Dorota Zurawa-Janicka, PhD

Summary of Professional Accomplishments

Department of General and Medical Biochemistry
Faculty of Biology
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Table of contents

1. Name

Dorota Zurawa-Janicka

2. Diplomas, degrees conferred in specific areas of science or arts, including the name of the institution which conferred the degree, year of degree conferment, title of the PhD dissertation

Master of Science in biology – Intercollegiate Faculty of Biotechnology of the University of Gdansk and Medical University of Gdansk, June 1998.

Ph.D. in biology – University of Gdansk, Faculty of Biology, Geography and Oceanology, November 2008. Title of doctoral dissertation: "Implication of HtrA1 and HtrA2 proteins in defense against estrogen-induced oxidative stress and nephrocarcinogenesis in Syrian hamster (*Mesocricetus auratus*)"

3. Information on employment in research institutes or faculties/departments or school of arts

01.04.1999 – 31.03.2000: 1st year-research-and-teaching assistant, University of Gdansk, Faculty of Biology, Geography and Oceanology, Department of Biochemistry

01.10.2004 – 30.09.2005: senior technician, University of Gdansk, Faculty of Biology, Geography and Oceanology, Department of Biochemistry

01.10.2005 – 31.01.2009: research-and-teaching assistant, University of Gdansk, Faculty of Biology, Geography and Oceanology, Department of Biochemistry

01.02.2009 – till now: assistant professor, University of Gdansk, Faculty of Biology, Department of General and Medical Biochemistry

4. Description of the achievements, set out in art. 219 para 1 point 2 of the Act

a) Title of the scientific achievement:

Molecular mechanism of action of human HtrA proteases and their implication in pathogenesis of cancer

b) Publications that are part of that achievement:

[1]. Zurawa-Janicka D., Skorko-Glonek J., Lipinska B. (2010). HtrA proteins as targets in therapy of cancer and other diseases. *Expert Opinion on Therapeutic Targets* 14(7): 665-679.

IF_{2010}=3.649; MNiSW=32

[2]. Zurawa-Janicka D.*, Kobiela J., Gałczyńska N., Stefaniak T., Lipinska B., Lachinski A., Skorko-Glonek J., Narkiewicz J., Proczko-Markuszewska M., Sledzinski Z. (2012). Changes in expression of human serine proteases *HtrA1*, *HtrA2* and *HtrA3* genes in benign and malignant thyroid tumors. *Oncology Reports* 28(5): 1838-1844.

IF_{2012}=2.297; MNiSW=20

[3]. Zurawa-Janicka D., Jarzab M., Polit A., Skorko-Glonek J., Lesner A., Gitlin A., Gieldon A., Ciarkowski J., Glaza P., Lubomska A., Lipinska B. (2013). Temperature-induced changes of HtrA2(Omi) protease activity and structure. *Cell Stress and Chaperones* 18(1): 35-51.

IF_{2013}=2.537; MNiSW=20

[4]. Jarzab M., Wenta T., **Zurawa-Janicka D.,** Polit A., Gieldon A. J., Wysocka M., Glaza P., Skorko-Glonek J., Ciarkowski J., Lesner A., Lipinska B. (2016). Intra- and intersubunit changes accompanying thermal activation of the HtrA2(Omi) protease homotrimer. *Biochimica et Biophysica Acta-Proteins and Proteomics* 1864: 283-296.

IF_{2016}=2.773; MNiSW=30

[5]. Giełdon A., **Zurawa-Janicka D.**, Jarzab M., Wenta T., Golik P., Dubin G., Lipinska B., Ciarkowski J. (2016). Distinct 3D Architecture and Dynamics of the Human HtrA2(Omi) Protease and Its Mutated Variants. *PloS One* 11(8): e0161526.

IF_{2016}=2.806; MNiSW=35

[6]. Zurawa-Janicka D.*, Wenta T., Jarzab M., Skorko-Glonek J., Glaza P., Gieldon A., Ciarkowski J., Lipinska B. (2017). Structural insights into the activation mechanisms of human HtrA serine proteases. *Archives of Biochemistry and Biophysics* 621: 6-23.

