Proteomic Analysis Suggests a Role for HtrA Serine Peptidase 1 in Immune Regulation and Thereby in Age-Related Macular Degeneration

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"Nothing is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."

"Life is not easy for any of us. But what of that? We must have perseverance and above all confidence in ourselves. We must believe that we are gifted for something and that this thing must be attained."

"I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale."

Marie Curie

Statement of Authorship

I hereby certify, to my best of knowledge, that this thesis contains no material that has been extracted totally or partially from a thesis, dissertation or research paper by which I have qualified for or been awarded another degree or diploma.

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Lili FENG

Abstract

Purpose. Single-Nucleotide Polymorphisms on chromosome 10 at the region 10q26 that harbors the three genes, PLEKHA1, ARMS2 and HtrA1, show strong association with the pathogenesis of age-related macular degeneration (AMD). In the last decade, evidence was mounted that mutations in the HtrA1 gene are a major causative factor of this disorder in Caucasian populations. The current study was undertaken in order to enhance the understanding the role of this HtrA1 in the pathogenesis of AMD.

Methods. A HtrA1 gene trap mouse model was employed to compare the differences in protein expression between the gene trap mouse and a wild type mouse. ARPE19 and HeLa cells were transfected with a plasmid coding for triple-tagged, proteolytically inactive HtrA1. Next, a Co-IP assay was used to isolate HtrA1 interacting proteins. Selected candidates were validated with Western blot. Potential substrates of HtrA1 were tested by an in vitro digestion assay.

Results. Our study indicated that HtrA1 as a secreted protein binds to various complement components, e.g. CFP, CFD, CFI, C1r, C1s, C2, C3 and C5 in the classical pathway, as well as to components of the lectin-mediated pathway and, in particular, to components of the alternative pathway. Several of these components have been implicated in the pathological process of age-related macular degeneration. Notably, a regulatory component of the innate immune activity, Ubiquitin-like protein ISG15 directly interacts with HtrA1 in our Co-IP. Furthermore, our in vitro digestion assay showed that Ubiquitin-like protein ISG15 is a substrate of HtrA1 protease.

Conclusion. The aberrant activation of the complement system by HtrA1 found in our assays underlies the importance of the immune system in the pathogenesis of AMD. Our results suggest that HtrA1 may exert its biological function through the regulation of the immune system.

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Abbreviations

AGRN	Agrin
AMD	Age-related macular degeneration
ARMS2	Age-related maculopathy susceptibility 2
BF	Complement factor B
BMI	Body mass index
BSA	Bovine serum albumin
C1r	Complement Component 1, R Subcomponent
C1s	Complement Component 1, S Subcomponent
C2	Complement component 2
C3	Complement component 3
C5	Complement component 5
CARASIL	Cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalophthy
CD59	CD59 Molecule, Complement Regulatory
CFD	Complement factor D
CFH	Complement factor H
CFHR	Complement factor H-related proteins
CFI	Complement factor I
CFP	Complement factor P
CNS	Central nervous system
CNV	Choroidal neovascularization
Co-IP	Co-immunoprecipitation

dFBS	Dialyzed fetal bovine serum
FBS	Fetal bovine serum
GWAS	Genome-wide association study
HtrA1	High-temperature requirement A Serine peptidase 1
ICPL	Isotope-coded protein label
IGFBP	Insulin-like growth factor binding protein
IGFBP6	Insulin-like growth factor binding protein 6
IGFBP7	Insulin-like growth factor binding protein 7
IL6	Interleukin 6
IP	Immunoprecipitation
ISG15	Interferon-stimulated gene 15
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LD	Linkage disequilibrium
LIPA	Lipase A, Lysosomal Acid, Cholesterol
MAC	Membrane attack complex
MS	Mass spectrometry
OR	Odds ratio
PCR	Polymerase chain reaction
PLEKHA1	Pleckstrin homology domain containing family A Member 1
PRRs	Pattern recognition receptors
PRSS23	Protease, Serine, 23
PVDF	Polyvinylidene difluoride

RPE	Retinal pigment epithelium
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SERPING1	Serpin Peptidase Inhibitor, Clade G (C1 inhibitor), member1
SERPINH1	Serpin Peptidase Inhibitor, Clade H (Heat Schock Protein 47), Member 1
SILAC	Stable isotope labeling of amino acids in cell culture
SNPs	Single nucleotide polymorphisms
VEGF	Vascular endothelial growth factor
VTN	Vitronectin
WARS	Tryptophanyl-TRNA Synthetas

1. Introduction

Age-related macular degeneration (AMD) is the major cause of permanent blindness in aged individuals (sixty years and older) in the developed world. It causes damages of the macula, a very small region in the center of retina, which is responsible for sharp, central and light vision in humans. People who suffer from AMD may have serious visual problems.

AMD may affect one or both eyes and the progression of the disease varies highly between individuals. As AMD progresses, patients will develop a common symptom, which is a blurred spot or area in the central vision. After some time (days to years), the blurred area will grow larger and patients will develop blank blots in the central vision area. Objects also may not appear as brilliant as before.

In the last three decades, there have been numerous epidemiological research studies on AMD. In a case-control meta-analysis study in Caucasian people aged sixty years and older, the incidence of early AMD was estimated to be 6.8% and late AMD 1.5% (Smith et al., 2001). More than 30 million people in the world suffer from visual impairment by the reason of AMD, which is estimated to account for more than US$300 billion in economic costs annually worldwide (Oneill, Jamison, McCulloch, & Smith, 2001).

The studies of AMD have been at the leading edge of the research of complicated diseases. The discovery of the Tyr402His SNP of complement factor H (CFH) that confers expanded epidemic risk to develop AMD was the first of its kind for a complicated disorder (Edwards et al., 2005; Haines et al., 2005).

1.1 Definition and classification of AMD

AMD is a disruption or deterioration of human eye's macula. The macula is a central spot of the retina---the photosensitive tissue lining the innermost of the eye, which is also the most important region of the retina that is accountable for the fundamental, colorful and sharp vision in human eyes (Fig. 1).

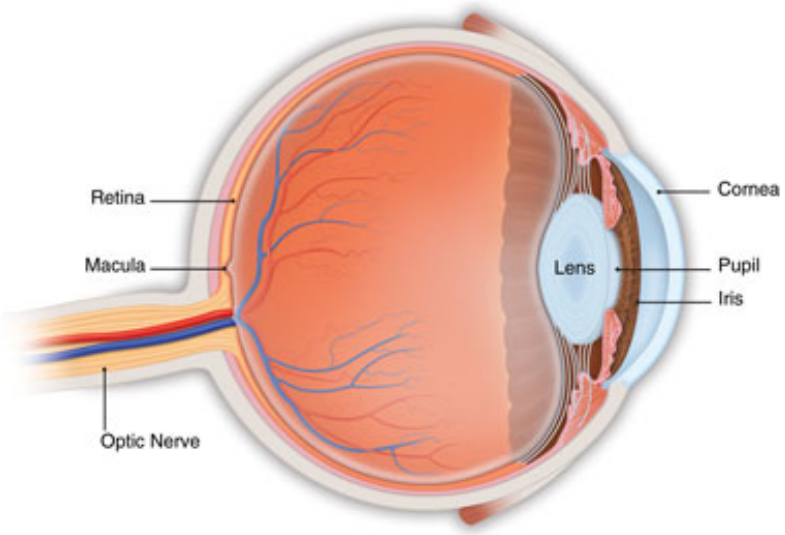


Figure 1. Human eye structure (longitudinal section) From the front to the back of human eye, there are cornea, iris and pupil, lens, vitreous humor, retina, optic nerve. Macula is the center spot of the retina, and it is responsible for sharp, colorful vision. Blue and red lines are retinal veins and arteries. *(Source: http://www.wnyretina.com)*

1.1.1 Macula

The macula is an oval-shaped/rounded highly pigmented yellow area located in the central retina, and it has a diameter about 5.5 mm (Fig. 2). The macula is usually histologically characterized as having more than two layers of retinal ganglion cells. The definite center of the macula is called fovea, which is the spot containing the highest concentration of retina cone photoreceptors in human retina and is accountable for the sharp and detailed vision.

The macula is normally straw yellow in color (also depends on the illumination), which is because of its content of lutein and zeaxanthin. These two molecules are yellowish and derived from daily diet. These pigments can absorb excessive harmful blue and ultraviolet light that enter the eye.

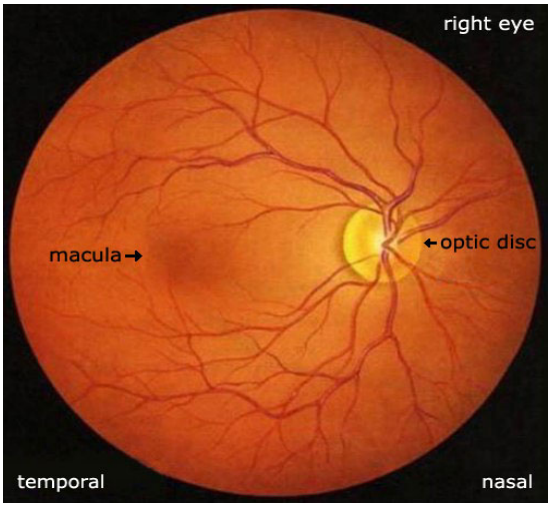


Figure 2. The localization of the macula as seen with an ophthalmoscope. In the center of the retina, the head of the optic nerve (optic disc or papilla, ~2 mm in diameter, arrowed) can be observed. Note the blood vessels radiating from this area. The reddish spot temporal to the papilla is the macula (arrowed). The area is devoid of blood vessels. *(Source; http://pubs.rsc.org)*

1.1.2 Photoreceptors

Photoreceptor cells are a type of specific neurons in the retina that have the capacity of photo-transduction. Photoreceptors have great biological value because they can transfer photon signals (visible electromagnetic radiation) into stimulating electrical signals. More precisely, specific proteins in the photoreceptors can absorb photons and trigger an action potential in the cell's membrane.

There are two types of photoreceptors in human retina. Depending on their cell body shape, they can be categorized into rod and cone photoreceptors. For human retina, there is only one type of rod photoreceptors; and three types of cone photoreceptors, depending on their light spectrum

sensitivity, they are separately named as short-wave cone (blue cone, 580nm), middle-wave cone (green cone, 530nm) and long-wave cone (red cone, 420nm) (Fig. 3).

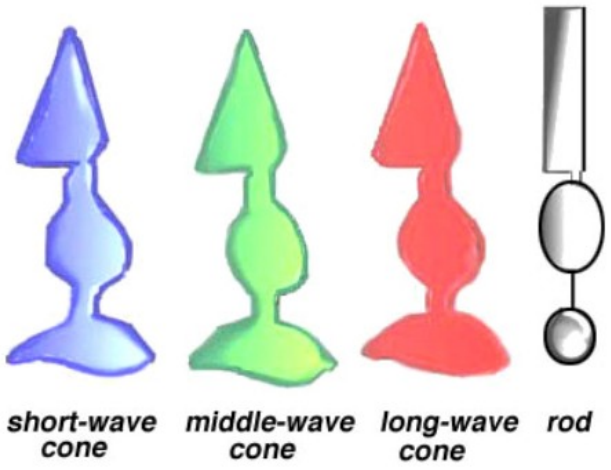


Figure 3. Human photoreceptors 3 types of cones (Blue-, Green- and Red-Cone) and 1 type of rods are show. They are defined by cell body shape and light spectrum sensitivity. *(Source: http://webvision.med.utah.edu)*

Both rods and cones contribute to the visual system to form vision. Cone activity acts upon photopic vision and functions when retina responses to a broader spectrum of light stimulation. Bright illumination is necessary for sharp accurate and color discrimination vision in human retina. Rods are extremely sensitive in dim-lit situations (scotopic vision, detection objects in low illumination), whereas cones are poorly sensitive. Normally, rods cannot resolve refined details of objects, and color discrimination is missing. Objects are seen in shades of gray.

In the last decade of the 20th century, the third type of photoreceptor cells was discovered: the photosensitive ganglion cells. These ganglion cells do not contribute to the vision directly, but they are closely related to the support of pupillary reflex and circadian rhythms (Foster et al., 1991).

