the efficiency of the UPS to clear misfolded proteins marked with ubiquitin decreases [26]. Ubiquitinated proteins accumulate in cells due to the UPS being unable to degrade these proteins [26]. In order to detect the ubiquitinated proteins, total protein samples from tissues in 1 mo., 3 mo., 6 mo., and 12 mo. mice were run by SDS-PAGE. The gels were then transferred onto nitrocellulose membranes. Membranes were stained for ubiquitin using Western Blot analysis. The gels were stained with Coomasie Blue. Following Western blot analysis, the intensities of the ubiquitinated proteins for each lane on the membranes were measured using ImageJ. The relative amount of ubiquitin is the ratio of the intensity of ubiquitin for each sample divided by the intensity of the total protein present in each sample in arbitrary units (au). There were tissues such as the cortex and liver that showed no ubiquitinated proteins. The tissues that had ubiquitinated proteins are shown in the following sections.

3.2.1 Protein Ubiquitination in the Diencephalon

Western blot analysis was done to determine the amount of ubiquitinated proteins present in the diencephalon of mice aged 1 mo., 3 mo., 6 mo., and 12 mo. (Figure 16 a). After measuring the intensities of the lanes using ImageJ, the relative amount of ubiquitin was obtained by dividing the intensity of each lane of the ubiquitin membrane by the intensity of the total protein for each sample (Figure 16 a). The bar graph in Figure 16 b) shows the relative amount of ubiquitinated proteins for each age group of mice in arbitrary units. As seen in the Figure 16 b) there is a significant increase in the relative amount of ubiquitinated proteins present in the diencephalon in the older mice compared to the younger mice.



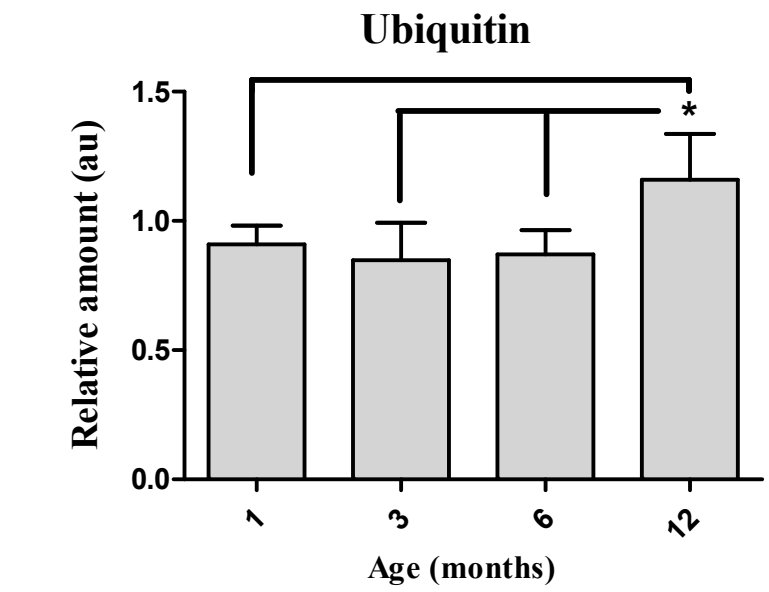


Figure 16 a) Ubiquitination in the Mouse Diencephalon Left Image of Western Blot of ubiquitin in the diencephalon of mice aged 1 mo., 3mo., 6mo., and 12 mo. **Right** Image of the total protein extracts of the same samples 1mo., 3mo., 6 mo., 12mo. **b) Graph of Ubiquitination in the Aging Mouse Diencephalon.** This graph shows the relative amount in arbitrary units (au) of ubiquitin in the diencephalon of mice aged 1 mo., 3 mo., 6 mo., and 12 mo. There was a significant difference of ubiquitinated proteins in the diencephalon of 12 mo. mice in comparison to 1 mo. ($p=0.0286$) 3mo. ($p=0.047$) 6mo. ($p=0.045$) (Mann-Whitney U Test, * Significant p-values were all $p< 0.05$, $n=4$).

3.2.2 Cerebellum

Ubiquitin expression in cerebellum samples from mice of 1 mo., 3 mo., 6 mo., and 12 mo. of age (Figure 17). As seen in the image, there are very little amounts of ubiquitinated proteins in the cerebellum. Due to the small amount of ubiquitin present in the cerebellum and also due to the poor quality of the membranes, the quantification of these extracts was not possible.

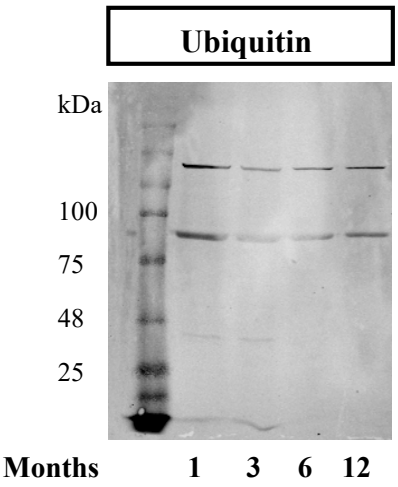


Figure 17 Western Blot of ubiquitin in the cerebellum. As seen in this image, the relative amount of ubiquitin in the cerebellum was very low and also difficult to quantify.

3.2.3 Heart

Western blot analysis of the amount of ubiquitinated proteins present in each heart sample is shown in Figure 18 a). The relative amount of ubiquitinated proteins (in arbitrary units) was measured for each age group of mice and there were no significant differences between mouse age groups (Figure 18 b). The Mann-Whitney U test was used as a statistical test to determine if there were significant differences.