IF_{2017}=3.118; MNiSW=30

[7]. Zurawa-Janicka D.*, Kobiela J., Slebioda T., Peksa R., Stanislawowski M., Wierzbicki P.M., Wenta T., Lipinska B., Kmiec Z., Biernat W., Lachinski A.J., Sledzinski Z. (2020). Expression of *HTRA* Genes and Its Association with Microsatellite Instability and Survival of Patients with Colorectal Cancer. *International Journal of Molecular Sciences* 21: 3947.

IF_{2020}=4.556; MNiSW=140

* - corresponding author

The Impact Factor and the number of the points awarded by the Ministry of Science and Higher Education are listed according to the specific year of publication.

Sum of the impact factors, according to Journal Citation Reports (JCR), evaluated for the specific year of publication: **21.736**

h–index according to Scopus – **6**

h–index according to Web of Science – **5**

c) Aim of the above publications and description of obtained results, including description of their possible utilization

The aim of above publications was to expand our knowledge about the molecular mechanism of action of the human HtrA proteases and their implication in carcinogenesis.

HtrA (High temperature requirement A) proteins comprise a family of evolutionarily conserved serine proteases, homologs of the HtrA protease from the bacterium *Escherichia coli*. They belong to the family of heat shock proteins (Heat shock proteins, HSPs). HSPs are induced under stress conditions to protect the cell against harmful effects of their action. HtrAs are widely present among prokaryotic and eukaryotic organisms, from bacteria to humans. Their characteristic feature is the presence of a protease domain with the catalytic triad composed of His-Ser-Asp, which is typical for serine proteases, and at least one PDZ (PSD-95, mammalian postynaptic density of 95kDa; DLG, *Drosophila* discs large tumor suppressor, ZO-1; zonula occludens 1) domain at the C-terminal end, implicated in protein-protein interactions. The protease domain adopts a chymotrypsin-like fold; it is composed of two perpendicularly arranged β-barrels, each β-barrel is formed by six antiparallel β strands connected with loops LA, L1, L2, L3 and LD, which act as regulatory elements **[Krojer *et al.*, 2010; Hansen *et al.*, 2013]**. Monomers form oligomeric structures with a trimer as the primary unit. HtrA proteases function as important cellular factors of protein quality control; they recognize irreversibly damaged or incorrectly folded proteins and degrade them. Some of them exhibit chaperone activity towards proteins with aberrant structure thus preventing the generation of toxic aggregates. Thereby, HtrAs facilitate survival of the cell under stress conditions. Moreover, the HtrA proteases exhibit proteolytic activity towards specific native proteins and, in this way, participate in regulation of many physiological processes. HtrAs of pathogenic bacteria function as virulence factors and important elements of pathogen's defense system against stress conditions induced in response to infection in host organism. HtrAs of eukaryotic organisms regulate cellular homeostasis and their dysfunction corelates with the development of pathological states. There are four homologous HtrA proteins in humans (HtrA1-4). HtrA1 and HtrA3 have been assigned to tumor suppressors due to their implication in promotion of apoptosis. They act as inhibitors of the TGF-β signaling pathway and participate in reorganization of extracellular matrix. HtrA2(Omi) protease is a mitochondrial protein. It is involved in the maintenance of mitochondrial homeostasis, stress response, and function as a proapoptotic protein which promotes apoptosis at multiple stages. In response to action of apoptosis-inducing

agents HtrA2 is released from mitochondria into the cytosol where it degrades antiapoptotic proteins including IAPs (Inhibitor of apoptosis proteins), and activates proapoptotic proteins. HtrA2 may also promote cell death due to its proteolytic activity, independently of caspase activation. Impaired activity of HtrA2 or its loss leads to dysfunction of mitochondria and is associated with neurodegenerative changes **[Skorko-Glonek *et al*., 2013]**. HtrA4 is the least characterized among human HtrA proteases; its implication in embryo implantation and pathogenesis of preeclampsia has been described **[Chen *et al*., 2009; Liu *et al*., 2018]**. Multiple lines of evidence reveal that dysfunction of HtrAs contributes to the development of several diseases including cancer, arthritis and neurodegenerative disorders – Parkinson's and Alzheimer's diseases **[Skorko-Glonek *et al*., 2013]**.