The human retina consists of around 120 million rods and 6 to 7 million cones, and 1.5 million ganglion cells; between 1% and 2% of them are lights sensitive. The retina is often described as two regions: the central retina and the peripheral retina. The central area (macula) is specialized for accurate vision and color discrimination (cones dominant). The peripheral part is primarily

designated for distinguishing large objects and movement (rods dominant). For the photoreceptor composition, the central retina is abundant in cones while the peripheral retina is dominantly composed of rods (Fig. 4).

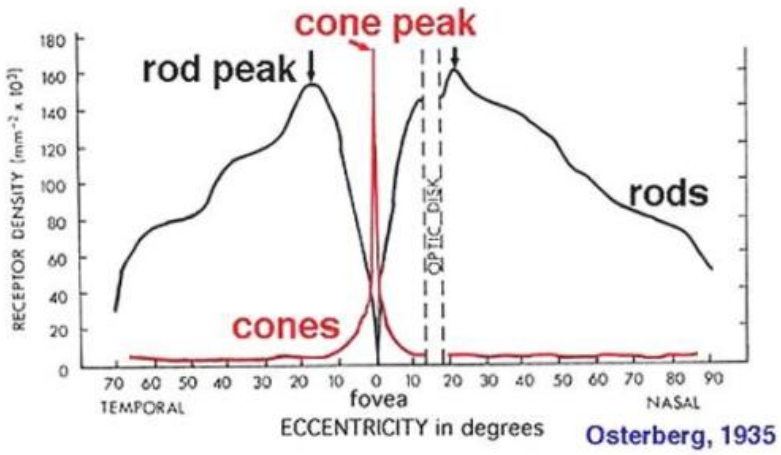


Figure 4. Distribution of cones and rods in human retina Measured density curves of the photoreceptor cells in human retina. Cones are shown an enormous density in the centralis of macula (fovea). For rods, they are absent for fovea, and their density rises from a few degrees away. Rods spread over large area of retina. For the optic disc, there are no photoreceptors because the optic nerve goes through it and it is the blind point in human retina (scotoma). *(Source: Osterberg, 1935)*

1.1.3 Classification of AMD

Phenotypically, AMD is categorized into two forms---'early' and 'late' AMD based on distinct clinical features (Casswell, Kohen, & Bird, 1985; Hageman et al., 2001). Early AMD is defined by the existence of few drusen (normally less than ten) with pigment changes, either hypopigmentation or hyperpigmentation (drusen, the white yellowish extracellular deposits that can be seen in funduscopy, a characteristic marker of AMD.) and thickening of the Bruch's membrane. Advanced AMD is associated with photoreceptor deterioration following either degeneration of the choroid vasculature and retinal pigment epithelium (RPE) cells or the invasion of choroid capillaries through the Bruch's membrane into the neuronal layer of retina. These features further sub-classify AMD in two categories: 'dry' AMD and 'wet' AMD (Ambati, Ambati, Yoo, Ianchulev, & Adamis, 2003; Casswell et al., 1985; Hageman et al., 2001).

1.1.3.1 Dry AMD

Dry AMD is the most prevailing subtype of AMD, more than 85% of the AMD patients belong to this subtype, which is manifested in the early stage of this disease. In this subtype, macular cells, especially RPE cells, progressively become thinner and degenerate (atrophy). RPE cells are crucial for the natural function of the cone and rod photoreceptors, which may also continuously degenerate.

Dry AMD progresses slowly as the number of the damaged cells increases, which usually takes several years for the deterioration of vision. Many patients suffering from dry AMD do not lose their precise visual perception completely (Fig. 5).

1.1.3.2 Wet AMD

About 10 to 15 percent of dry AMD patients may progress to the wet form, also called neovascular or exudative of AMD. The majority of vision impairment (~90%) appertains to wet/exudative AMD, which is a serious disease as it causes completely irreversible vision loss within a short period of time (weeks or days) (Bressel & McNair, 2002). Sporadically, if the visual loss is caused by hemorrhage from a newly forming small blood vessel, the visual loss can occur within hours.

In wet AMD, in addition to the RPE cells degeneration, new tiny, fragile blood vessels form from the small blood vessels in the choroid, which is defined as choroidal neovascularization (CNV). The newly formed micro-vessels break through the Bruch's membrane and grow into the macula. This process is very hazardous because the renascent vessels are very delicate and prone to rupture. The leaking blood and fluid can damage cone and rod photoreceptors and lead to scarring in the macula, which causes more serious visual loss (Fig. 5).

The categorization of AMD is established on the following characters as illustrated in Table 1.

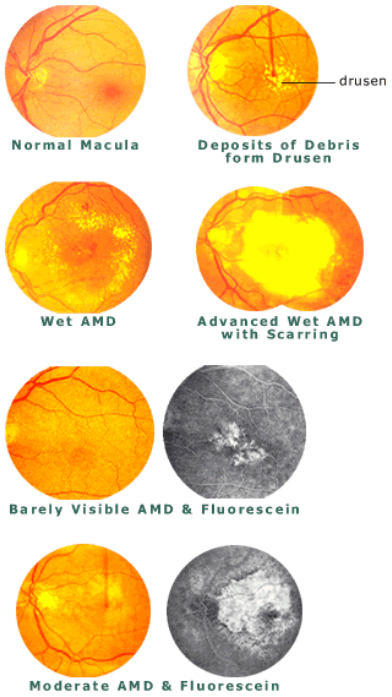


Figure 5. Different types of AMD as seen with an ophthalmoscope and fluorescence fundus angiography (FFA) Normal macula, Drusen, dry and wet AMD, advanced AMD with scarring, barely visible AMD and fluorescein, moderate AMD with fluorescein. *(Source: www.eyenutrition.com)*

Table 1. Standardized features of various forms of classical AMD

AMD (Prime types)	AMD (Subtypes)	Clinical features
Dry AMD	Drusen	A few drusen with pigment changes
	Geographic atrophy (GA)	Globular/oval regions of RPE atrophy with obvious choroidal vessels and rods/cones death
Wet AMD	Choroidal neovascularization (CNV)	Tiny blood vessels formation in choroid that ruptures RPE and gets into the sub-retinal area
	Disciform scar	CNV leaks and induces the formation of scars

1.2 Environmental Risk factors for AMD

In people with advanced age (over 50 years old), RPE cells do not work as well as in the young age. First, they do not provide enough nutrition for the cone and rod photoreceptors; second, they also do not clear waste materials and byproducts produced by cones and rods. As a result, small tiny yellowish extracellular deposits may form under the retina (Fig. 5). RPE cells and the nearby photoreceptors start to lose function and stop working. In other cases, some molecules (e.g. VEGF, vascular endothelial growth factor) trigger the formation of new capillaries beneath the retina.

Factors that predispose a person from developing AMD includes:

Age. AMD is most common in people over 60 years old. The risk of AMD increases as people age, especially after age 50. Cohort studies show, subjects aged between 60~80 years have 2~3-fold higher risk to develop late stage AMD compared to people younger than 60 years old (Friedman, Katz, Bressler, Rahmani, & Tielsch, 1999; VanNewkirk et al., 2000).

Family history. Hyman et al. first showed the genetic predisposition of AMD in a case-control study (L. G. Hyman, Lilienfeld, Ferris, & Fine, 1983). The association is further studied by familial aggregation, segregation analysis, twin studies and classical linkage studies (Klaver et al., 1998; Schick et al., 2003; Seddon, Book, Chong, Cote, & Santangelo, 2003; Silvestri, Johnston, & Hughes, 1994; Yates & Moore, 2000).

Race. The prevalence of AMD varies significantly among different racial populations. It is more common in Caucasians than in other races (1.91~3.5% compared to 0.19~1.4%) (Bressler, Munoz, Solomon, West, & Salisbury Eye Evaluation Study, 2008; Friedman et al., 1999). While the fundamental mechanism is still unidentified, Kawasaki et al. suggest that melanin might perhaps prevent the formation of lipofuscin, which is shown to be a crucial maker of senescence for cells and promotes oxidative damage in RPE cells (Kawasaki et al., 2008).

Smoking. Many cohort studies have indicated the relationship between smoking and accumulated risk of AMD and suggest that current smokers and people with smoking history develop AMD about 5 to 10 years earlier before nonsmokers (Berendschot, Willemse-Assink, Bastiaanse, de Jong, & van Norren, 2002; Delcourt, Diaz, Ponton-Sanchez, & Papoz, 1998). The exact mechanism underlying smoking and damaged retina, RPE or choroid vessels is still not clear, but data indicate that smoking may disturb the anti-oxidant metabolism of the outer retina (Berendschot et al., 2002).

Unhealthy diet. A poor diet that includes few fruits and vegetables may increase the risk to develop AMD (Weikel, Chiu, & Taylor, 2012).

Obesity. Being overweight increases the possibility to progress the early and intermediate AMD to the more severe form (wet AMD) faster. The underlying mechanism linking high body mass index (BMI) and AMD may include raised oxidative stress, higher lipoprotein density, and elevated chronic inflammation (E. J. Johnson, 2005; R. Klein, Klein, Tomany, & Cruickshanks, 2003).

Cardiovascular disease. Cardiovascular diseases, particularly high blood pressure, were found to be associated with severe form of AMD (L. Hyman, Schachat, He, & Leske, 2000).

Cholesterol level. Elevated cholesterol level may be associated with higher risk of AMD (Zhang et al., 2013).

Light Exposure. There exist conflicting opinions on the relationship of visible light or ultraviolet with AMD. Some studies showed that light exposure was associated with raised retinal pigment in male but not in female for early AMD (OR=1.44, 95%CI, 1.01-2.04) (Cruickshanks, Klein, & Klein, 1993).

Optic factors. These factors include darker iris color intensity, farsighted refraction and previous cataract medical procedure (Chakravarthy et al., 2010; Cugati et al., 2006; Sandberg, Tolentino, Miller, Berson, & Gaudio, 1993).

1.3 Genetic predisposition of AMD

1.3.1 Complement factor H (CFH)

CFH, which is located on chromosome 1q32, was found to be the first major gene associated with AMD and was validated in several distinct cohorts (Edwards et al., 2005; Haines et al., 2005; R. J. Klein et al., 2005). All these genome-wide association studies (GWAS) demonstrated that approximately half of the inheritance in AMD could be attributed to a single nucleotide polymorphism (SNP) located in exon 9 of the CFH gene. This SNP results in a coding variance Y402H, a tyrosine allele (Y, major allele) to histidine (H, minor allele) alteration (rs1061170, T>C). Individuals with a single copy of the risk allele 'C' showed an elevated risk of AMD with odds ratio (OR) ranging 2.4-4.6, while with two copies of the variant allele exhibited 3.3-7.4 OR change. Thakkinstian et al. further confirmed these results through a meta-analysis (Thakkinstian et al., 2006). Several other SNPs were also identified by other parallel case-control cohort studies, for example rs2274700 and rs203674 (Hageman et al., 2005). Most of these SNPs affect CFH transcription level and/or binding affinity to its ligands because they are located in the transcriptional regions of CFH.

Using human RPE cells, real-time PCR confirmed that these cells heavily express complement factor H (CFH) and complement factor H-related 1 (CFHL-1). Immunohistochemistry analysis of tissues from AMD patients revealed that the macula displayed intense CFH immune labeling compared to controls free of AMD, which strongly supports the role of complement activation in drusen formation and the progression of AMD (Hageman et al., 2005).

1.3.2 Complement factor B (CFB) and complement component 2 (C2)

Following the finding of CFH, several other genes involved in the complement cascade were investigated. Two paralogous genes, complement component 2 (C2) and the complement factor B (CFB) were found to be associated with AMD. These two genes located on chromosome 6p21 in the major histocompatibility complex III (MHC III). C2 and CFB are activators of the classical and the alternate complement pathway respectively.

Further screening of haplotype tagging SNPs in case-control cohort study showed four SNPs: rs641153 (R32Q), rs4151667 (L9H) in CFB gene and rs547154 (IVS10) and rs9332739 (E318D) in C2 gene were shown to have protective function for developing AMD. Several other cohorts from the US, UK and Australia replicated the finding of the connection between variants in BF/C2 and AMD (J. Maller et al., 2006; McKay et al., 2009; Richardson, Islam, Guymer, & Baird, 2009).