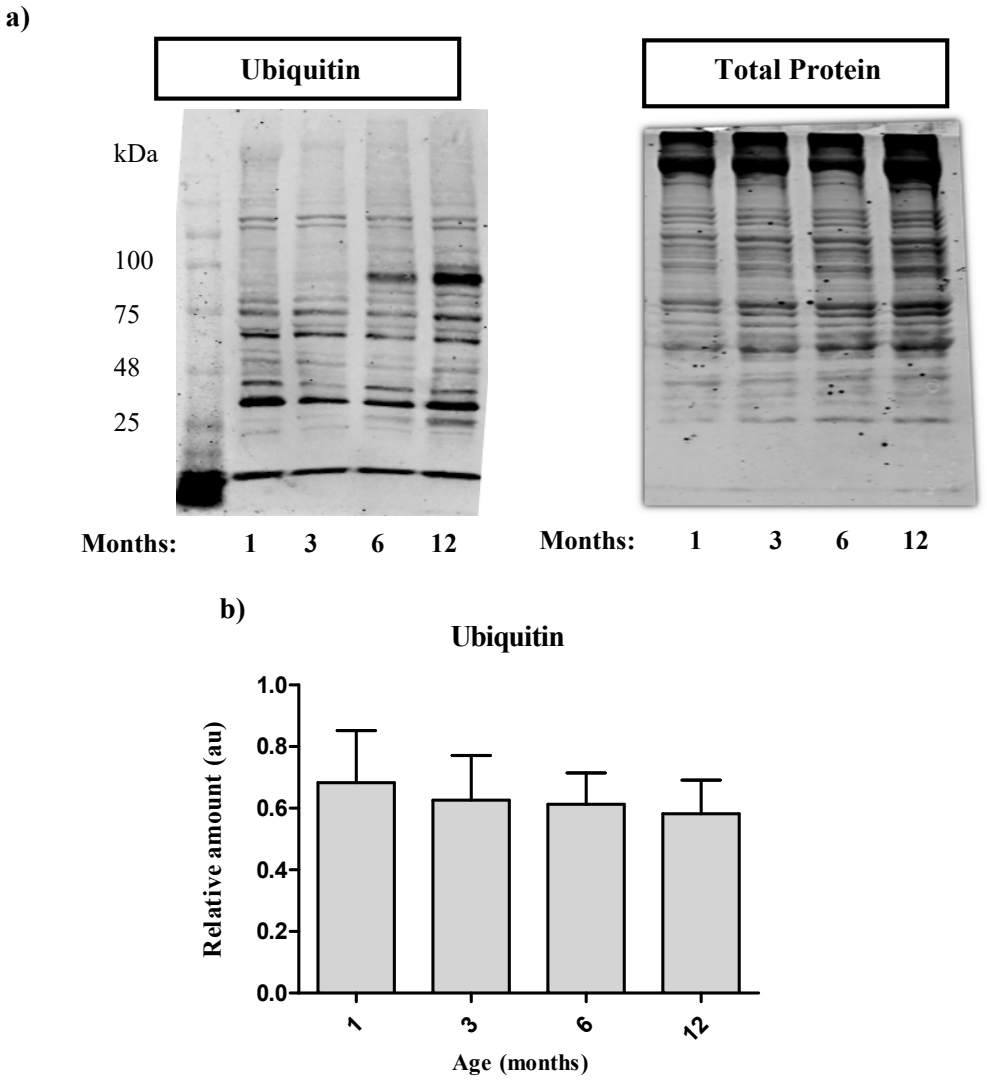


Figure 18 a) Ubiquitination in the Aging Mouse Heart. Image of Western Blot of ubiquitin in the heart of mice aged 1 mo., 3mo., 6mo., and 12 months. **b) Graph of Relative Ubiquitination in the Aging Mouse Heart.** This graph shows the relative amount in arbitrary units (au) of ubiquitin in the heart of mice aged 1 mo., 3 mo., 6 mo., and 12 mo. There were no significant differences between age groups (Mann-Whitney U Test, all $p > 0.05$, n=4).

3.2.4 Lungs

An image of ubiquitin expression in lung samples from mice of 1 mo., 3 mo., 6 mo., and 12 mo. of age as shown in Figure 19 a). As seen in the image, there was an increase in amount of ubiquitinated proteins. There was a gradual increase in the relative amount of ubiquitinated proteins present in the lungs in the older mice compared to the younger mice however, this difference was not significant (Figure 19 b).

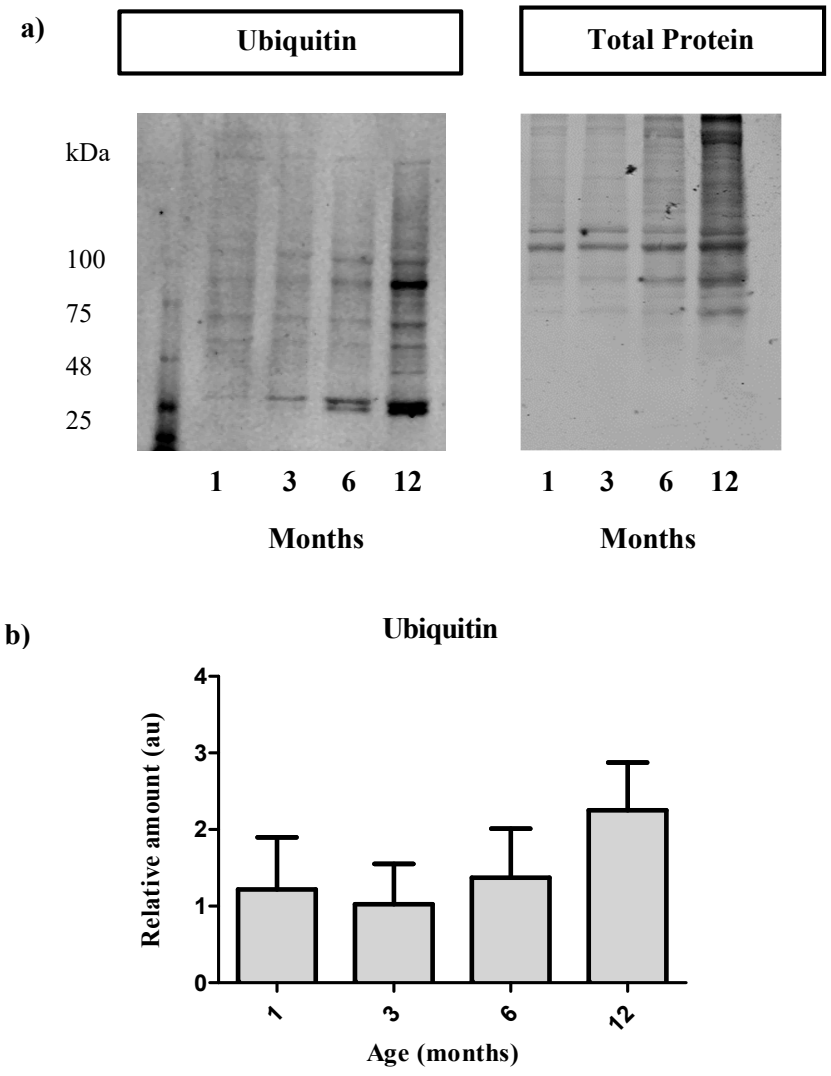


Figure 19 a) Ubiquitin expression in the Aging Mouse Lung. Image of Western Blot of ubiquitin in the lungs of mice aged 1 mo., 3mo., 6mo., and 12 months. **b) Ubiquitination in the Aging Mouse Lung.** This graph shows the relative amount in arbitrary units (au) of ubiquitin in the lungs of mice aged 1 mo., 3 mo., 6 mo., and 12 mo. There were no significant differences of ubiquitinated proteins in the lungs between ages (Mann-Whitney U Test, $p > 0.05$, n=4).

3.2.5 Kidneys

Ubiquitin expression with kidney samples from mice of 1 mo., 3 mo., 6 mo., and 12 mo. of age through Western blot analysis (Figure 20 a). As shown in Figure 20 b) the relative amount of ubiquitinated proteins for each age group of mice were measured. There was an increase in the relative amount of ubiquitinated proteins present in the kidneys in the older mice compared to the younger mice however, after statistical analysis, this difference was also not significant (Figure 20 b).