My research interest in the human HtrA proteases was a continuation of my PhD dissertation work, which involved implication of HtrA1 and HtrA2 proteases in stress response and nephrocarcinogenesis in Syrian hamster (*Mesocricetus auratus*). The results of my studies revealed that expression of the *HTRA* genes undergoes significant changes in response to estrogen treatment; estrogen-induced oxidative stress stimulated expression of *HTRA1* whereas prolonged estrogenization resulted in an enhanced expression of *HTRA2* and reduced expression of *HTRA1*. These results indirectly indicated an implication of the HtrA proteases in stress response and hormone-induced carcinogenesis.

The literature review concerning HtrA proteases became an introduction to my further investigation **[1]**. Although the knowledge about HtrAs was limited at that time, the collected data revealed a significant implication of these proteases in the maintenance of cellular homeostasis through their involvement in regulation of signaling pathways and stress response. On the other hand, results of published studies proved that HtrA proteases are one of the key regulators of apoptosis and anoikis – mechanisms of cell death whose disturbances open the way to neoplastic transformation and metastasis, and also revealed an association between the impairment of function or loss of HtrAs and development of pathological conditions. Such extremely diverse functions of HtrA proteases in cell physiology raised the question about the molecular mechanism of their action by which, depending on the presence of a specific factor, the protease is activated or deactivated, which in turn initiates the transition between the state promoting survival of the cell and directing it to apoptotic death. Understanding of this mechanism will improve the current basic knowledge concerning the HtrA proteases and could have applicative values; proapoptotic properties of HtrA proteases could be used in treatment of diseases.

In 2002, for the first time, the structure of human HtrA has been resolved. Based on crystallographic analysis, the structure of the HtrA2 protease in an inactive form has been resolved and published by **[Li *et al*., 2002]**. Also theoretical model of HtrA2 activation has been proposed. The model has been supported by very sparse experimental, biochemical and biophysical data. HtrA2 forms a pyramid-shaped homotrimer. Protease domains are located at the top of the trimer and comprise its core, and PDZ domains form the bottom. According to the model, the HtrA2 activity is allosterically regulated in a PDZ domain-dependent manner. In an inactive conformation, the C-terminal end of the PDZ domain interacts with the protease domain blocking an access to the catalytic site. Binding of a peptide to the hydrophobic groove of the PDZ domain causes significant

conformational changes leading to the exposure of the catalytic site by the PDZ domain. The protease adopts an active confirmation which is accompanied by the protease activation **[Li *et al*., 2002]**. Moreover, it has been postulated that activation by an allosteric peptide and activation by temperature increase might have similar effects **[Martins *et al*., 2003]**. This model became a starting point for further investigation aimed at experimental verification of the mechanism of HtrA2 regulation and understanding the molecular events accompanying the protease activation. The results of these studies are part of this achievement.

At the beginning, biochemical analysis of HtrA2 was performed. The obtained results indicated that HtrA2 exhibits kinetic properties characteristic for allosteric enzymes, and revealed that HtrA2 activity markedly increases with increasing temperature. These findings were consistent with the allosteric model of HtrA2 regulation **[Li *et al*., 2002]** and indirectly supported the postulated physiological role for HtrA proteins related to the response to action of stress-inducing factors including temperature elevated above the physiological value **[Clausen *et al*., 2011].** Moreover, the heat shock-treated HtrA2 cannot be further stimulated by an activating peptide that binds to the binding groove of the PDZ domain. This result argued that events accompanying the thermal activation of HtrA2 are similar to those induced by an allosteric peptide, and it was in agreement with the results of **[Martins *et al*., 2003].**