1.3.3 Complement component 3 (C3)

The complement component 3 (C3) is located on chromosome 19, and it plays a substantial role in the complement system and contributes to the innate immunity. As all other components, C3 is an essential cytoplasmic protein and its quantity and biological activity is pivotal for the construction of the terminal membrane attack complex (MAC), which leads to cell death.

Yates et al. demonstrated the connection of SNPs across the C3 gene using two separate case-control cohorts (Yates et al., 2007). They showed that, the SNP rs2230199, which is a functional polymorphism resulting from the change of arginine to glycine (R102G), was significantly associated with AMD in the two cohorts. The glycine variant, showed a higher OR compared to arginine homozygotes, OR=2.6 (95% CI, 1.6-4.1) for the heterozygotes and OR=1.7 (95% CI, 1.3-2.1), respectively. This might be related to the different functional properties of the gene. Subsequent studies in Caucasian cohorts confirmed Yates et al.'s finding, and they also showed that rs2230199 was the SNP associated with AMD (Bergeron-Sawitzke et al., 2009; Francis, Hamon, Ott, Weleber, & Klein, 2009; J. B. Maller et al., 2007). In other studies, instead of rs2230199, it was found that the SNP rs1047286 exhibited powerful and significant association with AMD, which is in the linkage disequilibrium (LD) with rs2230199 (Despriet et al., 2009; Spencer et al., 2008).

1.3.4 Chromosome 10q26 (ARMS2 and HtrA1)

Weeks et al. first demonstrated that gene located on chromosome 10q26 showed high linkage to AMD susceptibility (Weeks et al., 2000). After this, the association was confirmed by several other studies (Iyengar et al., 2004; Kenealy et al., 2004; Kortvely et al., 2010; Majewski et al.,

2003; Seddon et al., 2003). Another meta-analysis of several genome-wide scans validated the finding that genes located on chromosome 10q26 showed strongest susceptibility to AMD (Fisher et al., 2005).

There are three genes located on chromosome 10q26, viz. PLEKHA1/ARMS2/HtrA1 (Fig. 6). Jakobsdottir et al. indicated these genes are associated with AMD, but could not specify any individual gene (Jakobsdottir et al., 2005). Association studies with 93 SNPs over a domain covering PLEKHA1 and ARMS2 genes in German case-control cohorts demonstrated a region of powerful linkage disequilibrium between these two genes. Further AMD cases and controls sequencing pointed out the SNP rs1045216 in PLEKHA1 gene and rs10490924 in ARMS2 gene are significantly associated with AMD (Rivera et al., 2005). Later in 2006, an American cohort using case-control and family-based datasets validated the finding that rs10490924 contributes to AMD risk (S. Schmidt et al., 2006).

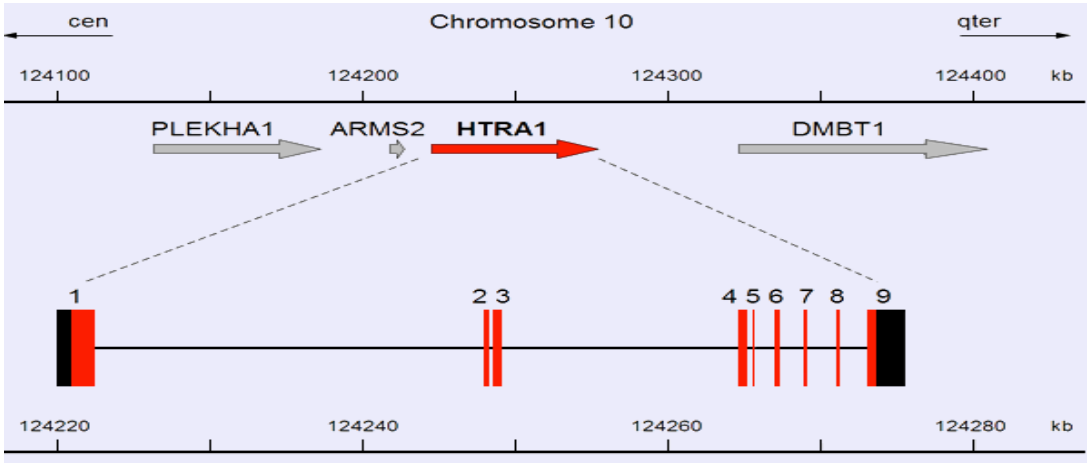


Figure 6. Localization and diagram of the chromosome 10q26 There are three genes located on chromosome 10q26: PLEKHA1/ARMS2/HtrA1. The numbers (e.g.124100) indicate the gene length in kilo base. Black boxes represent un-translated regions and red boxes indicate exons *(Source: http://atlasgeneticsoncology.org)*.

In an independent study, three distinct case-control cohorts consistently demonstrated the association between SNP rs10490924 and AMD (Ross et al., 2007). Fritsche et al. demonstrated that a new 443-bp in-del polymorphism located in the 3'UTR of ARMS2 was associated with changeable mRNA expression of ARMS2 (Fritsche et al., 2008). The ARMS2 protein was shown to be located in the mitochondria sector of the inner segment of photoreceptors.

At the same time, other studies indicated HtrA1 was a major susceptible gene on 10q26. A case-control cohort study which sequenced the gene of PLEKHA1 and HtrA1 identified a SNP located upstream of the promoter sequence in HtrA1 gene (rs11200638) showed a greater risk of developing AMD in comparison to wild type allele. The wild type allele is a part of CpG island that is the presumed binding site for the transcriptional factors, and the mutant allele was suggested to change the binding affinity. Two cell lines, ARPE19 and HeLaS3, were employed to confirm the binding between transcriptional factors in the promoter region of HtrA1. Cells that were transiently transfected with plasmids having the HtrA1 promoter variant showed higher protein expression for the mutant allele compared to the wild type allele (Dewan et al., 2006; Yang et al., 2006).

Yang et al. further confirmed the HtrA1 gene variation in a Chinese cohort, wherein, a significantly higher HtrA1 mRNA expression level was detected for the mutant allele in the RPE and lymphocytes of AMD patients. Also, immunohistochemistry staining of HtrA1 showed intensive labeling in AMD patients' drusen (Yang et al., 2006). Several other groups using different ethnic cohorts replicated the association of rs11200638 with high risk of AMD (H. Chen et al., 2008; Chowers et al., 2008; Deangelis et al., 2008; Kondo, Honda, Ishibashi, Tsukahara, & Negi, 2007; Tam et al., 2008; Weger et al., 2007; Yoshida et al., 2007).

1.4 HtrA1

High-temperature requirement A Serine peptidase 1 （HtrA1） is a 50-KDa secreted protein and a member of serine proteases in the high-temperature requirement A family, which is characterized by a highly conserved structure with a trypsin-like protease and a PDZ domain. Actually, the HtrA1 protein is comprised of four main domains, in addition to the very beginning of the signaling peptide (22 amino acids), from amino-terminus to carboxyl-terminus are insulin-like growth factor binding protein (IGFBP) domain, Kazal domain, trypsin-like peptidase domain (proteolysis center) and PDZ domain (Fig. 7).

Figure 7. Domain structure of HtrA1 protein SP: signal peptide, IGF-BD: insulin-like growth factor binding domain, KM: Kazel-like domain, PROTEOLYTIC: proteolysis center, PDZ: PDZ domain. HtrA1 protein has 480 amino acids.

As a secreted protein, HtrA1 is ubiquitously expressed in a diverse range of normal human tissues, such as the blood, placenta, central nervous system (CNS) and liver (Zong et al., 2013). Several cells can express HtrA1, and it can be found in the epidermis, neuronal and vascular endothelia cells (De Luca et al., 2003). (Fig. 8) HtrA1 mutations have been associated with several diseases, such as cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL), age-related macular degeneration (AMD), cancers (ovarian cancer, endometrial cancer, melanoma and liver cancer) and arthritic diseases.

Previous studies have demonstrated that HtrA1 expression levels in drusen, damaged RPE cells and choroidal neovascular membranes are significantly increased (An, Sen, Park, Gordish-Dressman, & Hathout, 2010; Nakashizuka et al., 2008; Yang et al., 2006).

Consistently, several studies have well demonstrated that HtrA1 is a strong genetic risk factor for the pathologic process of AMD. One of the SNPs, which is located in the promoter region of HtrA1 gene on the chromosome 10q26, has been found to be the most significant genetic risk factors for the pathogenesis of AMD (Tong et al., 2010; Yang et al., 2006). Even though the mechanism of the macula damage in AMD is not clear, many research results suggested that HtrA1 protease might destroy the stabilization of the extracellular matrix; or suppress the transforming growth factor–beta (TGF-beta) signaling pathway and thereby influences angiogenesis. A paper demonstrated that HtrA1 mRNA expression level is significantly higher in cultured RPE cells homozygous for the risk rs11200638 allele, and many molecules involved in the complement pathway, like vitronectin, clusterin, fibromodulin, are substrates of HtrA1 protease (Parmeggiani et al., 2012).

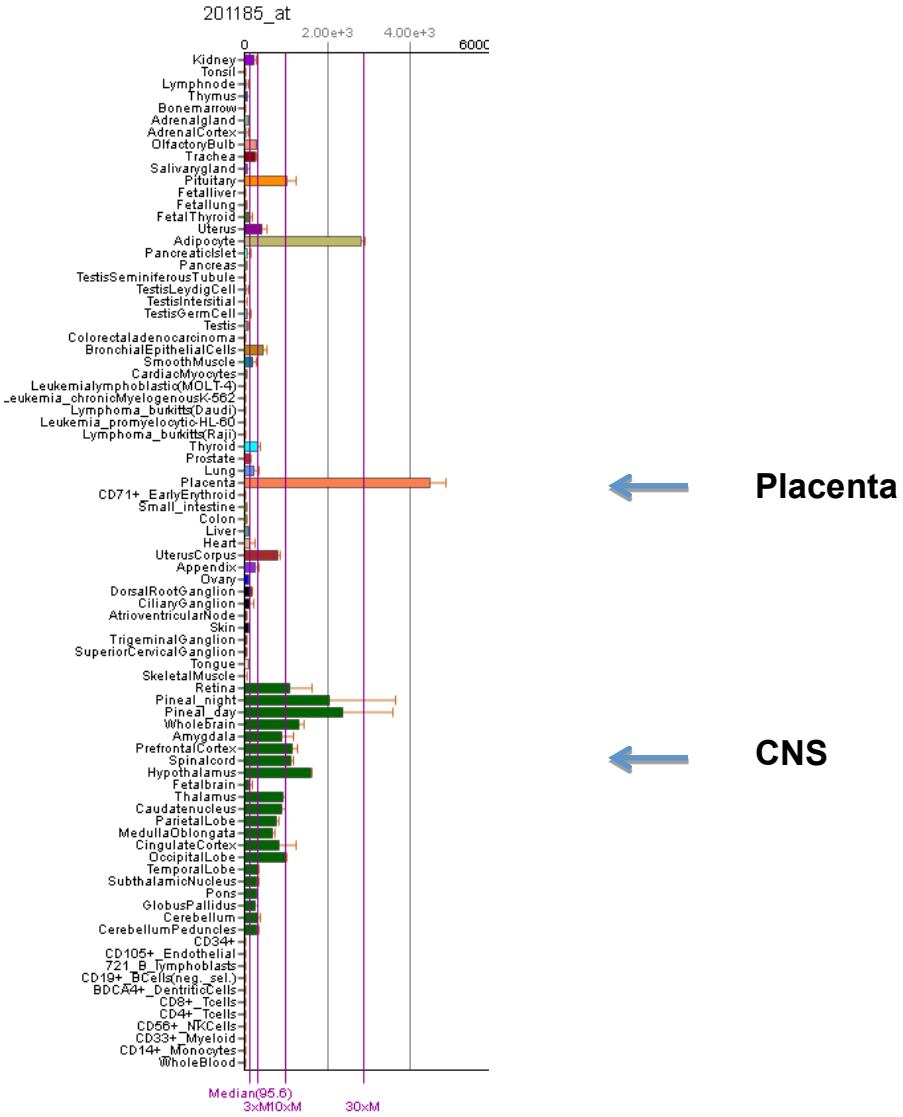


Figure 8. Relative expression levels of HtrA1 protein in human tissues The peak expression can be observed in placenta and central nervous system (CNS). Human retina is a part of central nervous system. *(Source: http://www.genecards.org)*

1.5 Quantitative Proteomics

Proteomics technology has being making great advances in the last few decades. With the help of powerful and sensitive mass spectrometers, superb databases, and the advancement of bioinformatics, a great amount of researches have emerged in the last few years. But the quantification of differences among two or more biological states is still the most challenging task in proteomics.