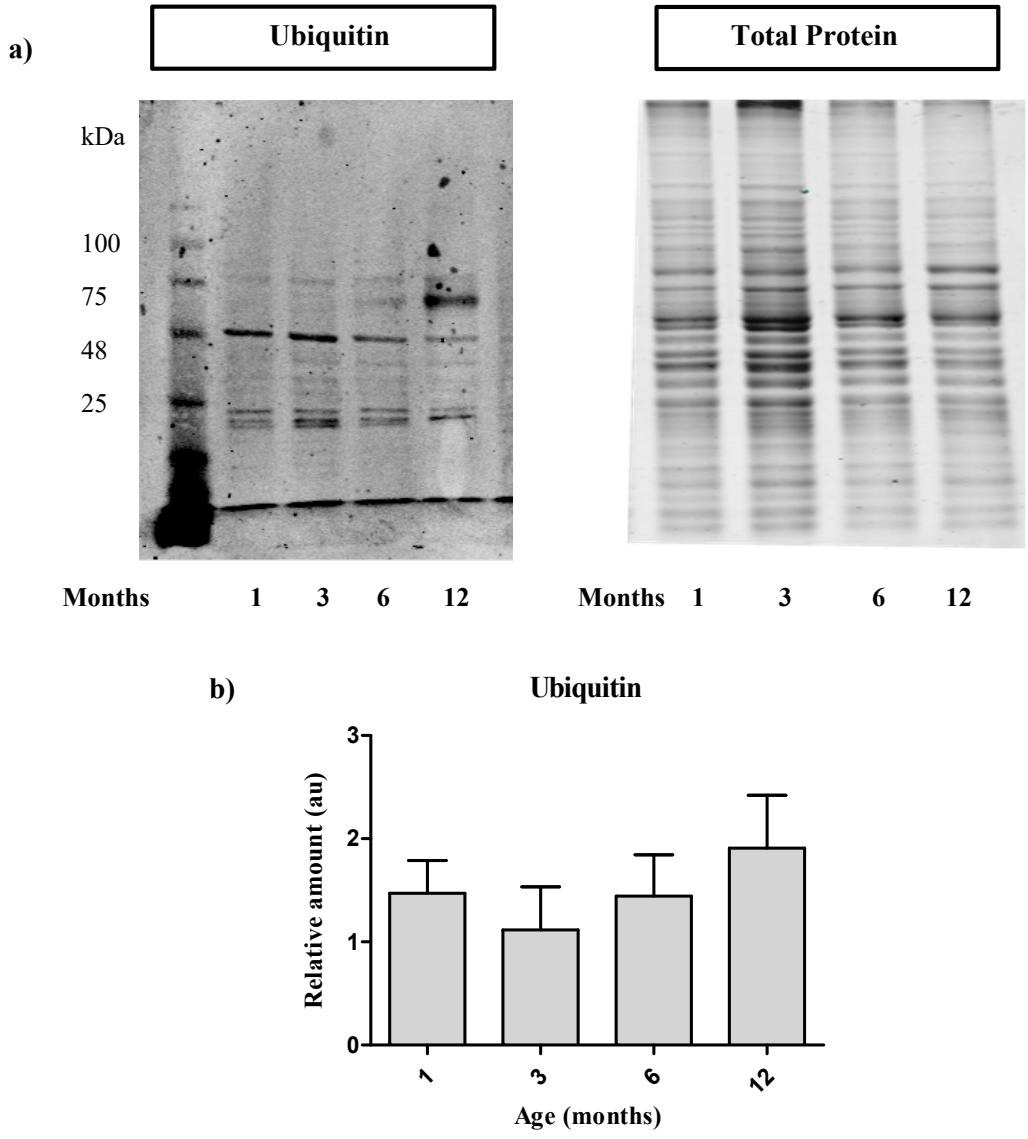


Figure 20 a) Western Blot of Ubiquitin in Kidneys. Image of Western Blot of ubiquitin in the kidneys of mice aged 1 mo., 3mo., 6mo., and 12 mo. **b) Relative Ubiquitination in the Aging Mouse Kidneys.** This graph shows the relative amount in arbitrary units (au) of ubiquitin in the kidneys of mice aged 1 mo., 3 mo., 6 mo., and 12 mo. Statistical analyses revealed that the difference between age groups was not significant

3.4.6 Summary of Ubiquitin Western Blot Results

Tissue	Increase of Ubiquitinated Proteins with Age?	Were the differences significant?
Diencephalon	**Yes**	**Yes, *1 mo. versus 12 mo. (p=0.0286), 3mo. versus 12 mo. (p=0.047) 6mo. versus 12 mo. (p=0.045).**
Cortex	**No**	**No**
Cerebellum	**No**	**No**
Heart	**No**	**No**
Lung	**Yes**	**No**
Kidney	**No**	**No**
Liver	**No**	**No**

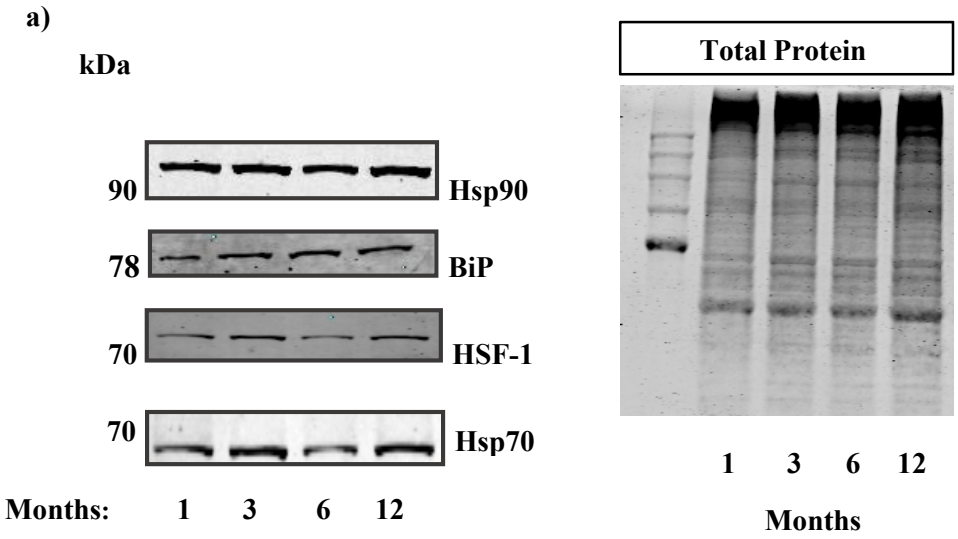
Table 4. Summary of Western blot analysis results of relative ubiquitinated protein amounts in each mouse tissue tested in this study.

3.3 Chaperone expression in aging mice

Protein chaperones such as Hsp70, Hsp90, and BiP are part of the heat shock response and are able to refold proteins that are misfolded [47,48]. Hsp90 works together with Hsp70 in order to maintain protein quality control [47,48]. Heat shock factor 1 (HSF-1) is a transcriptional factor which activates the transcription and production of HSPs [48]. In aging, there is a decline in heat shock response due to declines in protein chaperone levels [48]. Therefore, the next step was to analyze the expression of chaperone proteins Hsp70, Hsp90, and BiP as well as HSF-1 in the same mouse tissues as in previous sections through western blot analysis. Total protein samples were run by SDS-PAGE and then antibodies for each chaperone were used to see chaperone expression. Hsp70, Hsp90, and BiP was tested in the diencephalon, cortex, and heart. Expression of chaperone proteins was only seen in two brain regions, the diencephalon and cortex. Relative amounts of chaperone expression were calculated by measuring the intensities of the bands for each chaperone and then dividing these values by the intensities of total protein present in each sample (the same procedure as for ubiquitin) using ImageJ. Housekeeping genes were not used as internal standards due to several aging studies citing that housekeeping gene levels vary between ages [49]. The relative amounts are in arbitrary units (au). Statistical significance was determined by the Mann-Whitney U Test.

3.3.1. Chaperone expression in the Diencephalon

Western blot analysis was done to see the expression of chaperones Hsp70, Hsp90, BiP, and HSF-1 in the diencephalon of mice aged 1 mo., 3mo., 6 mo., and 12 mo. Figure 21 a) shows images of the bands from the western blot membranes. Images of the membranes were taken by an Odyssey® IR scanner. Figure 21 b) shows a graph of the average relative amounts of each chaperone in the diencephalon. There was increase in HSF-1 expression in older mice compared to younger mice, however after statistical analyses, this difference was not significant. Hsp90 levels increased between 1 mo. mice and 12 mo. mice. Hsp70 levels increased between 1 mo. and 6 mo. mice and then decreased between 6 mo. and 12 mo. mice. BiP levels remained unchanged between age groups. Mann-Whitney U tests between age groups determined that there were no significant differences in chaperone expression.



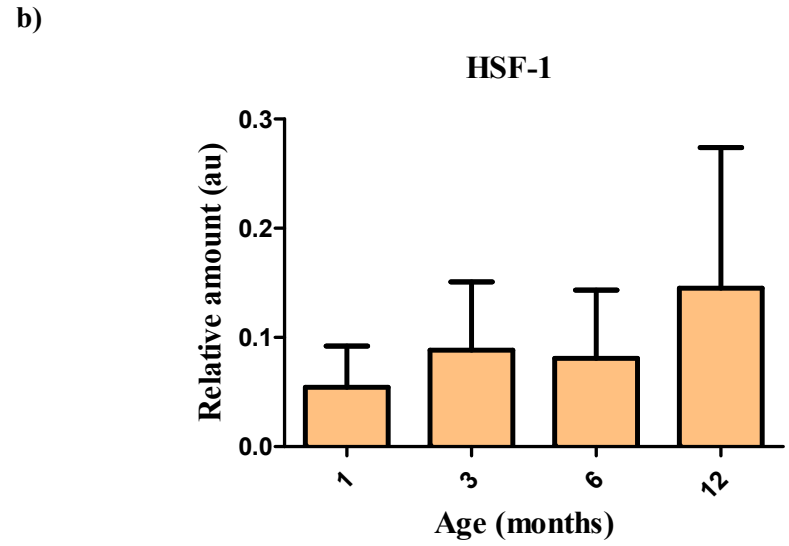


Figure 21 a) Chaperone Expression in the Diencephalon. Image of Western blot membranes of HSF1, Hsp90, Hsp70, and BiP expression in the Diencephalon of mice aged 1mo., 3 mo., 6 mo., and 12mo. **b) Graph of the relative amount of HSF1.** There was increase in HSF1 expression in older mice (12mo.) compared to younger mice, however after statistical analyses, this difference was not significant (Mann-Whitney, $p>0.05$, $n=4$).

c)