According to the theoretical model of HtrA2 regulation, the structural changes accompanying activation of the protease occur at the interface between the protease (PD) and PDZ domains, and remove the inhibitory effect of the PDZ domain from the catalytic site. Consequently, activity of the protease increases. Based on previous studies on the mechanism of the *E. coli* HtrA regulation **[Sobiecka-Szkatula *et al*., 2009]**, I decided to verify the HtrA2 activation model by monitoring temperature-induced structural changes using spectroscopic techniques. A set of single-Trp HtrA2 mutants with Trp residues located at the interface between the PDZ and protease domains, including the peptide binding groove was prepared. Due to spectroscopic properties, Trp served as a specific molecular probe; it allowed tracking local conformational changes in the surrounding microenvironment induced by temperature increase. Analysis of wavelength maxima of Trp fluorescence and parameters of acrylamide quenching of Trp fluorescence revealed that under temperature up-shift the PDZ-protease domain interface becomes more exposed to aqueous medium and that conformational changes depend on temperature and are most pronounced in the temperature range from 30 to 35^{o}C. Furthermore, the obtained data showed that the structural rearrangements occur gradually; firstly, in the PDZ domain and subsequently, in the protease domain. The above results also suggested that during thermal activation the HtrA2 structure relaxes. Cooperation with Agnieszka Polit, PhD from the Department of Biochemistry, Biophysics and Biotechnology of the Jagiellonian University provided opportunities to verify this assumption. To gain information concerning the potential structural changes occurring in the HtrA2 molecule during temperature up-shift the method of dynamic light scattering (DLS) was used. Analysis of hydrodynamic parameters revealed that in the temperature range from 20 to 45^{o}C HtrA2 forms a uniformly sized oligomer and its hydrodynamic radius increases gradually with temperature increase. These results support the idea that during temperature increase the HtrA2 oligomers undergoes relaxation accompanied by an increase in size.

It has been proposed that interactions of hydrophobic amino acid residues located at the PDZ-protease domain interface are crucial for the maintenance of the HtrA2 structure – these include hydrophobic residues on strands β11 and β12 of the protease domain, and β14 and α5 of the PDZ domain **[Li *et al*., 2002]**. I decided to make an attempt to verify this assumption experimentally. The obtained results indicated that substitutions of selected hydrophobic residues belonging to the β11 and β12 strands with hydrophilic ones (I325D, I329N, F331Y) resulted in a loss of the protease activity. Moreover, two substitutions (V226K, R432L) aiming at weakening the interaction between the PDZ and protease domains resulted in a significant increase of activity. These results confirmed the importance of hydrophobic interactions involving amino acid residues located at the PDZ-protease domain interface for structural integrity of HtrA2. Furthermore, they proved indirectly that the weakening of the interdomain interactions can significantly influence the opening of the structure and increase activity of HtrA2 during thermal activation. These results presented above accounted for the **publication [3]**.

Subsequently, studies on HtrA2 thermal activation were continued with the attempt to understand the molecular events creating this mechanism. The research was focused on interactions important for stabilization of the protease domain structure within the monomer and interactions between adjacent subunits within the trimer, as well as the role of regulatory loops. Based on the analysis of the HtrA2 crystal structure, regions crucial for interdomain interactions were selected (the α5 helix and β14 strand of the PDZ domain with the C-terminal β-barrel of PD, and the β13 strand and α7 helix of the PDZ domain with the N-terminal β-barrel of PD). To monitor temperature-induced structural changes between the selected elements of these regions, the tryptophan-induced quenching (TriQ) method was used. TriQ utilizes the ability of Trp to quench fluorescence of a bimane probe and occurs over short distances when the fluorophore and the Trp residue are in proximity of 5-10 Å **[Mansoor *et al*., 2010].** The TriQ-based studies showed that the temperature-induced structural rearrangements occur inside the monomer (the L3 loop increases its distance from the PDZ domain, the β14 strand of the PDZ domain moves towards both β-barrels, the β13 strand increases its distance from loops LC and LB of the N-terminal β-barrel, the loops flanking the β15 strand moves away from the protease domain), and importantly, they take place between adjacent subunits (the distance between the α5 helix of the PDZ domain and the LD* and L1* loops of the protease domain of the adjacent subunit increases) (* indicates a structural element of an adjacent subunit). There was an assumption that changing interactions between structural elements of the PDZ and protease domains could influence the activity of the protease. To verify this assumption, we prepared a set of HtrA2 muteins by replacing the selected amino acid residues crucial for the maintenance of interdomain contacts with amino acid residues with different physicochemical properties. Analysis of proteolytic activity of these muteins showed that the substitutions that weaken the interactions between the PDZ and protease domains inside the monomer and between the PDZ domain and the protease domain of the adjacent subunit (PD*) promote protease activity. This result suggests that destabilization of the PDZ domain interaction with the protease domain inside the monomer as well as with the structural elements of PD* promotes activation of HtrA2, and is consistent with the results obtained using the TriQ method. Furthermore, the α5 helix of the PDZ domain may play an important role in the structural