Stable isotope labeling in combination with mass spectrometry has developed to become a high throughput method to determine and quantify large number of proteins within complex protein mixture. But using this new technology to illustrate the underlying mechanism of AMD still remains a challenge. For this research project, isotope-coded protein label (ICPL) and stable isotope labeling by amino acids in cell culture (SILAC) were employed in an attempt to explore this complex disorder.

1.6 Aims of the Study

To sum up, growing evidence indicates that mutation of the HtrA1 gene plays a pivotal role in the pathological process leading to age-related macular degeneration. Therefore, it is highly interesting to find out how HtrA1 gene or protein expression variation influences the pathogenesis of AMD. The purpose of this study was to elucidate the exact molecular components or the pathological pathway that link HtrA1 to the pathogenesis of age-related macular degeneration. More specifically, we aim at:

1) Identifying binding partners or regulators of HtrA1.
2) Defining the physiological substrates for HtrA1.
3) Gaining insights into the pathological pathway leading to AMD.

2. Materials and methods

2.1 Experimental Design

2.1.1 Analysis of HtrA1-deficient mouse sera

All animal experiments followed the guidelines of the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the animal care committee of University Tübingen.

Five months old HtrA1 gene trap mice (from Dr. Chio Oka, Nara Institute of Science and Technology, Japan) were used as mouse model; similar aged wild type C57BL/6 mice served as controls. For each experiment two wild type mice and two knockout mice were sacrificed and the serum samples were used to compare the protein expression difference using isotope-coded protein label (ICPL) labeling and Mass Spectrometry analysis.

2.1.2 Identification of binding partners of HtrA1 using cultured human cell lines

ARPE19 (a human retinal pigment epithelial cell line) cells and HeLa (a cervical cancer cell line) cells were used to overexpress HtrA1 protein followed by co-immunoprecipitation of HtrA1 along with its binding partners. Western Blot and in vitro digestion assays were used to validate the potential candidates. (Experimental workflow Fig. 9)

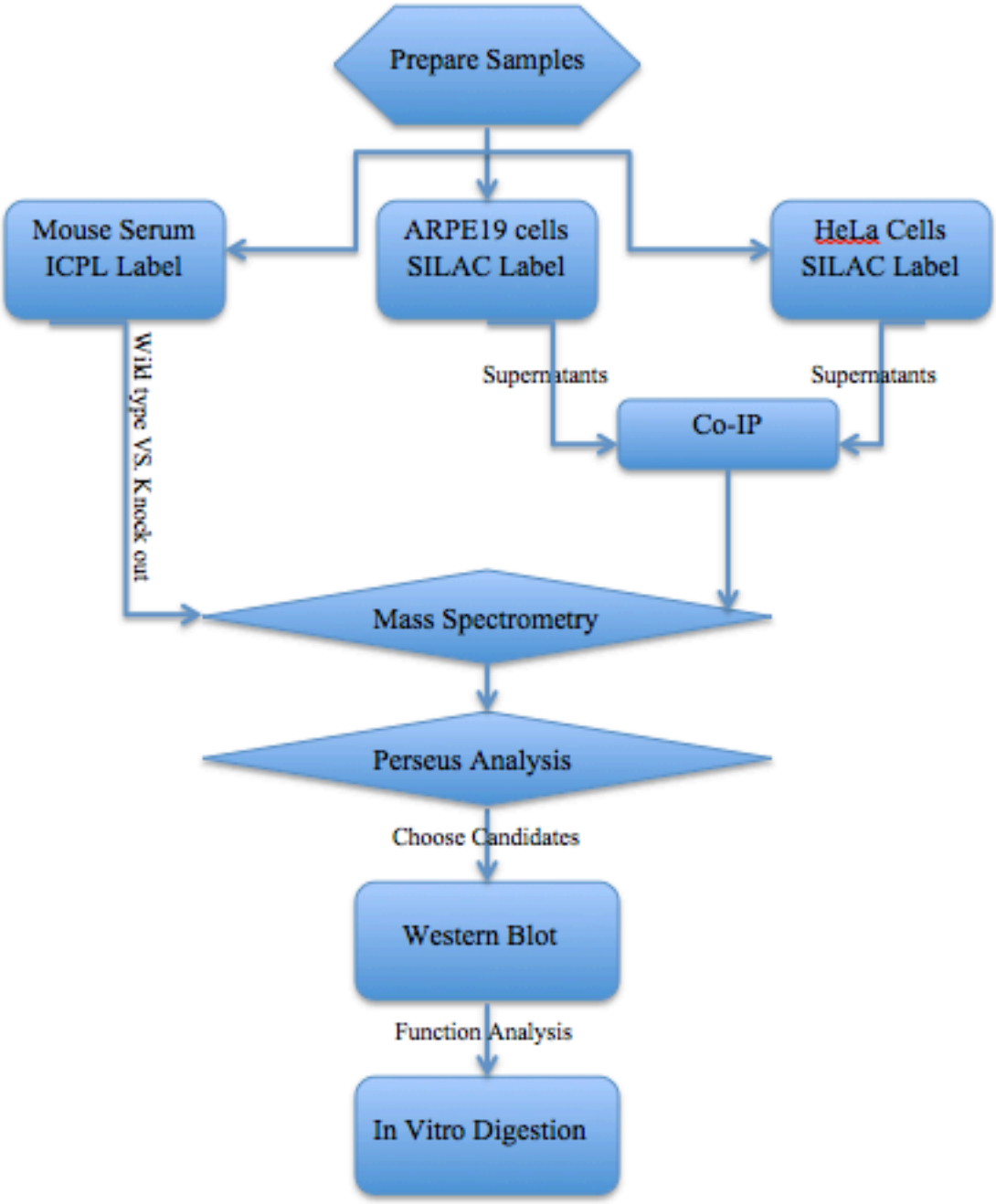


Figure 9. Experimental workflow The entire project includes three main parts: prepare samples, run experiments and evaluate results. Samples include: mouse serum sample (ICPL labeling), and ARPE19 cell and HeLa cell (SILAC labeling). The main experimental steps include: mass spectrometry analysis, co-immunoprecipitation, western blot and in vitro digestion. Evaluation steps include quantification of the significant proteins, identification of new interactions and validation of interesting candidates.

2.2 ICPL labeling

Isotope-coded protein label (ICPL) is capable of performing quantitative proteome profiling on a comprehensive scale. Since ICPL is dependent on isotope labeling at the free amino acids of intact proteins, it is appropriate to apply to a large proportion of protein samples, including bodily fluids and extracts from tissues. After labeling, the samples can be mixed and simplified by any separation method presently used in protein chemistry. After trypsin cleavage of the protein fractions the ratios of peptides from different proteome conditions can be evaluated by simple liquid chromatography-tandem mass spectrometry (LC-MS/MS) based mass spectrometry analyses (Colome et al., 2010; Kellermann, 2008; A. Schmidt, Kellermann, & Lottspeich, 2005).

A kit from SERVA and Bruker (SERVA ICPL^{TM} Kit) was employed for the ICPL labeling. Around 80 µg Albumin/IgG depletion serum samples from both wild type and knockout animals were used for ICPL labeling, resulting in a modification of lysine amino groups in the forms of ICPL_0 and ICPL_6. After the ICPL labeling two samples were merged and digested by trypsin following the kit protocol.

2 µl (about 80 µg) of serum samples were used for each experiment. 0.5 µl of reduction solution was added to each sample and proteins were reduced for 30 min at 60°C. Samples were cooled to room temperature (RT) and were centrifuged to remove condensed solution from the lid. 0.5 µl of freshly prepared alkylation solution was added to each sample, following this, samples were immediately wrapped in aluminum foil for light protection and were kept at 25°C for 30 min. Reaction was stopped by using 0.5 µl stop solution and then incubated for 15 min at RT. 3 µl of ^{12}C-Nic-reagent solution (ICPL_0) and ^{13}C-Nic-reagent solution (ICPL_6) was added separately to wild type and knockout serum samples (Fig. 10). Both samples were treated with argon to exclude oxidation, vortexed for 10 sec and sonicated for 1 min in an ultrasound bath and then collected by centrifugation. Subsequently, samples were incubated for 2 h at RT. 2 µl of stop solution was then added to each sample and incubated for 20 min at RT to destroy excess reagent. Both samples were combined and vortexed thoroughly. Finally 2 µl of 2N NaOH was added to adjust the pH and eliminate possible esterification products. The samples were then incubated for 20 min and treated with 2 µl of 2 N HCl to neutralize them. (Fig. 11)

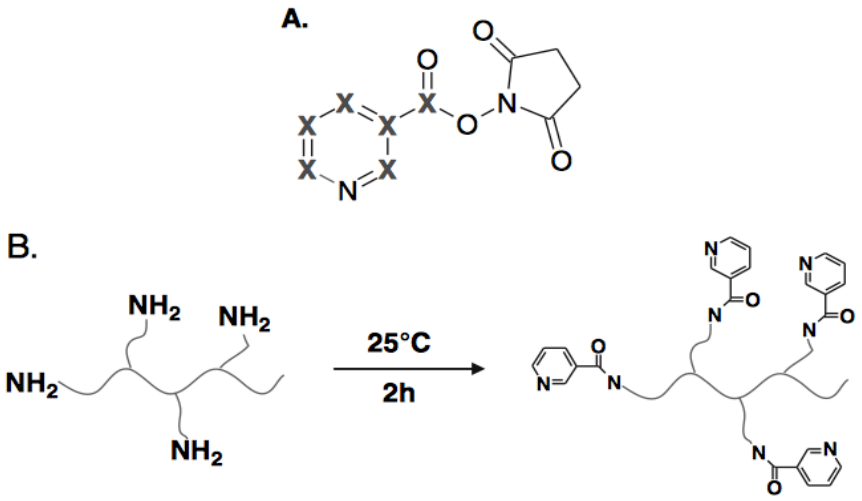


Figure 10. Molecular structure and reaction scheme of the ICPL-label A represents molecular structure of the isotope-coded protein label-label and B shows the reaction scheme of the isotope-coded protein label-label. X corresponds to 12C/13C isotopes ICPL: isotope-coded protein label *(Kellermann, 2008)*

2.3 Cell Culture

ARPE19 cells and HeLa cells were used for this research. ARPE19 cells were cultured in a 1:1 mixture of Dulbecco's modified Eagles medium and Ham's F12 medium supplemented with heat inactivated fetal bovine serum (FBS, 10%, v/v), 100U/mL penicillin and 100µg/mL streptomycin. Culture media were changed every 2-3 days; cells were passaged when they reached 80~90% confluence with a sub cultivation ratio of 1:2. After passaging, instead of normal culture medium, cells were cultured with stable isotope labeling of amino acids in cell culture (SILAC) 1:1 mixture of Dulbecco's modified Eagles medium and Ham's F12 medium supplemented with either light amino acids (Lysine, Arginine, and Proline) or heavy amino acids ($^{13}C_6$, $^{15}N_2$ Lysine, $^{13}C_6$, $^{15}N_4$ Arginine, and Proline), containing 10% dialyzed fetal bovine serum (dFBS), and 100U/mL penicillin and 100µg/mL streptomycin. After five or more divisions in the SILAC labeling media, proteins achieve at least 97% isotope labeling. Both ARPE19 heavy and light cells were subjected to the following experiments when they reached similar confluence. The same procedure was applied to HeLa cells using the appropriate medium (RPMI-1640). (Fig. 11)