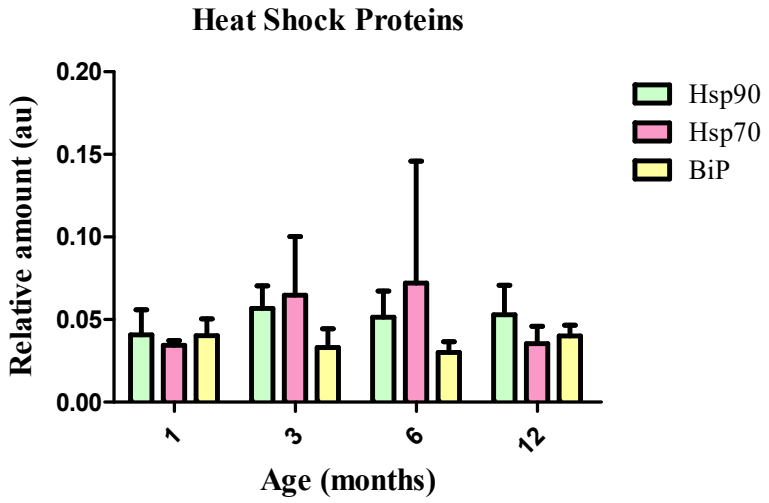


Figure 21 c) Graph of relative amounts of Hsp90, Hsp70 and BiP in the diencephalon of mice of four age groups. There was an increase in Hsp90 expression. In terms of Hsp70 expression, there was an increase between 1 mo. and 6 mo. and then a decrease between 6 mo. and 12 mo. BiP levels remained relatively unchanged. The differences in expression of Hsp90, Hsp70 and BiP between age groups were also not significant (Mann- Whitney U Test, $p>0.05$, $n=4$).

3.3.2. Chaperone Expression in the Cortex

Figure 22 a) shows an image of a western blot membrane showing the expression of Hsp90 in the cortex. The graph of the average relative amounts of each chaperone in the cortex. There was a gradual increase in Hsp90 expression in older mice compared to younger mice, however after statistical analyses, this difference was not significant (Figure 22 b). Other chaperone proteins such as Hsp70 and BiP were also tested however, these did not show any expression in the cortex.

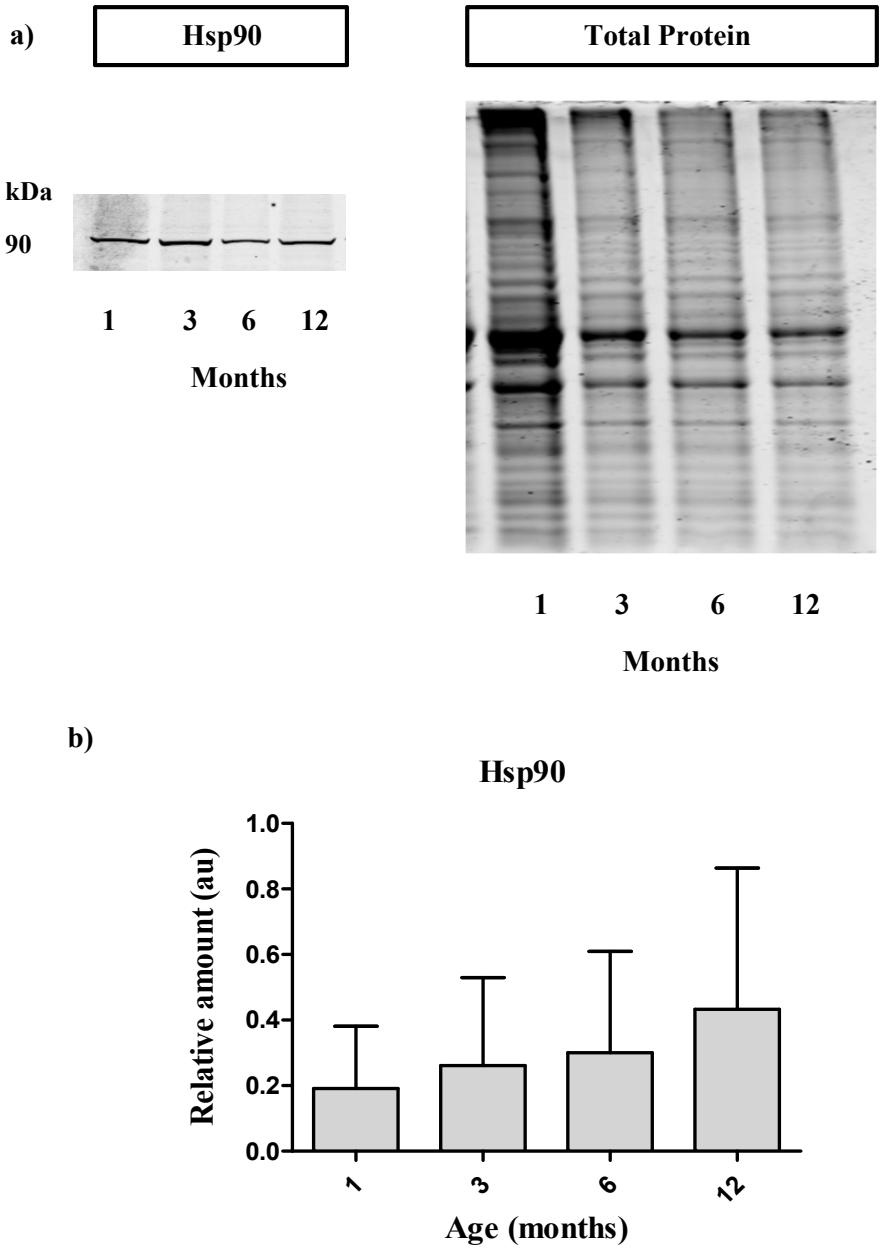


Figure 22 a) Hsp90 expression in the Cortex. Image of Western Blot membrane showing the expression Hsp90 in the cortex of mice aged 1 mo., 3 mo., 6 mo., and 12 months. **b) Relative amount of Hsp90 in the mouse cortex.** Graph of the relative amount of Hsp90 expressed in the cortex of the mice aged 1 mo., 3 mo., 6 mo., and 12 mo. There was an increase of Hsp90 in the cortex of 12 month old mice in comparison to the younger age groups. Statistical analyses determined the difference between age groups was non- significant (Mann-Whitney U Test, $p > 0.05$, n=4).

3.3.3 Summary of Western Blot Analysis of Chaperone Expression

Tissue	Was chaperone expression present?	Which proteins were present?	Were the differences significant?
Diencephalon	**Yes**	**HSF-1** **Hsp70** **Hsp90** **BiP**	**No**
Cortex	**Yes**	**Hsp90 only**	**No**

Table 5. Summary of Western blot results of relative chaperone protein amounts in each mouse tissue tested in this part of the study.

3.4 Protein Carbonylation in aging mice

Protein carbonylation has been considered a marker for aging [23]. As mice age, the amount of carbonyl content in tissues increases [24]. This is due to increased oxidative stress by reactive oxidative species (ROS) [24]. In this study, the carbonyl content measured in the diencephalon, heart, lung and kidney of 1 mo. and 12 mo. mice. There were no differences between the age groups in all four tissues. The protein carbonyl content present in the diencephalon, heart, lung, and kidney of mice aged 1 mo. and 12 mo. was obtained by measuring the absorbance of each sample at 370nm divided by the total protein content of each sample previously quantified by BCA. Figure 23 A-D shows the graphs of the protein carbonyl content for each tissue. Overall, there were no statistical differences in protein carbonyl content in 12 month mice compared to 1 month old mice in the diencephalon, heart, lung, and kidney (Mann-Whitney U Test, $p > 0.05$ for all tissues, $n=2$).