rearrangements occurring inside the subunit and within PD*, and therefore presumably may be a crucial element in the sequence of events that happens during thermal activation of HtrA2. Because in the inactive conformation of HtrA2 the arrangement of the catalytic triad is not optimal for catalysis **[Singh *et al*., 2011]**, the conformational changes occurring in the L1* loop with the serine residue of the catalytic triad may facilitate proper positioning of the triad elements and formation of a functional active site.

The results presented above contributed to the publication **[4]**, in which a model of HtrA2 thermal activation has been proposed. According to this model, under temperature up-shift the PDZ domain changes its position towards the protease domain and this change occurs in two planes. Moreover, structural rearrangements affect the monomer and are transferred to the adjacent subunit in the HtrA2 trimer. Under temperature up-shift the L3 loop moves away from the PDZ domain, which slides in the plane formed by the PDZ-protease domain interface in the direction opposite to the catalytic site and, subsequently, rotates lifting its edge overhanging the catalytic site. Consequently, the catalytic site becomes exposed and the access to the peptide binding groove in the PDZ domain increases. Simultaneously, structural rearrangements are transferred to the adjacent subunit, in particular, they include the L1* loop where the serine residue of the active site of PD* is located.

The proposed model of HtrA2 thermal activation did not answer the question whether the sequence of events accompanying activation by the peptide is similar to the changes that occur during temperature rise. The answer to this question was challenging because the crystal structure of HtrA2 in an active form was unknown – only the structure of HtrA2 S306A with the active site serine replaced with alanine was published **[Li *et al*., 2002]**. Moreover, there were unresolved regions in this structure – the flexible linker located between the PDZ and protease domains and the L3 loop that is essential for activation. In collaboration with Artur Giełdon, PhD (currently: PhD, DSc) from the Team of Jerzy Ciarkowski, PhD, DSc, ProfTit from the Department of Theoretical Chemistry of the University of Gdansk and Grzegorz Dubin, PhD (currently: PhD, DSc, ProfTit) from the Malopolska Center of Biotechnology of the Jagiellonian University, the missing structures were restored and the HtrA2 structure in an active form was modeled. Also, the structures of two HtrA2 variants: HtrA2 V226K and HtrA2 V325D which I constructed and characterized biochemically were modeled. Positions 226 and V325D are part of the regions of hydrophobic interactions of amino acid residues located at the PDZ-protease interfaces. These interactions are crucial for the maintenance of the protease structure. The V325D substitution resulted in a loss of HtrA proteolytic activity, the V226K substitution resulted in its increase. The V226 residue is a part of the loop between the β5 and β5 strands of PD. In an inactive conformation it is locates in front of the peptide binding groove of the PDZ domain and restricts ligand access to it. Thus, a substitution that changes interactions in this region could favor the opening of the structure during activation. This variant and the V325D variant were included in the *in silico* studies aimed at understanding the sequence of molecular events accompanying activation of HtrA2 by a peptide but also at undertaking to answer the question about the molecular basis of the increase in activity caused by the V226K substitution. To obtain this goal, these structures modeled with and without the peptide ligand GWTMFWV bounded to the peptide binding groove of the PDZ domain

were subjected to molecular dynamic simulations. The crystal structure of the PDZ domain with this peptide was described previously **[Zhang *et al.*, 2007]**.