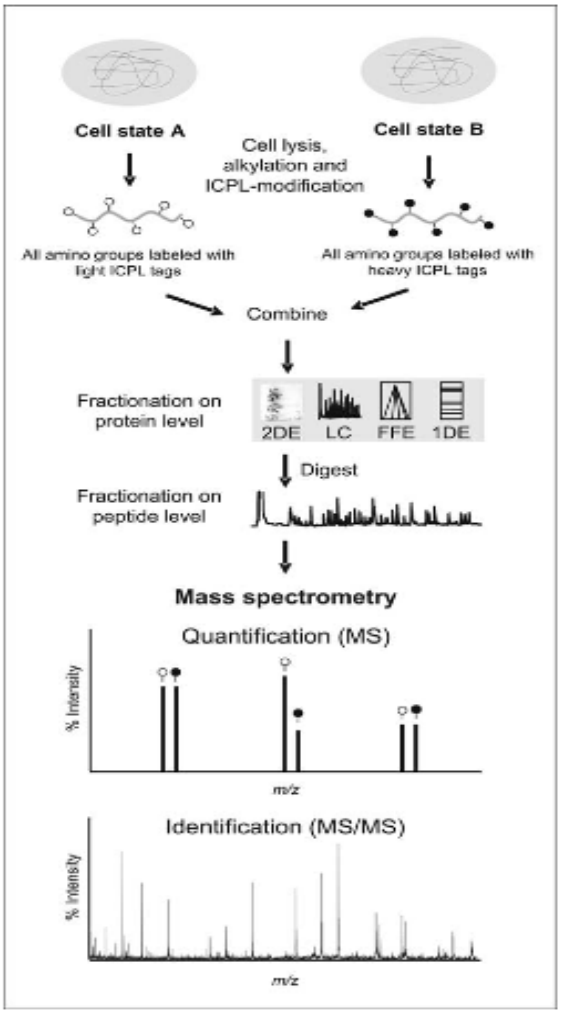


Figure 11. General flow chart of quantitative proteomics Quantitative proteomics in this project includes SILAC and ICPL labeling. The general workflow of ICPL is shown. 1) two different samples 2) ICPL-labeling, including ICPL_0 (light) and ICPL_6 (heavy) 3) fractionation (two-dimensional electrophoresis, 2DE; liquid chromatography, LC; free-flow electrophoresis, FFE; one-dimensional electrophoresis, 1DE) 4) in gel digestion 5) mass spectrometry. Fractionation was used to ensure an unbiased reliable result that represents an accurate composition of all proteins in initial samples. SILAC: stable isotope labeling of amino acids in cell culture ICPL: isotope-coded protein label *(Kellermann, 2008)*

2.4 Transfection

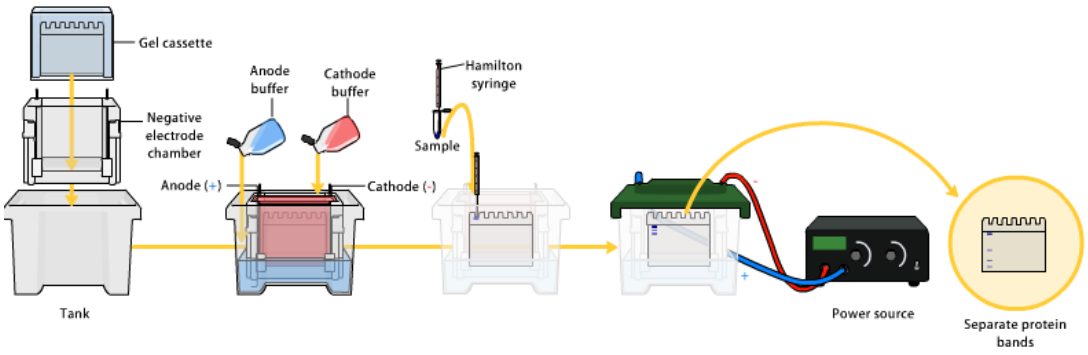
In order to overexpress HtrA1 protein, Lipofectamine® LTX with Plus Reagent Kit (Invitrogen, Life Technologies) was used. For a 10 cm dish, 10µg plasmid coding for triple-tagged, catalytically inactive (S328A) HtrA1 was used. After mixing serum free medium, plasmid and Plus Reagent, the mixture solution was kept for 5 min at RT; then Lipofectamine LTX reagent was added，mixed and kept at RT for 30 min. At the end, plasmid mixture was added to the

dishes containing at least 80% confluent cells. After 8 to 12 h, the whole transfection medium was changed to serum free medium. Cells and suspension were harvested after 48 h. For the control dish, the same amount of plasmid coding for fluorescent green (GFP) was used.

2.5 Western Blotting

Protein samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (12%) and then transferred into polyvinylidene difluoride (PVDF) membrane and probed by anti-HtrA1 mouse monoclonal antibody (R&D system) and anti-ISG15 antibody (mouse monoclonal antibody). The membranes were washed 3 times and incubated with perioxidase-conjugated secondary antibodies (1:7500) for 1 h at RT and washed 3 times again. Enhanced chemiluminescence Western blotting detection reagents (Pierce) were used to determine HtrA1 and ISG15 protein levels (Fig. 12).

A



B

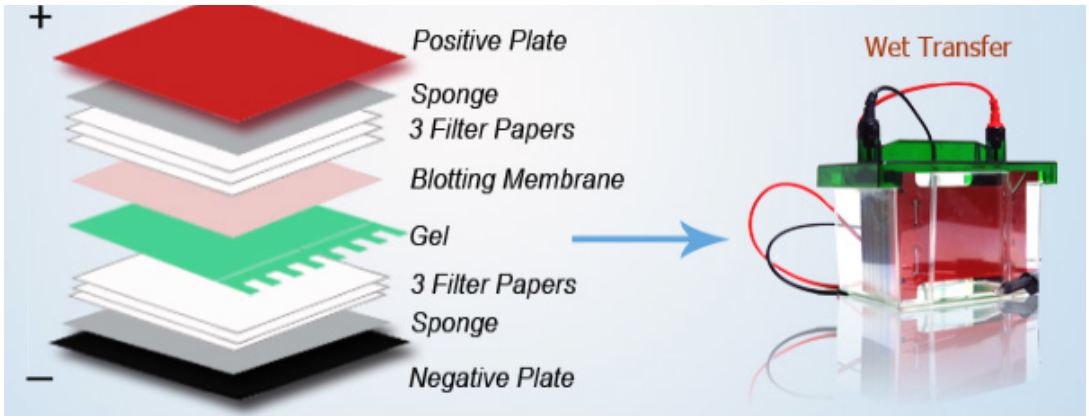


Figure 12. Western Blot Western blot is a widely used protein analytical technology in molecular biology research. It uses gel electrophoresis to separate natural or denatured proteins. The separated proteins are then transferred to PVDF membrane and detected by the specific antibodies. A represents gel electrophoresis, (the electrophoresis buffer, anode and cathode buffer are the same) and B represents transfer installation. *(Source: http://en.wikipedia.org)*.

2.6 Co-Immunoprecipitation

In order to use co-immunoprecipitation (Co-IP) to obtain HtrA1's interacting proteins, ARPE19 and HeLa cells' supernatants were used. Supernatants were collected and pre-cleaned with Protein A-Agarose and Protein L-Agarose (Santa Cruz Biotechnology) for 2 h at 4°C under gentle agitation. Then the samples were centrifuged at 1,000×g for 5 min at 4°C. Beads were discarded and suspension was reserved. Then 5 µl of mouse monoclonal HtrA1 antibody (R&D) was added for 2 h at 4°C under gentle agitation. Afterwards, 20 µl of Protein G Sepharose beads (Santa Cruz biotechnology) were added overnight at 4°C under gentle agitation. After incubation for 16 h, the beads were recovered by centrifugation at 1000×g, for 5 min and gently washed 3 times with washing buffer (NaCl, 150mM, NP-40, 0.2%, Tris-Cl, 50 mM, pH 7.5). At the end, the precipitated proteins were eluted with 5×SDS Laemmli buffer and incubated for 30 min at 50°C and then 5 min at 96°C and were resolved in a 10% SDS-PAGE gel and detected by Coomassie staining and western blotting until finally ready for MS analysis.

2.7 In vitro digestion

RPMI-1640 cell culture medium (with L-Glutamine and Phenol Red) (Sigma) was used as the digestion buffer. HtrA1 human recombinant purified protein (from Prof. Michael Ehrmann, University Duisburg-Essen) was used as the enzyme for the in vitro digestion. Recombinant ISG15 purified from *E.coli* and conjugated to an N-terminal Calmodulin tag (151 amino acids) was used as a putative interacting partner/substrate. Different time points (3 h, overnight) and different enzyme to substrate ratios (1:1, 1:10) was selected to test the interaction between the two proteins.

2.8 Proteomics and Mass spectrometry (MS)

Mass spectrometry was used to identify the interacting proteins of HtrA1. HtrA1 and ISG15 were both identified from several mass spectrometry results in the Medical Proteome Center (MPC), University Tübingen. The protein bands were cut from SDS-PAGE (NuPAGE® Bis-Tris gels, 10%, life technologies) and digested with trypsin. Obtained peptides were analyzed with LC-MS/MS then searched against human proteome (Fig. 13).

MaxQuant 1.4.1.2 software, which is a quantitative proteomics data package designed specifically for analyzing sizeable mass-spectrometric datasets and is particularly aimed at high-resolution MS data, was used for data acquirement.

Perseus 1.4.0.16, which is designed to perform all downstream bioinformatics, was employed to perform the statistical analysis.

Figure 13. Mass spectrometry Mass Spectrometry is advantageous due to its high capacity, resolution and accuracy.

3. Results

3.1 Mouse Serum ICPL labeling

80μg serum samples from either wild type or HtrA1 knockout mice were used for ICPL labeling. Then NuPAGE® 10% Bis-Tris Plus Gel was used to decrease the complexity of the samples. The statistically significant candidates after the MS analysis are shown in the table below (Table 2). Among all of the statistically significant candidates, those with the fold-change over 1.5 deserve attention for future research. Compared with the wild type mouse, complement factor P (CFP) and complement factor D (CFD) were found to be up regulated (1.81 fold and 1.69 fold respectively); complement component factor I (CFI, 0.68 fold), complement C5 (C5, 0.65 fold), complement C3 (C3, 0.60 fold), complement factor H related protein C (CFHR, 0.56 fold), complement component 2 (C2, 0.50 fold) were found to be down regulated in the serum of HtrA1 knockout mouse (Table 2).

3.2 Co-IP

3.2.1 ARPE19 Cells Co-IP

Before performing Co-IP experiments, ARPE19 cells were firstly labeled with SILAC. Then, either heavy ARPE19 cells were transiently transfected with the plasmid coding for triple-tagged, catalytically inactive HtrA1 and light ARPE19 cells were transfected with the plasmid coding for GFP, or vice versa. After transfection (8 to 12 hours), transfection media were changed to serum free media and were incubated for 48 hours. Afterwards, medium was collected from each dish. Then anti-flag M2 monoclonal antibody (sigma) was used for precipitation and heavy/light samples were combined separately. Statistically significant candidates after MS analysis are shown in table 3 (fold change > 1.5). TMEM10 (3.42 fold), SERPING1 (3.25 fold), ISG15 (2.17 fold), complement C1s (2.07 fold), complement C1r (2.03 fold), CD59 (1.94 fold), IGFBP6 (1.84 fold), WARS (1.84 fold), IGFBP7 (1.77 fold) were found to show high affinity with HtrA1 in transfected ARPE19 cells (Table 3).

3.2.2 HeLa Cells Co-IP-FLAG

HeLa cells' SILAC labeling and Co-IP experiments were similar to the ARPE19 cells above. As antibody, anti-flag M2 monoclonal antibody (Sigma) was used. The control cells were not transfected. Statistically significant candidates after MS analysis are shown in table 4 (fold change > 1.5). Interleukin 6 (IL6, 10.09 fold), ISG15 (9.67 fold), PRSS23 (2.51 fold), AGRN (2.49 fold), SERPINH1 (2.16 fold), LIPA (2.11 fold), VTN (2.07 fold) were found to show high affinity to HtrA1 in transfected HeLa cells (Table 4).