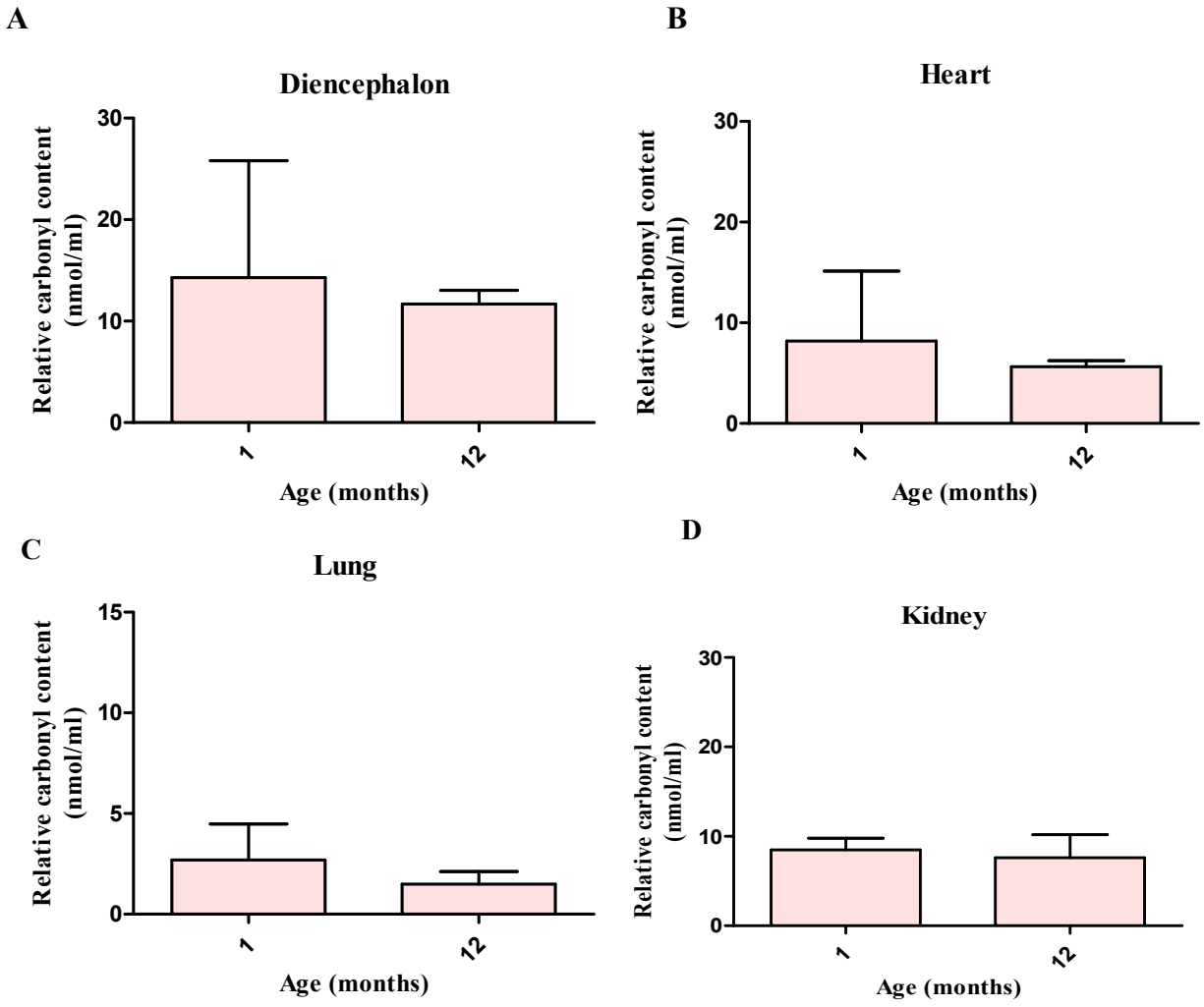


Figure 23- A. Carbonyl Content in the Diencephalon. There was no significant difference in carbonylated proteins in 1 mo. and 12 mo. mice. **B. Carbonyl Content in the Heart**. There was no significant difference in carbonylated proteins between 1 mo. and 12 mo. mice **C. Carbonyl content in the lungs.** There was no difference difference between the 1 mo. mice and 12 mo. mice was not significant. **D. Carbonyl content in the kidneys**. The difference between the age groups was not significant. (Mann-Whitney U test, all $p > 0.05$ for all tissues, n=2).

3.5 Summary of All Results

Tissue	Increase of Insoluble Protein in Older Mice?	Were the differences significant?	Increase of Ubiquitinated Proteins in older mice?	Was there expression of HSR proteins?	Carbonyl Content increase in older mice?
Diencephalon	**Yes**	**Yes**	**Yes, significant**	**Yes**	**No**
Cortex	**Yes**	**No**	**No**	**Yes**	**Not tested**
Cerebellum	**No**	**No**	**No**	**No**	**Not tested**
Heart	**Yes**	**No**	**No**	**No**	**No**
Lung	**Yes**	**No**	**Yes, not significant**	**No**	**No**
Kidney	**No**	**No**	**No**	**Not tested**	**No**
Liver	**No**	**No**	**No**	**Not tested**	**Not tested**

Table 6. Summary of all the results of this study

Part IV: Discussion, Conclusions & Future Studies

4.1 Insoluble Protein Profiles and Protein Aggregation in Aging Mice

In aging, cellular mechanisms involved in maintaining protein integrity such as the UPR and UPS become inefficient in degrading misfolded proteins [26]. As a result, misfolded proteins accumulate in cells and lead to further aggregation of more misfolded proteins [26]. In this study, the amount of insoluble proteins in several mouse tissues were analyzed using SDS-PAGE. Our results showed a significant difference in the amount of insoluble proteins between mice aged 1 month and 12 months in the diencephalon. In addition, there was an increase of insoluble proteins in the cortex, heart and lungs however, the difference between mouse age groups was not significant. This may be due to the oldest mouse group in this study being only 12 months of age which is equivalent to a 40 to 50-year-old human adult [29]. Mice only enter senescence at 18 months and have an average lifespan of 24 months [50]. In most aging studies, the oldest mice are usually between 18 -24 months [50]. If older mice were available for this study, perhaps the differences between age groups would have been greater. Also, the difference in the amounts of insoluble proteins seen among the tissues may indicate that the rate of aging varies among different tissues. A study by Zahn and colleagues showed that different mouse tissues age at different rates through microarray analysis of various genes, with expression levels varying between tissues during aging [51]. Therefore, based on our results and past aging studies, certain mouse tissues age faster than others at different rates.

All of the brain tissues, the diencephalon, cortex and cerebellum, had detergent- insoluble proteins in this study. However, in the diencephalon and cortex there was an increase in protein aggregates whereas in the cerebellum, insoluble protein levels remain unchanged. Recent epigenetic studies have shown that the cortex ages more quickly than the cerebellum [52]. The amount of DNA methylation, a biomarker of aging, is the lowest in the cerebellum compared to other brain areas such as parts of the cortex and diencephalon [52].

In addition, protein aggregation in the brain is very common, with protein aggregates being hallmarks of several neurodegenerative diseases [53]. In Huntington´s Disease, there are polyglutamine (polyQ) aggregates that are concentrated in the hypothalamus, a region of the diencephalon responsible for controlling appetite, blood pressure, hormone release, and sleep [53]. There have been recent studies that have looked at detergent-insoluble proteins in aging mice. Detergent-insoluble proteins from heart tissues in mice were shown to increase significantly with age and also in hypertension [47]. In mouse kidneys, specifically in glomerulus, there was a gradual accumulation of protein aggregates and oxidized proteins in older mice [54]. Perhaps having more mice (increasing the n value in our study) as well as older mice would provide more statistically significant results as those seen in past studies.

4.2 Ubiquitinated Proteins in Aging Mouse Tissues

The ubiquitin proteasome (UPS) system degrades damaged and abnormal proteins by tagging these proteins with ubiquitin for degradation via the proteasome [26,28]. In aging, the activity of the proteasome declines, incapacitating cells from efficiently removing damaged proteins [26]. As a result, large amounts of ubiquitinated proteins appear in cells because the amount of protein misfolding overwhelms proteasomal degradation of these proteins [26]. In this study, total protein samples from the diencephalon, cortex, cerebellum, heart, lungs, and kidneys were incubated with an anti-ubiquitin antibody in order to determine the amount of ubiquitinated proteins present in these samples. The amount of ubiquitinated proteins present in the diencephalon was significantly greater in 12 month old mice in comparison to mice with 1 month, 3 months, and 6 months of age. In the cortex and cerebellum, very few ubiquitinated proteins were found, that were unquantifiable. In terms of the other tissues, in the kidneys and lungs there was an increase in ubiquitinated proteins in older mice however the difference was not significant. In contrast, there was no significant difference in total ubiquitin expression in the heart.