The obtained results of the *in silico* studies showed that binding of the peptide triggers rotation of the PDZ domain towards the protease domain by an angle of approximately 30-50^{o} in a lid-like fashion. In the trimer these movements progress sequentially during the 50 ns simulation and resemble a flower bud opening. It results in the exposure of the enzyme's catalytic center. The PDZ domains opened dynamically in the wild-type (unmutated) protease, whereas this movement was not observed in the inactive HtrA2 V325D protein. Moreover, in the absence of a peptide the PDZ domain movement relative to the protease domain seemed to be more subtle and the PDZ-protease domain interfaces were only slightly exposed. Taking into account the results of the *in silico* analysis and the results of the *in vitro* studies it can be suggested that while the mechanisms of HtrA2 activation by temperature up-shift and peptide binding share a common general scheme, they may differ in details. In the course of the study, the crystal structure of the HtrA2 S306A V226K protein (exhibiting an increased activity) was solved. However, its overall architecture resembles that of the structure of HtrA2 S306A. The molecular basis of the increased activity of this HtrA2 mutein has not been elucidated. These results were collected and published in **[5]**.

The results presented and discussed in publications **[3]**, **[4]**, **[5]** provided additional arguments for the PDZ domain-dependent allosteric regulation of HtrA2 activity. Upon activation, conformational changes at the PDZ-protease domain interface occur and lead to removal of the inhibitory effect of the PDZ domain from the catalytic center. Also, the access to the peptide binging groove in the PDZ domain increases. These rearrangements, combined with the regulatory loops' movements, transfer the allosteric signal to the adjacent subunit and result in the formation of a catalytically competent active site. The mechanisms of thermal activation and activation by an allosteric peptide may have a similar scheme.

The review article **[6]** which is a part of the presented cycle summarizes the current knowledge regarding structure and mechanisms of regulation of the human HtrA proteases. The results published in articles **[3]**, **[4]**, **[5]**, which I co-authored, have been included in this review.

Aside from the molecular mechanism of action of the human HtrA proteases, my interests concerned their implication in carcinogenesis. Literature review suggested that these proteins may play essential roles in mechanisms of cancer pathogenesis and could be used as potential markers in diagnostics and cancer treatment **[1]**. Cooperation with Tomasz Stefaniak, MD (currently: MD, DSc) and Jaroslaw Kobiela, MD (currently: MD, DSc) from the Team of Zbigniew Sledzinski, MD, DSc, ProfTit form the Department of General, Endocrine and Transplant Surgery of the Medical University of Gdansk provided opportunities for research in this field. I focused on the pathogenesis of thyroid tumors and pathogenesis of colorectal cancer. The former ones are the most common tumors of the endocrine system. This group comprises benign lesions and malignant tumors, including papillary and follicular carcinomas which exceed approximately 90% of thyroid malignancies **[Fagin & Wells, 2016]**. Colorectal carcinoma is one of the most frequently diagnosed cancer worldwide, with higher incidence in developed countries. Colorectal cancer is the fourth leading cause of cancer-related deaths **[Arnold *et al.*, 2017; Kuipers *et al.*, 2015]**. Our studies aimed at answering the question on the relationship

between HtrA1-3 proteins, including the HtrA3 isoforms and pathogenesis of these cancers. HtrA3 protein was previously identified as a pregnancy-related protease **[Nie *et al.*, 2003]**. It is believed to function as a tumor suppressor in the development of some types of cancers **[Skorko-Glonek *et al.*, 2013]**. Two HtrA3 isoforms produced by the alternative RNA splicing may exist in the cell: a long (HtrA3L) and short (HtrA3S) one. HtrA3S lacks the C-terminal PDZ domain which in HtrA3L is replaced by a unique amino acid sequence **[Nie *et al.*, 2003]**. The question arose about the meaning of the presence of these isoforms in the cell; their physiological roles in cell physiology and development of pathologies were unknown. The lack of the PDZ domain makes HtrA3S unique among proteins of the HtrA family. Therefore, the question about the relationship between the HtrA3 isoforms and oncogenesis became even more intriguing.