3.2.3 Hela Cells Co-IP-c-MYC

This experiment was done the same way as experiment 3.2.2 except changing anti-flag M2 monoclonal antibody (sigma) to anti-myc antibody. Statistically significant candidates after MS analysis are shown in table 5 (fold change > 1.5). ISG15 (9.5 fold), IL 6 (8.86 fold), AGRN (2.76 fold), PRSS23 (2.45 fold) SERPINH1 (2.29 fold), LIPA (2.03 fold) were found to show high affinity to HtrA1 protein in transfected HeLa cells (Table 5).

Table 2. ICPL labeling experiment of mouse serum

Protein	Symbol	Fold-Change	Significance
Up-Regulated			
Major urinary protein 11	Mup11	2.69	0.0000
C-X-C motif chemokine 4	Cxcl4	2.34	0.0003
Anti-HIV-1 reverse transcriptase single-chain variable	Gm1409	2.25	0.0007
Trem-like transcript 1 protein	Treml1	2.23	0.0008
Major urinary protein 3	Mup3001	2.11	0.0019
Macrophage colony-stimulating factor 1 receptor	Csf1r	1.94	0.0063
Carbonate dehydratase II	Ca2	1.87	0.0107
Glia-derived nexin	Serpine2	1.84	0.0125
Fructose-bisphosphate aldolase	Aldoa	1.84	0.0128
Complement factor P	CFP	1.81	0.0160
Adenylate kinase isoenzyme 1	Ak1	1.80	0.0168
von Willebrand antigen 2	Vwf	1.75	0.0236
Adipsin	CFD	1.69	0.0357
Down-Regulated			
Coagulation factor XIII A chain	F13a	0.71	0.0495
Ig gamma-2B chain C region	Igh-3	0.70	0.0456
Complement component factor i	CFI	0.68	0.0280
Albumin 1	Alb1	0.66	0.0192
Complement C5	C5	0.65	0.0161
C3a anaphylatoxin	C3	0.60	0.0055
Complement factor H-related protein C	CFHR	0.56	0.0022
Anti-MOG Z12 variable light chain	anti-MOG	0.56	0.0021
Alpha-2-macroglobulin	A2m	0.51	0.0004
Complement component 2	C2	0.50	0.0003
Chemokine (C-X-C motif) ligand 7	Cxcl7	0.45	0.0000
Histidine-rich glycoprotein HRG	Hrg	0.42	0.0000

Table 2 shows the results of the ICPL labeling experiments of the wild type and HtrA1 knockout mouse. Proteins showing different concentrations in these two samples are listed here. Data shown are the proteins either up regulated or down regulated in knockout mouse. The protein identification probability was set to 95% and the peptide identification probability was set to 80% by Scaffold. The highlighted proteins (CFP, CFD, CFI, C5, C3, CFHR and C2) have function in the immune system.

Table 3. Co-IP experiment of HtrA1 protein in ARPE19 cells

Protein	Symbol	Fold-change	Significance
HtrA serine peptidase 1	HtrA1	85.39	0.0000
Transmembrane emp24-like trafficking protein 10	TMED10	3.42	0.0000
Ribosomal protein S28	RPS28	3.28	0.0000
Serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	SERPING1	3.25	0.0000
Pleiotrophin	PTN	2.28	0.0041
Heparan sulfate proteoglycan 2	HSPG2	2.26	0.0045
Annexin A5	ANXA5	2.18	0.0067
ISG15 ubiquitin-like modifier	ISG15	2.17	0.0068
Nucleobindin 1	NUCB1	2.17	0.0068
Annexin A2	ANXA2	2.14	0.0081
Complement C1s subcomponent	C1S	2.07	0.0109
Calreticulin	CALR	2.06	0.0117
Peptidylprolyl isomerase B (cyclophilin B)	PPIB	2.03	0.0133
Complement C1r subcomponent	C1R	2.03	0.0133
Ribosomal protein S19	RPS19	1.96	0.0188
CD59 molecule, complement regulatory protein	CD59	1.94	0.0204
beta-2-microglobulin	B2M	1.91	0.0243
Extracellular matrix protein 1	ECM1	1.88	0.0276
Interferon-induced protein with tetratricopeptide repeats 3	IFIT3	1.87	0.0284
Dickkopf-related protein 3	DKK3	1.86	0.0304
Interferon-induced protein with tetratricopeptide repeats 1	IFIT1	1.85	0.0317
Endoplasmic reticulum resident protein 29	ERP29	1.85	0.0318
Insulin-like growth factor-binding protein 6	IGFBP6	1.84	0.0324
tryptophanyl-tRNA synthetase	WARS	1.84	0.0326
Ribosomal protein L29	RPL29	1.83	0.0354
H2A histone family, member Y	H2AFY	1.82	0.0358
Integrin beta-1	ITGB1	1.82	0.0362
Follistatin-like protein 1	FSTL1	1.81	0.0380
Stromal cell derived factor 4	SDF4	1.79	0.0409
Receptor accessory protein 5	REEP5	1.78	0.0440
heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	HSPA5	1.78	0.0443
Annexin A1	ANXA1	1.78	0.0445
Annexin A3	ANXA3	1.77	0.0456
Insulin-like growth factor-binding protein 7	IGFBP7	1.77	0.0462
Tropomyosin 1 (Alpha)	TPM1	1.75	0.0496

Table 3 shows the result of precipitation of HtrA1 protein complex in SILAC labeled ARPE19 cells. ARPE19 cells were transiently transfected with plasmid coding for HtrA1. Data show the proteins belonging to HtrA1 complex or associated with HtrA1 protein. Proteins shown here were identified in at least two out of three independent experiments. The proteins listed here displayed all significant interactions with a fold change of over 1.5. The protein identification probability was set to 95% and the peptide identification probability was set to 80% by Scaffold. The highlighted proteins (TMED10, SERPING1, ISG15, C1S, C1R, CD59, IGFBP6, WARS and IGFBP7) are important candidates associated with HtrA1 protein.

Table 4. Co-IP-Flag experiment of HtrA1 protein in HeLa cells

Protein	Symbol	Fold-change	Significance
	HtrA1	43.53	0.0000
Interleukin 6	IL6	10.09	0.0000
	ISG15	9.67	0.0000
	PDIA6	3.28	0.0000
heat shock protein 90kDa beta (Grp94), member 1	HSP90B1	2.74	0.0000
protease, serine, 23	PRSS23	2.51	0.0000
Agrin	AGRN	2.49	0.0000
tryptophanyl-tRNA synthetase	WARS	2.31	0.0000
serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	SERPINH1	2.16	0.0000
lipase A, lysosomal acid, cholesterol esterase	LIPA	2.11	0.0000
vitronectin	VTN	2.07	0.0000
transketolase	TKT	1.99	0.0000
non-metastatic cells 2, protein (NM23B) expressed in	NME2	1.97	0.0000
prolyl 4-hydroxylase, beta polypeptide	P4HB	1.95	0.0000
UDP-glucose glycoprotein glucosyltransferase 1	UGGT1	1.65	0.0011
heat shock 70kDa protein 4	HSPA4	1.65	0.0011
	FAM50A	1.63	0.0014
carbonyl reductase 1	CBR1	1.61	0.0020
sperm associated antigen 9	SPAG9	1.60	0.0022
peptidylprolyl isomerase A (cyclophilin A)	PPIA	1.59	0.0026
	GLO1	1.53	0.0058

Table 4 shows the result of the precipitation of HtrA1 protein complex in SILAC labeled HeLa cells. HeLa cells were transiently transfected with plasmid coding for HtrA1. Anti-flag M2 monoclonal antibody was used for precipitation. The data shown displayed the proteins belonging to HtrA1 complex or associated with HtrA1 protein. Proteins shown here were identified in at least two out of three independent experiments. The proteins listed here displayed all significant interactions with a fold change over 1.5. The protein identification probability was set to 95% and the peptide identification probability was set to 80% by Scaffold. The highlighted proteins (IL6, ISG15, PRSS23, AGRN, SERPINH1, LIPA and VTN) are important candidates associated with HtrA1 protein.

Table 5. Co-IP-c-Myc experiment of HtrA1 protein in HeLa cells

Protein	Symbol	Fold-change	Significance
	HtrA1	54.52	0.0000
ISG15 ubiquitin-like modifier	ISG15	9.50	0.0000
Interleukin 6	IL6	8.86	0.0000
peroxiredoxin 4	PRDX4	4.30	0.0000
protein disulfide isomerase family A, member 6	PDIA6	3.52	0.0000
Agrin	AGRN	2.76	0.0000
heat shock protein 90kDa beta (Grp94), member 1	HSP90B1	2.66	0.0000
protease, serine, 23	PRSS23	2.45	0.0000
tryptophanyl-tRNA synthetase	WARS	2.32	0.0000
serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	SERPINH1	2.29	0.0000
vitronectin	VTN	2.22	0.0000
prolyl 4-hydroxylase, beta polypeptide	P4HB	2.19	0.0000
lipase A, lysosomal acid, cholesterol esterase	LIPA	2.03	0.0000
transketolase	TKT	1.94	0.0000
non-metastatic cells 2, protein (NM23B) expressed in	NME2	1.86	0.0000
heat shock 70kDa protein 4	HSPA4	1.71	0.0002
programmed cell death 10	PDCD10	1.71	0.0014
carbonyl reductase 1	CBR1	1.68	0.0003
granulin	GRN	1.55	0.0020
heat shock 105kDa/110kDa protein 1	HSPH1	1.55	0.0086
glyoxalase I	GLO1	1.53	0.0029
peptidylprolyl isomerase A (cyclophilin A)	PPIA	1.53	0.0109

Table 5 shows the result of the precipitation of HtrA1 protein complex in SILAC labeled HeLa cells. HeLa cells were transiently transfected with HtrA1 plasmid. An anti-myc antibody was used for precipitation. Data shown are the proteins either belonging to HtrA1 complex or associated with HtrA1 protein. Proteins shown here were identified in at least two out of three independent experiments. The proteins listed here displayed all significant interactions with a fold change of over 1.5. The protein identification probability was set to 95% and the peptide identification probability was set to 80% by Scaffold. The highlighted proteins (ISG15, IL6, AGRN, PRSS23, SERPINH1 and LIPA) are important candidates associated with HtrA1 protein.

3.3 IP and WB to confirm the interaction

HeLa cells were transiently transfected with the plasmid coding for triple-tagged, catalytically inactive HtrA1 shown in the 2nd lane (4mg/10cm dish); for the 1st lane, cells were left blank, without any transfection. Cell lysates were subjected to immunoprecipitation (anti-HtrA1, rabbit) and western blot (anti-HtrA1, mouse and anti-ISG15, mouse) analysis. The 1st lane was empty, while in the 2nd lane; both the band for HtrA1 and the band for ISG15 were detected (Fig. 14).

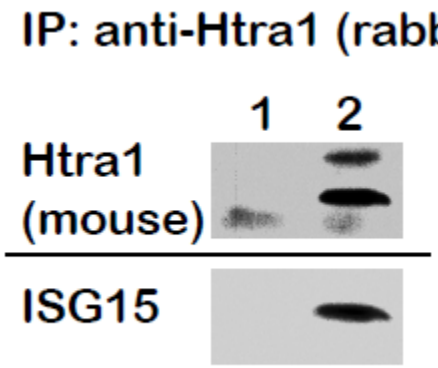


Figure 14. ISG15 interacts with HtrA1 Western blot analysis of HtrA1 (rabbit polyclonal antibody) precipitation from HeLa cells that were transiently transfected with plasmid coding for HtrA1. Bands shown in the 2nd lane are HtrA1 (upper band, mouse monoclonal antibody) and ISG15 (lower band, mouse monoclonal antibody), while no band was found to show in the 1st lane.