A significant increase of ubiquitinated proteins was only found in the diencephalon, which is a region of the brain responsible for controlling bodily functions such eating, sleeping, blood pressure, hormone release and many other functions [55]. Studies have shown that the diencephalon and its subregions are subject to degeneration and aggregates in several age-related diseases [55,56]. Atrophy of the diencephalon is a characteristic of Parkinson´s Disease and Lewy Body dementia [55,56]. In particular, Lewy bodies contain mainly α-synuclein aggregates that are highly ubiquitinated and located in the diencephalon [56,57]. The hypothalamus, a region located in the diencephalon, plays an important role in controlling lifespan through the hypothalamic-pituitary-growth hormone axis [55-57]. The hypothalamic-pituitary-growth hormone axis regulates physiological bodily reactions to stress and inflammation in this region via NFκB signaling speeds up aging in mice by affecting their memory and physical endurance [58]. Currently, there are no studies that looked at normal aging and protein ubiquitination specifically in the diencephalon. However, there have been studies in nearby regions such as the hippocampus. In a study done by Yang and colleagues, the amount of polyubiquitinated proteins in the hippocampus was higher in 12 and 20 month old mice compared to 3 month old mice [59]. Levels of ubiquitinated proteins decreased with caloric restriction by activating autophagy, a pathway known to clear protein aggregates [59]. There is a scarcity of literature that investigates the amount of ubiquitination present in normal aging tissues and most studies focus on age-related and neurodegenerative diseases. For this reason, our results are providing some insight into how protein ubiquitination levels change in mouse tissues with normal aging.

4.3 Chaperone Expression, Heat Shock Response, & Aging

In aging, there is a decline in heat shock response where chaperone protein levels such as Hsp70 decrease [8,17,60]. Chaperone levels were analyzed in the diencephalon, heart and cortex. In the diencephalon, Hsp70 increased between 1 and 6 month old groups and decreased between 6 months and 12 month old groups. However, changes in Hsp70 expression were not significant. Studies in *c.elegans* as well as in rats have shown that the production of HSPs including Hsp70 decrease with aging [17,55,61]. Levels of chaperones decrease due to either dysregulation of transcription or sequestering by aggregates [60]. The decline in heat shock response has been shown to occur in the adult stage and is more evident in neuronal tissues as well as cardiac and skeletal muscle [8,30,33]. In addition, tissue culture and mouse studies have shown that Hsp70 levels decrease as protein aggregation increases [30, 31].

Heat shock factor 1 (HSF-1) is a transcriptional factor which activates the transcription and production of heat shock proteins [8,9,61]. In the diencephalon, which was the only tissue that showed any HSF-1 expression in our study, there was an increase in HSF-1 expression in older mice. There are no current research papers that have studied the levels of HSF-1 or Hsp70 in the diencephalon during normal aging. Our results have given some information of how HSF-1 and Hsp70 levels change with age in mice. Studies in flies and nematodes have shown that the heat shock response decreases during aging and when HSF-1 activity is increased, longevity is lengthened [8,18,62]. For instance, in *c. elegans*, the inhibition of HSF-1 leads to a decreased lifespan whereas overexpression of HSF-1 extends lifespan [9,18]. Therefore, the results from this study, although not statistically significant, indicates that the heat shock response is being activated in the older mice in order to promote expression of HSPs to bind to misfolded proteins present in the diencephalon. When misfolded proteins accumulate in cells, there is a release of HSF-1 from the multi-chaperone complex with Hsp90, Hsp70, and Hsp40 [9,10,61]. HSF-1 trimerizes and enters the nucleus where it binds to heat shock elements present in HSP genes and activates transcription of these genes to produce HSPs [9,10].

In both of the brain regions, the diencephalon and cortex, there was an increase in Hsp90 expression in this study. In normal conditions, without stress, there is expression of Hsp90 in all tissues since it is constantly involved in protein folding [15,63,64]. Hsp90 works together with Hsp70 in order to maintain protein quality control [16,63,64]. Hsp90 has been shown to have high levels of expression in patients with neurodegenerative diseases such as Parkinson´s Disease [65]. Increases in Hsp90 levels occur in the aging brain in order to compensate the loss of proteasome and is crucial in stabilizing and clearing aggregated proteins such as a-synuclein, huntingtin, and tau [65].

4.4 Protein Carbonylation in Aging

Protein carbonylation consists of reactive ketones or aldehydes which are formed when free radicals react directly with proteins [66]. This modification may result in the affected proteins losing their function. Protein carbonylation has been considered a marker for aging [66]. As mice age, the amount of carbonyl content in tissues increases [66, 67]. This is due to increased oxidative stress by reactive oxidative species (ROS) [67]. In this study, the carbonyl content measured in the diencephalon, heart, lung and kidney of 1 month old mice and 12 month old mice. There were no differences between the age groups in all four tissues. Perhaps the main reason why carbonyl content remains unchanged may be once again due to the oldest mice in this study being only 12 months old. Mice that are 12 months old are middle-aged and therefore, significant modifications are not as visible in this stage compared to older mice [50]. In a study by Tanase and colleagues, spleen and bone marrow protein aggregates were characterized with SDS-PAGE and the carbonyl content was measured in mice aged 3 months, 12 months and 22 months [68]. Results revealed that carbonyl content was significantly higher in 22 month old mice compared to 3 month old mice [68]. However, the difference between the 3 month old mice and 12 months old was not significant [68]. These results suggest that protein carbonylation increases with age but only significantly increases when mice are older than 12 months of age.

4.4.1 Protein Carbonylation in Tissues

Many studies have found that the brain is the organ most vulnerable to oxidative stress [69]. Therefore, most age-related protein carbonylation studies have been done on the brain specifically in regions such as the cortex and hippocampus [69]. Several age-related diseases are associated with an increase in carbonylation including Alzheimer´s Disease, Parkinson´s Disease and diabetes [69]. Normal and abnormal levels of ROS affect brain tissues due to the brain consuming large amounts of oxygen and having less antioxidant mechanisms than other tissues [69]. In terms of protein carbonylation in cardiac aging, studies on aging mice have shown that protein carbonyl content increases with age [69]. Also, in myocardial ischemia, a common cardiovascular disorder, there is a decrease in proteasomal activity resulting in an increase in carbonylated proteins present in heart tissue [70]. Future studies should look to include older mice as well as attempt other methods for measuring carbonyl content with antibody detection in order to further confirm the results in this study.

4.5 Concluding Remarks and Future Studies

To conclude, this study has found that there are insoluble proteins, possible protein aggregates, that increase as mice age. These insoluble proteins were more visible in the brain, with a significant increase in the amount of insoluble proteins being seen in the diencephalon of older mice. In addition, there was an increased amount of insoluble protein in the cortex, heart, and lungs however, the differences were not significant. The amount of ubiquitinated proteins also increased significantly in the diencephalon of older mice. Future studies should look to include mice that are 18 months and older, that have entered senescence, in order to confirm that insoluble protein levels continue to increase in older mice. Perhaps if mice that are older than 12 months are used, a greater difference between age groups might be seen in terms of insoluble, ubiquitinated and carbonylated protein levels. Considering the results of this study, it would be interesting to measure phosphorylated eIF2α (eIF2α-P) and ATF6 levels expression through western blot analysis to see if these proteins which are part of the UPR pathway are also increased in older mice. The next step would be to identify the proteins that are present in the insoluble fraction and total protein of the diencephalon through mass spectrometry. This would provide further information about which proteins have more tendency to aggregate in the diencephalon. Also, measuring carbonyl content of proteins using a more common method through Western blot analysis would further confirm our results from the carbonylation section. Based on the results seen in the diencephalon, it would be interesting to see if surrounding brain regions such as the hippocampus and striatum show similar results. Most studies done about these brain regions also focus more on the neurodegenerative diseases, therefore it would be novel to see what happens during normal aging. Also, using microarrays to analyze gene expression in tissues would provide more information about the aging process of each organ and how levels of gene expression alter with age.

References

1. López-Otín C, Blasco MA, Partridge L, Serrano M, and Kroemer G. The Hallmarks of Aging. Cell. 2013; 153:1194–217.

2. Morimoto RI. Proteotoxic stress and inducible chaperone networks in neurodegenerative disease and aging. Genes Dev. 2008; 22:1427–38.

3. Chen B., Retzlaff M., Roos T., and Frydman J. Cellular strategies of protein quality control. Cold Spring Harbor Perspectives in Biology 2011; 3: 1-14.