Forty patients treated for benign and malignant thyroid tumors were included in the study aimed at analysis of the implication of HtrAs in thyroid carcinogenesis. In tumor and control (macroscopically unchanged thyroid tissue) thyroid tissue samples expression of the *HTRA1-3* genes was examined by evaluation of the HtrA proteins' levels using the immunoblotting technique. It was found that levels of HtrA2 and HtrA3S proteins were elevated in malignant tumor tissue compared to control and benign tumor tissues, whereas the HtrA3L protein level was higher in malignant tumor tissue compared to benign lesion tissue. These results suggest an association between the expression of the *HTRA* genes, including *HTRA3* variants, and thyroid carcinogenesis. Interestingly, the HtrA3L level was increased in control tissue originating from malignant tumor patients compared to control tissue from patients with benign lesions, which suggest that the higher level of HtrA3L in thyroid tissue may be correlated with the development of thyroid carcinoma. Furthermore, papillary and follicular thyroid malignancies differed in *HTRA1* and *HTRA3S* expression; the HtrA1 protein level was decreased in papillary carcinoma, while the HtrA3S protein level was increased in follicular carcinoma. It suggests that expression of the *HTRA1/3S* genes may be associated with the histological type of thyroid cancer. The obtained results showed for the first time that in the course of thyroid carcinogenesis expression of the *HTRA3* variants may vary and depend on the histological type of the tumor. Therefore, it can be assumed that these isoforms may have different functions in the pathogenesis of thyroid cancer. The collected results contributed to **publication [2]**.

When I started researching the participation of HtrA proteases in the pathogenesis of colorectal cancer, I teamed up with the Team of Zbigniew Kmiec, MD, DSc, ProfTit from the Department of Histology and Rafal Peksa, MD from the Department of Pathophysiology of the Medical University of Gdansk. The results of this cooperation contributed to **publication [7]**. Sixty-five colorectal cancer patients were included in the study. From each patient the colorectal tumor tissue and control tissue (macroscopically unchanged colonic mucosa) samples were collected. In these samples the expression of *HTRA1-3* genes was determined; mRNA level was evaluated by real-time PCR and protein level by immunoblotting. The obtained results indicated that the *HTRA1* mRNA level was elevated in tumor tissue, specifically in primary lesions of metastasizing cancer (combined stages III and IV according to the TMN scale **[Amin *et al.*, 2017]**) while the *HTRA2* mRNA level was decreased in tumor tissue. Moreover, the protein levels of HtrA1 and HtrA2 were reduced in colorectal tumor tissue, specifically in tumors from patients with advanced disease. These results suggest that among

HTRA genes, expression of *HTRA1* and *HTRA2* changes during colorectal carcinogenesis, and furthermore, these changes may be associated with the metastatic potential of the tumor. Searching for the cause of the observed changes in *HTRA1/2* expression we checked whether there is a link between the mRNA levels of *HTRA* genes and microsatellite instability (MSI). MSI is associated with a dysfunction of the DNA mismatch repair system and underlies one of the three molecular pathways responsible for the development of colorectal cancer (the others are: the chromosomal instability pathway (CIN) and the CpG island methylator phenotype pathway (CIMP)) **[Mundade *et al*., 2014]**. In the study the microsatellite status of each colorectal tumor was examined by determination of the polymorphism of microsatellite sequences belonging to the panel of markers recommended for defining the microsatellite stability status of colorectal cancer **[Boland *et al*., 1998]**. Based on this analysis tumors were characterized as microsatellite stable (MSS) and microsatellite instable with low microsatellite instability (MSI-L) and high microsatellite instability (MSI-H). Then possible correlations between microsatellite status of a tumor and expression of *HTRA* genes were determined. It was found that the *HTRA1* and *HTRA2* transcripts' levels were reduced in tumors with MSI-H compared to MSS ones. Moreover, in case of *HTRA1*, the transcript level was increased in MSS tumors compared to the transcript level in control tissue. These results indicated that the mRNA levels of *HTRA1/2* decrease with increasing MSI, and suggest that the observed changes in the expression of these genes may be, at least partially, associated with the phenomenon of microsatellite instability. An interesting observation resulted from the analysis of the patient survival curves; the patients with a low protein level of HtrA1 or HtrA2 had a lower survival rate, moreover, the decrease of levels of both HtrA1 and HtrA2 proteins had a negative cumulative effect on the survival time of the patients. Furthermore, no significant changes in expression of *HTRA3L* and *HTRA3S* were observed. Taking into account the results of the previous study **[2]**, it can be assumed that the implication of each HtrA3 isoform in neoplastic transformation may be different and may depend on the type of tumor. The results presented in **[7]** serve as an interesting starting point for detailed further studies on the characteristics of HtrAs in colorectal cancer development and their usefulness as markers in diagnostics and treatment of this type of cancer.