3.4 In vitro digestion

3.4.1 Comparison of ISG15 and ISG15+HtrA1

In order to test the interaction between ISG15 and HtrA1, in vitro digestion assay was used. HtrA1 protease was expressed and purified from a eukaryotic expression system (from Prof. Michael Ehrmann, University Duisburg-Essen). Recombinant ISG15 was purified from E. Coli and fused to N-terminal Calmodulin Tag (151 a.a.) and has a molecular weight of 34 KDa (PROSPEC). The 1st tube (left lane) contained 100ng of human ISG15 recombinant protein, 1 µL of ddH2O and 18 µL of RPMI-1640 cell culture medium (without serum); the 2nd tube (right lane) instead of ddH2O, contained 1 µg (1 µg/ µL) of HtrA1 protein. Tubes were incubated for 3 h at 37°C, 5% CO2, and then a 5-fold SDS loading buffer was added to stop the reaction. Subsequently, samples were incubated for 5 min at 96°C and were analyzed with SDS-PAGE. After transferring to PDVF membrane, proteins were probed with anti-HtrA1 (mouse) and anti-ISG15 (mouse) antibodies. In the left lane, ISG15 was found to show in the expected

position; in the right lane, except for HtrA1 and ISG15 bands, another band was observed below the ISG15 band (arrowhead) (Fig. 15).

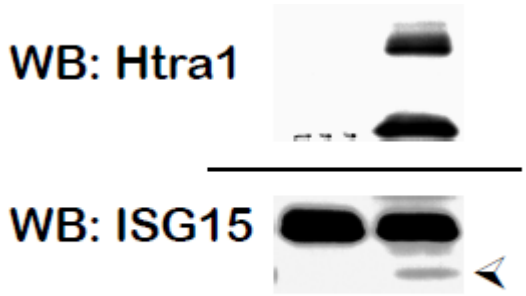


Figure 15. ISG15 is cut by HtrA1 protease Western blot analyzes HtrA1 and ISG15 protein from the in vitro digestion samples. Bands shown in the right lane are HtrA1 (upper band) and ISG15 (lower band), below ISG15 band an extra band is found to show (arrow head). No HtrA1 band was found to show in the left lane.

3.4.2 Comparison of HtrA1, ISG15 and HtrA1+ISG15

The experiment setup was similar to 3.4.1. The 1st tube (left lane) contained a mixture of 1 µg of HtrA1 (1 µg/µL), 1 µL of ddH_2O, 18 µL of RPMI-1640 medium; the 2nd tube (middle lane) contained a combination of 1 µg of ISG15, 1 µL of ddH_2O and 18 µL of RPMI-1640 medium; the 3rd tube (right lane) contained a mixture of 1 µg HtrA1 (1 µg/µL), 1 µg ISG15 and 18 µL RPMI-1640 medium. All tubes were incubated at 37°C, 5% CO_2 overnight (16 h). After that, SDS-PAGE gel (12%) was used and bands were detected with coomassie blue staining. The image (Fig. 16) below shows the results: the left lane showed only HtrA1 bands (asterisk); the middle lane showed ISG15 bands (arrow head), two highly intensive bands were found to show; the right lane showed the combination, HtrA1 bands were found to show more intensive compared to bands in the left lane (asterisk); ISG15 bands were found to appear in the same position but obviously weaker (arrow head). 7 extra bands were found to show in the right lane (numbers).

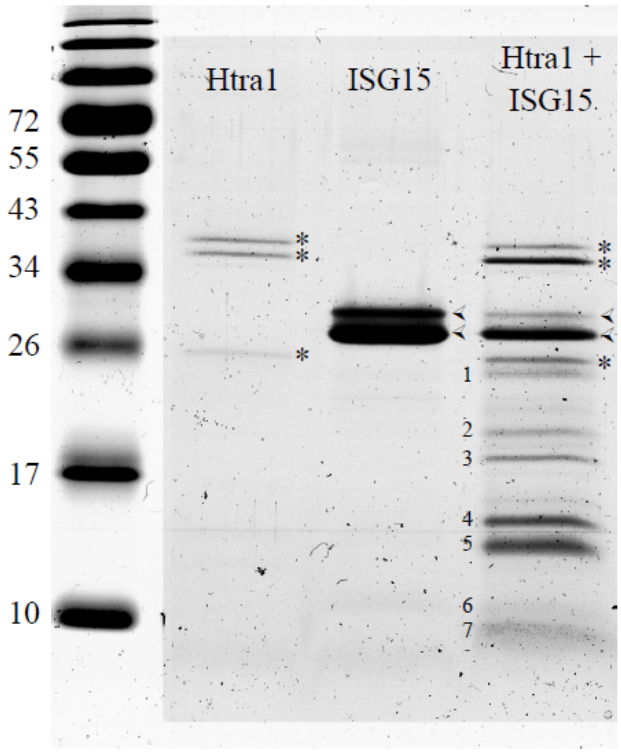


Figure 16. ISG15 is a substrate of HtrA1 Coomassie blue staining analyzes HtrA1 and ISG15 from the in vitro digestion samples. HtrA1 bands are found to show in the left and right lanes (asterisk), ISG15 bands are found to show in the middle and right lane (arrow head). 7 extra bands in the right lane are found to show (numbers).

3.4.3 Digestion with control

Bovine serum albumin (BSA) was used as a negative control as it is not a substrate for HtrA1. Human serum, which is known for its complexity, some differences were expected (between with and without HtrA1 protease). The digestion condition was the same as the above (3.4.2) and results are shown below (Fig. 17): The first three lanes showed similar results as in experiment 3.4.2. For lanes 4 and 5, lane "4" contained 1 μg BSA, 1 μL ddH_2O and 18 μL RPMI-1640 medium; lane "5" contained 1 μg BSA, 1 μg HtrA1 and 18 μL RPMI-1640 medium. Compared with lane "4", the BSA band showed the same pattern as in the lane "5" and the HtrA1 bands were found to show the similar pattern as in the lane "1". Lane "6" and "7" showed serum alone and serum with HtrA1 protease respectively. Similar bands were observed in lane 6 and 7 except for the HtrA1 bands that were also found to show in the first lane.

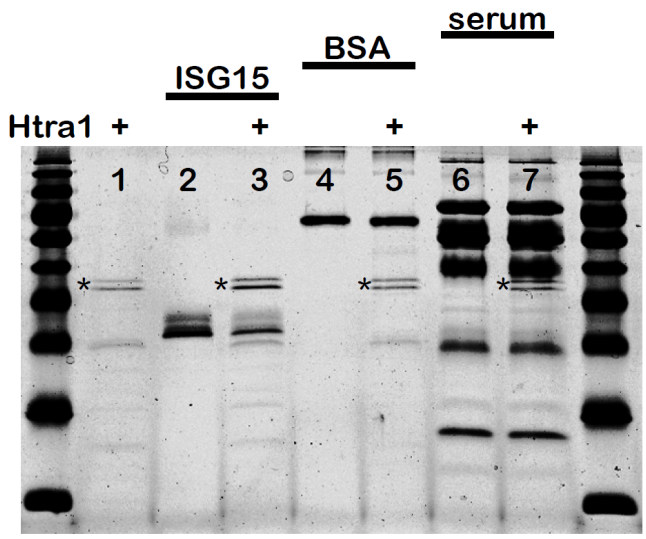


Figure 17. ISG15 is a substrate of HtrA1 (with contrast) Coomassie blue staining analyzes HtrA1 and ISG15 from the in vitro digestion samples. BSA and human serum samples were used as control. HtrA1 bands are shown in lane 1, 3, 5, and 7 (asterisk), ISG15 bands are shown in lane 2 and 3. BSA bands are shown in lane 4 and 5. Serum samples are shown in lane 6 and 7.

4. Discussion

The prevalence of AMD (around 0.05% in people younger than 50 years old and 11.8% in people older than 80 years old) is going to double in the forthcoming decades because of an expected shift in the aging population worldwide (Friedman et al., 2004). The mechanisms responsible for the pathogenesis of AMD are largely unknown.

Several biological pathways have been implicated in the pathological process of AMD.

(1) The first well accepted theory is senescence, which is shown by lipofuscin accumulation in RPE cells. Mechanisms for RPE cells aging could be explained by oxidative or photo-oxidative stress. RPE cells and outer segment of photoreceptors are exposed to one of the highest fluxes of oxygen in human body. Meanwhile, outer segments are exposed to light, especially blue and ultraviolet light. Direct damage of the cellular membrane and proteins are considered to be the causative factors of aging in RPE cells (Rozanowska et al., 1995).

(2) Except for aging, the second popular hypothesis is the formation of drusen, considered as the hallmark of the pathogenesis of AMD. Although few small hard drusen can be found in over 90% of normal population over 50 years old, the presence of numerous large hard drusen or soft drusen in the macula, especially accompanied with hypo- or hyper-pigmentation is considered as a great risk factor for developing serious late-stage AMD.

(3) Choroidal neovascularization (CNV) is considered to be another major mechanism that destroys AMD patients' normal visual function. Patients with CNV, especially in the macula, can have widely varied degree of scotoma and distortion. Newly formed small vessels, which are fragile, curled and easily leaky, usually bleed and form macular scars. Therefore, CNV is a dominant cause of visual impairment for AMD patients.

(4) Despite the fact that AMD is assumed to have multifaceted etiological processes, immune dysfunction is a recurring theme in the pathological process of AMD. (See chapter 4.2) (Fig.18)

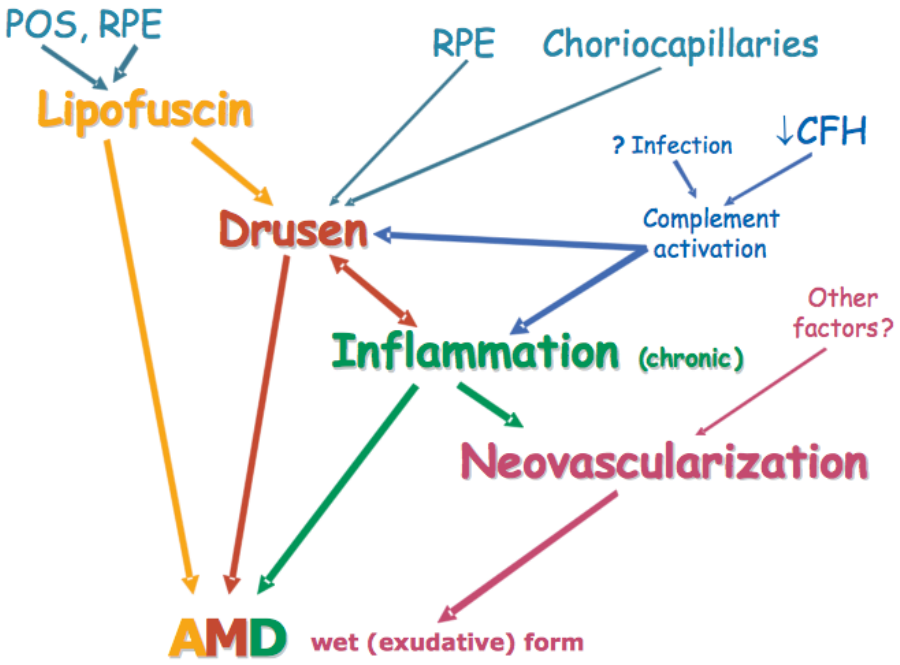


Figure 18. Four main pathogenic pathways in the pathological process of wet AMD Lipofuscin formation, Drusen formation, Inflammation (chronic) and Neovascularization. Photoreceptor outer segments (POS), Retinal pigment epithelium (RPE), Complement factor H (CFH) (Nowak, 2006)

4.1 Immune system and AMD

The integrity of the blood-retinal barrier is crucial for the immune privilege in human eye. This is due to the endogenous immunosuppressive components and the lack of functional intraocular lymphatics (Streilein, 1995; Sugita, 2009). Although a functional retinal immune system is pivotal for vision maintenance, a great number of evidence also shows the over-activation of the immune system, especially the innate immune system, plays a crucial role in the pathogenesis of AMD (Detrick & Hooks, 2010).

Among all immune activation pathways, the complement pathway is a well-studied and generally accepted as a contributor to the pathogenesis of AMD. The complement system is an ancient part of the host immune defense and a specific pattern recognition sensor for damage signals. It contains three pathways: (a) the classical pathway (b) the lectin pathway and (c) the alternative pathway, which can be activated by a variety of complement component molecules (Gasque, 2004) (Fig. 19).