4. David, DC. Aging and the Aggregating Proteome. Frontiers in Genetics. 2012; 3: 1-6.

5. Contreras KC, Mukherjee A, and Soto C. Role of Protein Misfolding and Proteostasis Deficiency in Protein Misfolding Diseases and Aging. International Journal of Cell Biology 2013:1-10.

6. Lindquist S. The Heat-Shock Response. Annual Reviews in Biochemistry. 1986; 55:1151–11591.

7. Martínez I, Toda D, Vida C, Ortega E, La M De. Hsp70 basal levels, a tissue marker of the rate of aging and longevity in mice. EXG [Internet]. Elsevier Inc.; 2016; 84:21–8. Available from: http://dx.doi.org/10.1016/j.exger.2016.08.013

8. Calderwood SK, Murshid A, Prince T. The Shock of Aging: Molecular Chaperones and the Heat Shock Response in Longevity and Aging – A Mini-Review. Gerontology. 2009; 55:550–8.

9. Morley JF, Morimoto RI. Regulation of longevity in *Caenorhabditis elegans* by heat shock factor and molecular chaperones. Mol Biol Cell. 2004; 15:657–64.

10. Hartl FU, Bracher A, Hayer-Hartl M. Molecular Chaperones in Protein Folding and Proteostasis. Nature. 2011; 475: 324-332.

11. Rüdiger S, Buchberger A, & Bukau B. Interaction of Hsp70 chaperones with substrates. Nature Structural Biology. 1997; 4: 342−349.

12. Lotz, GP, Legleiter J, Aron R, Mitchell EJ, Huang SY, Ng C, Glabe C, Thompson LM, and Muchowsk PJ. Hsp70 and Hsp40 functionally interact with soluble mutant huntingtin oligomers in a classic ATP-dependent reaction cycle. Journal of Biological Chemistry. 2010; 285: 38183−38193.

13. HSPIR Hsp90. [Internet] HSPIR, 20 Jan. 2014. Web. 06 Dec. 2016 Available from: http://pdslab.biochem.iisc.ernet.in/hspir/hsp90.php

14. Pearl LH, and Prodromou C. Structure and mechanism of the Hsp90 molecular chaperone machinery. Annual Review Biochemistry, 2006; 75: 271-294.

15. Scheufler, C. Structure of TPR domain–peptide complexes: critical elements in the assembly of the Hsp70–Hsp90 multichaperone machine. Cell 2000; 101: 199−210

16. Wandinger, SK, Richter, K. & Buchner, J. The Hsp90 chaperone machinery. Journal of Biological Chemistry. 2008; 283:18473−18477.

17. Colotti C, Cavallini R, Vitale A, Donati M, Maltinti S, Del RE, Bergamini DG. Effects of Aging and Anti-Aging Carbonyl and Heat Shock Protein Levels and Expression. Biogerontology. 2005; 6:397-406.

18. Wang M., and Kaufman, RJ. The Impact of the Endoplasmic Reticulum Protein Folding Environment on Cancer Development. Nature Reviews Cancer. 2014;14: 581-597.

19. Moreno JA, Radford H, Peretti D, Steinhert JR, Verity N, Martin MG, Halliday M, Morgan J, Dinsdale D, Ortori CA, Barrett DA, Tsaytler P, Bertolotti A, Willis AE, Bushell M, and Mallucci GR. Sustained translational repression by eIF2α-P mediates prion degeneration. Nature. 2012; 485:507-511.

20. Malhotra JD, and Kaufman RJ. The Endoplasmic Reticulum and the Unfolded Protein Response. Seminars in Cell and Developmental Biology. 2007; 18: 716-731.

21. Bertolotti A, Zhang Y, Hendershot LM, Harding HP, Ron D. Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. Nature Cell Biology. 2000; 2:326–32.

22. Haze K, Yoshida H, Yanagi H, Yura T, Mori K. Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. Molecular Biology Cell 1999;10:3787–99.

23. Levine RL, Stadtman ER. 2006. Carbonylated proteins and their implication in physiology and pathology. In: Dalle-Donne I, Scaloni A, Butterfield DA, editors. Redox proteomics: From protein modifications to cellular dysfunction and diseases. United States of America: Wiley-Interscience. 123–168.

24. DeGraff AMR, Hazoglou MJ, Dill KA, Graff AMR De, Hazoglou MJ, Dill KA. Highly Charged Proteins : The Achilles ' Heel of Aging Theory Highly Charged Proteins : The Achilles ' Heel of Aging Proteomes. Struct Des [Internet]. Elsevier Ltd; 2016;1–8. Available from: http://dx.doi.org/10.1016/j.str.2015.11.006.

25. Nuss JE, Choksi KB, DeFord JH., and Papaconstantinou J. Decreased Enzyme Activities of Chaperones PDI and BiP in Aged Mouse Livers. Biochem Biophys Res Commum. 2008; 365(2):355-361.

26. Low P. The Role of Ubiquitin-Proteasome System in Ageing. Gen. Comp. Endocrinol. 2011; 172(1): 39-43.

27. Min, J. N. et al. CHIP deficiency decreases longevity, with accelerated aging phenotypes accompanied by altered protein quality control. Molecular Cellular Biology. 2008; 28: 4018–4025.

28. Murata S, Yashiroda H, Tanaka K. Molecular Mechanisms of Proteasome Assembly. Nature Ver. Mol. Cell Biol. 2009;10(2): 104-15.

29. Labbadia J, Morimoto RI. Proteostasis and Longevity: when does aging really begin? Prime Reports. 2015;6(1):435–64.

30. David DC, Ollikainen N, Trinidad JC, Cary MP, Burlingame AL. Widespread Protein Aggregation as an Inherent Part of Aging in C. elegans. PLoS Biol. 2010;8(8):1–23.

31. Rubinsztein DC, Mariño G, and Kroemer G. Autophagy and aging. Cell. 2011; (146): 682–95.

32. Taylor RC. & Dillin A. Aging as an Event of Proteostasis Collapse. Cold Spring Harbor Perspective in Biology. 2011;3: 1-17.

33. Kenyon, CJ. The Genetics of Ageing. Nature. 2010; 464: 504-512.

34. Ben-Zvl A, Miller EA, and Morimoto, RI. Collapse of proteostasis represents early molecular event in Caenorhabditis elegans. PNAS. 2009; 106 (35): 14914-14919.

35. Suh Y, Atzmon G, Cho M. O, Hwang D, Liu B, Leahy D. J, Barzilai N. & Cohen P. Functionally significant insulin-like growth factor I receptor mutations in centenarians. Proc. Natl Acad. Sci. USA. 2008; 105: 3438– 3442.

36. Willcox BJ. FOXO3A genotype is strongly associated with human longevity. Proc. Natl Acad. Sci. USA 2008; 105 (13):987–992.

37. Flachsbart F, Caliebe A, Kleindorp R., Blanche H, von Eller-Eberstein H, Nikolaus S, Schreiber S, and Nebel, A. Association of FOXO3A variation with human longevity confirmed in German centenarians. Proc. Natl Acad. Sci. USA 2009; 106: 2700–2705.

38. Leger F, Fernagut PO, Canron MH, Leoni S Vital C Bezard E Vital A. Protein aggregation in the aging retina. Journal of Neuropathology Experimental Neurology. 2011; 70(1):63-68.

39. Tartaglia GG, Pechmann S, Dobson CM, and Vendruscolo M. Life on the Edge: a link between gene expression and aggregation rates of human proteins. Trends in Biochemical Sciences. 2007; 32(5): 204-206.

40. Yuan R, Peters LL, and Paigen B. Mice as a Mammalian Model for Research on the Genetics of Aging. ILAR. 2011; 52:4-15.

41. Ayyadevara S, Mercanti F, Wang X, Mackintosh SG, Tackett AJ, Prayaga SVS. Aging and Hypertension Mouse Heart Have Similar Proteomic Profiles. Hypertension. 2016; 67:1–8.

42. Olson BJSC. Assays for Determination of Protein Concentration. Current Protocols in Pharmacology. 2016; 73: A.3.A.1-A.3A.32.

43. Hellman U. Sample Preparation by SDS/PAGE and in-gel digestion. Proteomics and Functional Genomics. 2000; 88: 43-54.

44. Oswald, N. How SDS-PAGE works. Techniques Protein Expression and Analysis: Bitesize Bio. 9 July 2016. Accessed from: http://bitesizebio.com/580/how-sds-page-works.