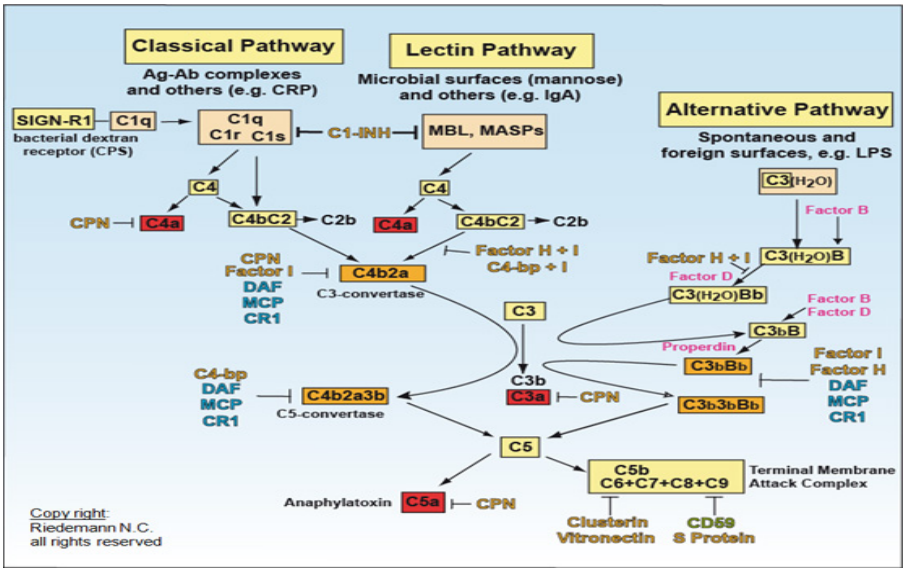


Figure 19. Complement activation pathway three complement pathways: Classical pathway, Lectin pathway, and Alternative pathway.

Genome-wide association studies (GWAS) show several genes in the alternative pathway associated with AMD, including the complement factor H (CFH), the complement factor-H-like protein 1 (CFHL1), the complement factor-H-related proteins 1-5 (CFHR1-5) (Friese et al., 1999; Raychaudhuri et al., 2011; Spencer et al., 2008) and other genes (C2, CFB, C3) that are discussed in the introduction section.

The SNPs in CFH gene have been demonstrated to be strongly associated with AMD (Edwards, 2008; Gasque, 2004; Lotery & Trump, 2007; Swaroop, Branham, Chen, & Abecasis, 2007). In human retina, CFH is expressed in RPE, choroid and drusen (P. T. Johnson et al., 2006; Mandal & Ayyagari, 2006). Previous research has demonstrated that the phagocytosis of the outer segments of oxidized photoreceptors could damage synthesis and secretion of CFH in RPE cells (M. Chen, Forrester, & Xu, 2007; Wu, Lauer, Sick, Hackett, & Campochiaro, 2007).

Drusen are extracellular deposits that are comprised of lipid-rich and protein-rich debris (Hageman et al., 2001). Proteomic, biomedical and histological researches have shown that human drusen mainly contain albumin, apolipoprotein E (ApoE), complement components and factors (including C1q, C3, C5, C5b, C6, C7, C8 and C9), vitronectin, immunoglobulin and

amyloid-beta (Crabb et al., 2002; Hollyfield, Salomon, & Crabb, 2003; L. V. Johnson et al., 2002). Drusen extracts (e.g. C1q) can induce inflammasome activation (Doyle et al., 2012).

In this study, although alternations of CFH protein were not found, the complement factor P (CFP) and complement factor D (CFD) were found to be up regulated; the complement factor I (CFI), complement component 5 (C5), complement component 3 (C3), complement component 2 (C2) and the complement factor H related protein C were found to be down regulated in the HtrA1 knockout mouse. SNPs of CFD gene were found to be associated with AMD in a cohort study by Stanton and colleagues (Stanton et al., 2011). Furthermore, in the ARPE19 cell culture experiment, similar results were found: the level of complement C1s, complement C1r and CD59 (complement regulator protein) was altered in HtrA1 overexpression cells. All these results provide evidence for the systemic activation of the complement pathway, implying that chronic inflammation plays an important role in the pathological process of age-related macular degeneration. This also highlights the role of complement system in the prevention of endogenous damage insults that result in AMD.

4.2 ISG15, HtrA1 and AMD

Interferon stimulated gene 15 (ISG15) is an ubiquitin-like modifier induced by type I interferon. This protein was first discovered in 1979 as an interferon-alpha and beta-regulated protein (Farrell, Broeze, & Lengyel, 1979). Several years later, another group purified this protein, both from human and bovine cells, which they then referred to as the 15 KDa protein induced by interferon (Korant, Blomstrom, Jonak, & Knight, 1984). Later, another group showed that immediately after its expression, ISG15 is proteolytically cut at its C-terminus thus exposing a LRLRGG sequence, which is identical with the Ubiquitin C-terminus (Potter, Narasimhan, Mende-Mueller, & Haas, 1999).

ISG15 conjugates with various intracellular proteins in a process called ISGylation. ISGylation occurs through a series of enzymatic reactions; this includes E1 activating enzyme, E2 conjugating enzyme and E3 ligase and is similar to the ubiquitin conjugation pathway. The functional outcome of ISGylation is yet to be completely clarified. Unlike ubiquitin conjugation, there is no evidence that ISGylation can lead to the proteasome-mediated degradation of target

proteins. Several functions have been ascribed to ISG15, such as chemotactic activity, direction of ligated proteins to intermediate filaments, cell communication and antiviral activity during infections. Even the conjugation form is the major form of this ubiquitin-like molecule (UBLs), the unconjugated ISG15 can also be found in the serum (Loeb & Haas, 1994; Morales & Lenschow, 2013).

The precipitation results (both ARPE19 and HeLa cell experiments) indicated similar variations of ISG15 protein. The ARPE19 cell experiment showed a 2.17 fold change while the two independent HeLa cell experiments showed a 9 fold change in both experiments (9.67 and 9.50 respectively in Flag-IP and c-Myc-IP). Therefore, a hypothesis that ISG15 might interact with HtrA1 protein can be proposed. Even previous studies have shown that ISG15 can conjugate with a huge amount of target proteins; there is no finding about the ISG15's function in age-related macular degeneration or with HtrA1 protein.

HtrA1 is a serine protease, which has proteolysis activity and degradation function. Result 3.3 showed that ISG15 protein was precipitated together with HtrA1 protein. If our results showed the interaction between ISG15 and HtrA1, is ISG15 a substrate for HtrA1 protease? Or might ISG15 simply regulate HtrA1's proteolysis activity to other substrates?

To answer the question above, in vitro digestion assay was used. From results 3.4, I can conclude that ISG15 is cut by HtrA1 and it is a substrate of HtrA1. However, even 7 clear bands in the "HtrA1+ISG15" lane were observed in result 3.4.2, the exact cutting position within ISG15 protein is still unknown. Due to HtrA1's auto proteolysis activity, three clear bands in the HtrA1 lane were observed in these experiments. In experiment 3.4.3, BSA and human serum were used as control. Our results confirmed that BSA is not a substrate of HtrA1. Human serum, which contains a huge amount of proteins, cytokines and enzymes, difference between the lanes with and without HtrA1 protease was expected. But maybe because of the resolution power of the Coomassie staining, no difference was found.

Although ISG15 is a substrate of HtrA1 protease, the exact function of the interaction is still unknown. In the future, I plan to examine its function in the HtrA1 gene trap mouse or AMD

patients' specimen. Combining all the results gained in this study, I suppose that ISG15 may function as the regulator of the immune system and result in the activation of the immune system (innate immunity), chronically destroy RPE cells, Bruch's membrane and photoreceptors, and finally lead to AMD.

4.3 ICPL label and HtrA1 mouse model

In this project, the HtrA1 gene trap mouse model was used to investigate the HtrA1 protein function in mouse serum. Serum samples from both wild type mice and knockout mice were compared.

In order to quantitatively compare the protein expression difference in these two mouse lines, ICPL assay was employed (see Chapter 2.2). From our results, complement factor P and complement factor D were found to be up regulated in HtrA1 gene trap mouse; complement component factor I, complement C5, complement C3, complement factor H related protein C, and complement component 2 were found to be down regulated (see Table 2). All of these proteins are the components or regulators of the alternative complement pathway. This may imply that HtrA1 protein can regulate the activity of the mouse complement pathway. However, there was a weakness in the experimental design: I did not check the HtrA1 expression pattern in the gene trap mouse. In future experiments, I plan to check it in these two mouse lines.

From all results, it can be concluded that HtrA1 gene as a risk gene for age-related macular degeneration can modulate the immune system activity in HtrA1 knockout mouse. Mutations in HtrA1 gene may influence the activity of complement pathways, through either the expression level or the activity of complement components, and finally disturb the normal immune function.

ISG15, as a new substrate of HtrA1 protease, may function as a regulator in the immune system; or through its conjugation with ligands (e.g. complement components), influences the normal function of immune system. In the future, I plan to use HtrA1 knockout mouse model and AMD patients' donor eyes to investigate the exact function of ISG15.

5. Zusammenfassung

ZIEL: Einzelnukleotid-Polymorphismen (englisch: Single-Nucleotide Polymorphisms, SNP) in dem chromosomalen Bereich 10q26, der die drei Gene PLEKHA1, ARMS2 und HtrA1 enthält, zeigen eine starke Assoziation mit der Pathogenese der Altersbedingten Makuladegeneration (AMD). In den letzten zehn Jahren haben sich zahlreiche Anzeichen dafür gehäuft, dass eine Hauptursache der Erkrankung in einer Mutation des HtrA1 Gens liegen könnte. Mit dieser Studie sollte daher die pathologische Rolle des Gens bzw. Proteins weiter aufgeklärt werden.

METHODEN: Ein HtrA1 "gene trap" Mausmodell wurde verwendet, um die Proteinexpression zwischen der gene trap Maus und der Wildtyp Maus zu vergleichen. Des Weiteren wurden ARPE19 Zellen und HeLa Zellen mit einem HtrA1-Plasmid transfiziert, das einen dreifach Tag trägt und für proteolytisch inaktives HtrA1 kodiert. Mittels Co-Immunopräzipitation wurden dann die Proteine präzipitiert, die mit HtrA1 interagieren. Interessante Kandidaten wurden mit Western Blot überprüft. Potentielle HtrA1 Substrats, wurden mittels in vitro Verdau getestet.

ERGEBNISSE: Die Ergebnisse dieser Studie zeigen, dass HtrA1 als ein sekretiertes Protein an verschiedene Komponenten des Komplementsystems binden kann, und zwar an die Komponenten CFP, CFD, CFI, C1r, C1s, C2, C3 und C5 aus dem klassischen Weg, sowie an Komponenten des Lektin-Weges und besonders an Komponenten des alternativen Weges. Einige dieser Proteine stehen im Zusammenhang mit der Pathogenese der Altersbedingten Makuladegeneration. Insbesondere konnten wir zeigen, dass das Ubiquitin-like protein ISG15 bei Co-Immunopräzipitation direkt mit HtrA1 interagiert. Bei einem in vitro Verdau war Ubiquitin-like protein ISG15 auch ein Substrat für HtrA1.

AUSWERUNG: Die Aktivierung des Komplementsystems durch HtrA1, die wir unseren Assays gefunden haben, unterstützt die Vermutung, dass das Immunsystem eine entscheidende Rolle bei der Pathogenese von AMD spielt. Die Ergebnisse dieser Arbeit lassen vermuten, dass HtrA1 die Aktivität des Immunsystems reguliert.

6. Publications or Presentations

Lili Feng, Elod Kortvely, Andreas Vogt, Karsten Boldt, Marius Ueffing. **Quantitative proteomic identification of novel interacting partners for the serine protease HtrA1** Poster and oral presentation at the annual meeting of the Association and Research in Vision and Ophthalmology, Seattle, May 2013 (Lili Feng was awarded the great potential young scientist in ARVO 2013)

7. Curriculum Vitae

Education

Institution	Years	Degree	Fields of study
Southeast University, China	2000-2005	Bachelor	Clinical Medicine
Southeast University, China	2007-2010	Master	Ophthalmology
University of Tuebingen, Germany	October 2010-Present	Dr. Med Candidate	Ophthalmology (AMD)

Professional Experience

Institution	Years	Title
ZhongDa Hospital, China	2004-2005	Internship
ZhongDa Hospital, China	2008-2010	Resident/Teaching Assistant

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