45. Mahmood T, Yang PC. Western Blot: Technique, Theory, and Trouble Shooting. 2012. North American Journal of Medical Science. 2012; 4 (9): 429-434.

46. Corder GW, Foreman DI. Nonparametric Statistics: A Step-by-Step Approach. Wiley. 2014.

47. Bobkova NV, Evgen M, Garbuz DG, Kulikov AM, Morozov A. Exogenous Hsp70 delays senescence and improves cognitive function in aging mice. PNAS. 2015;112(52).

48. Oosten-hawle P Van, Porter RS, Morimoto RI. Regulation of Organismal Proteostasis by Transcellular Chaperone Signaling. Cell [Internet]. Elsevier Inc.; 2013;153(6):1366–78. Available from: http://dx.doi.org/10.1016/j.cell.2013.05.015

49. Boda E, Pini A, Hoxha E, Parolisi R, and Tempia F. Selection of reference genes for quantitative real-time RT-PCR studies in mouse brain. Journal of Molecular Neuroscience. 2009; 37:238-253.

50. Dutta S, Sengupta P. Men and mice: Relating their ages. Life Sci [Internet]. Elsevier Inc.; 2015
51. Zhan JM. AGEMAP: A gene expression database for aging in mice. PLoS Genetics. 2007; 3:2326-2337.
52. Horvath S, Mah V, Lu AT, Woo JS, Choi OW, Jasinska AJ, et al. The cerebellum ages slowly according to the epigenetic clock. 2015;7(5):294–305.
53. Kotliarova S, Jana NR, Sakamoto N, Kurosawa M, Nekooki M, Doi H, et al. Decreased expression of hypothalamic neuropeptides in Huntington disease transgenic mice with expanded polyglutamine-EGFP fluorescent aggregates. J Neurochem. 2005; 93:641–53.

54. Wanner N, Hartleben B, Herbach N, Goedel M, Stickel N, Zeiser R, et al. Unraveling the Role of Podocyte Turnover in Glomerular Aging and Injury. Journal of the American Society of Nephrology. 2014; 25:707–716.

55. Morton, G. J., Cummings, D. E., Baskin, D. G., Barsh, G. S. & Schwartz, M. W. Central nervous system control of food intake and body weight. Nature. 2006; 443, 289–295.

56. Vallortigara J, Hortobagvi T, Attems J, Aarsland D, Ballard CG, O´Brien J, and Francis PT. Correlations between synaptic proteins expression and clinic-pathological features in dementia with Lewy bodies and Parkinson´s disease dementia. Society for Neuroscience. 2012; 621: 02

57. Tortosa GE, Newell K, Irizarry MC, Sanders JL, Hyman BT. Alpha-Synuclein immunoreactivity with Lewy bodies: morphological staging and comparison with ubiquitin immunostaining. Acta Neuropathology. 2000; 99: 352-357.

58. Zhang G, Li J, Purkayastha S, Tang Y, Yin Y, Li B, et al. Hypothalamic Programming of Systemic Aging Involving IKKβ/NF-κB and GnRH. Nature. 2013;497(7448):211–6.

59. Yang F, Chu X, Yin M, Liu X, Yuan H, Niu Y, et al. mTOR and autophagy in normal brain aging and caloric restriction ameliorating age-related cognition deficits. Behav Brain Res. 2014;1–9

60. Morley JF, Morimoto RI. Regulation of longevity in *Caenorhabditis elegans* by heat shock factor and molecular chaperones. Mol Biol Cell. 2004; 15:657–64.

61. Oosten-hawle P Van, Porter RS, Morimoto RI. Regulation of Organismal Proteostasis by Transcellular Chaperone Signaling. Cell [Internet]. Elsevier Inc.; 2013;153(6):1366–78. Available from: http://dx.doi.org/10.1016/j.cell.2013.05.015

62. Moskalev A, Shaposhnikov M, Turysheva E. Life span alteration after irradiation in *Drosophila melanogaster* strains with mutations of Hsf and Hsps. Biogerontology. 2009; 10:3–11. .

63. Taipale M., Krykbaeva I, Koeva M, Kayatekin C, Westover KD, Karras GI, Lindquist S, Quantitative analysis of HSP90-client interactions reveals principles of substrate recognition. Cell 2012; 150:987-1001.

64. Nielsen M. Linking HSP90 target occupancy to HSP70 induction and efficacy in mouse brain. Pharmacol Res. Elsevier Ltd; 2015.

65. Baldo B, Weiss A, Parker CN, Bibel M, Paganetti P, Kaupmann K. A screen for enhancers of clearance identifies huntingtin as a heat shock protein 90 (Hsp90) client protein. Journal of Biological Chemistry. 2012; 287:1406-1414.

66. Levine RL, Stadtman ER. Carbonylated proteins and their implication in physiology and pathology. In: Dalle-Donne I, Scaloni A, Butterfield DA, editors. Redox proteomics: From protein modifications to cellular dysfunction and diseases. United States of America: Wiley-Interscience. 2006: 123–168.

67. Cabiscol E, Tamarit J, Ros J. Protein carbonylation: proteomics, specificity and relevance to aging. 2014; 21–48.

68. Tanase M, Urba AM, Zolla V, Cleme CC, Huang L, and Morozova K. Role of Carbonyl Modifications on Aging-Associated Protein Aggregation. Nature. 2016;6(January):1–14.

69. Perluigi M, Di Domenico F, Giorgi A, Schinina ME, Coccia R, Cini C, Bellia F, Cambria MT, Cornelius C, Butterfield DA, Calabrese V. Redox proteomics in aging rat brain: Involvement of mitochondrial reduced glutathione status and mitochondrial protein oxidation in the aging process. Journal of Neuroscience Research. 2010; 88: 3498–3507.

70. Dai D, Chen T, Johnson SC, Szeto H, Rabinovitch PS. Cardiac Aging: From Molecular Mechanisms to Significance in Human Health and Disease. 2012;16(12):1492–526.

Appendix 1

- ELB Buffer (10ml)

  H20 8,95ml

  Triton X-100 50 µl

  HEPES 1M pH7 500 µl

  NaCl 5M 500 µl

- ELB Complete

  ELB 4,645 ml

  DTT 1M 5 µl

  NaF 1M 5 µl

  EDTA 0,5M 20 ul

  EGTA 100.0 mM 50 µl

  Na_3VO_4 100.0 mM 50 µl

  Roche 50x 20 µl

  PMSF 40mM 25 µl

- BCA Protein Assay Kit (Pierce, Rockford IL)

- Stacking (Upper) and Resolving (Lower) Gel

Reagents	Lower (10%)	Upper (4%)
H_20	3.6 ml	3.464 ml
Tris HCl	pH 8.8 3.75 ml	pH 6.8 1ml
Acrylamide (29.1)	2.5 ml	0.5 ml
SDS	100 µl	50 µl
APS	100 µl	50 µl
TEMED	10 µl	10 µl

- APS (Ammonium Persulfate) 10%

  Dissolve 1 g of APS in 10 ml of deionized H_20.

- SDS (Sodium Dodecilsulfate) 10%

  Dissolve 1g of SDS in 10 ml of deionized H_20

- Loading Gel Buffer (6x)

  Tris HCl/SDS 6.05g Tris, 0.5g SDS for 100ml pH 6.8

  For 7 ml of loading gel buffer (6x)

  Glycerol 87% 3ml

  SDS 1g

  DTT 0.93g

  Bromophenol 6mg

- Coomasie Blue (0.25%)

  50% Methanol

  10% Acetic Acid

  0.25g/100ml Coomasie Blue

  ddH_20

- Destaining Solution

  Ethanol 100ml

  Acetic Acid 75ml

  Distilled H20 825ml

- 10x Running Buffer

  Glycine 144g

  Tris Base 30.2g

  SDS 10g

  Distilled H_20 Fill until 1L

Western Blot

- 1x Transfer Buffer

  Transblot Turbo Transfer buffer 200ml

  Ethanol 200ml

  Nanopure H_20 600ml

- 1x TBS solution

  Tris base 6.05g

  NaCl 8.76g

  Distilled H_20 800ml

- 1x TBST (Tween 20)

  TBS 100ml

  Distilled H_20 900ml

  Tween-20 1ml