Summarizing, major discoveries presented in the papers comprising my scientific achievements are:

• Finding that during temperature up-shift conformational changes occur at the interface between the PDZ and protease domains and lead to the relaxation of the HtrA2 structure. These changes occur gradually, first, in the PDZ domain and subsequently, in the protease domain.

• Finding that hydrophobic interactions of amino acid residues located at the interface between domain surfaces are important for stabilization of the HtrA2 structure, and that during thermal activation the interactions between amino acid residues of interdomain contacts change and result in opening of the structure and increasing the access to the peptide binding groove of the PDZ domain and the protease catalytic site.

• Finding that during thermal activation the PDZ domain changes its position relative to the protease domain inside the monomer, specifically a prominent change affects the L3 regulatory loop, and that the PDZ domain changes its position with respect to the protease domain of the adjacent

subunit which is accompanied by structural changes in the L1* loop containing the active site serine. Finding that the α5 helix is an important mediator of changes within the monomer and between the subunits in the trimer.

• Stating that the mechanism of thermal activation of the HtrA2 protease may follow a similar scheme as activation by an allosteric peptide.

• Proposing the scheme of HtrA2 regulation in which the PDZ domain changes its position relative to the protease domain during activation. The PDZ domain moves along the PDZ-protease domain interface in the direction opposite to the catalytic site. This movement turns into a rotation. As a result, the edge of the PDZ domain exposes the catalytic site and the access to the peptide binding groove increases. At the same time, the conformational changes are transferred to the adjacent subunit, specifically, they concern the L1* regulatory loop, which results in adoption of an active conformation by the catalytic site.

• The conclusion that expression of *HTRA1-3* genes, including *HTRA3* variants, changes during the development of thyroid cancer, which indirectly suggests the relationship between HtrAs and the pathogenesis of thyroid cancer, and a different function of HtrA3 isoforms in the development of this type of cancer.

• Finding that expression of *HTRA1/2* genes changes during colorectal carcinogenesis and may be, at least partially, correlated with microsatellite instability, and that the decrease in the HtrA1/2 protein levels had a negative and cumulative effect on the survival time of the colorectal cancer patients, and also suggestion that changes in *HTRA1/2* expression may correlate with the metastatic potential of the tumor.

• Conclusion that expression of *HTRA3L* and *HTRA3S* may be different during carcinogenesis and may depend on the type of tumor.

The results of the studies described above provide an insight into the molecular details of the HtrA2 protease activation and complement the knowledge about the regulation of proteins belonging to the HtrA family. This knowledge may serve as the starting point for research on the development of molecules selectively modulating the HtrA2 protease activity based on allosteric effects. Taking into account the proapoptotic properties of HtrA2 such molecules could be used in the treatment of diseases, including cancer. Moreover, the results of the research supplement the current knowledge on the relationship between HtrAs and cancer pathogenesis, and may be a basis for further research on the role of these proteases in mechanisms of carcinogenesis and their usefulness as potential cancer biomarkers.

Additional literature (except publications that are part of the scientific achievement)

Amin M.B., Edge S., Greene F., Byrd D.R., Brookland R.K., Washington M.K., *et al*. (2017). AJCC Cancer Staging Manual, 8th ed. American Joint Committee on Cancer: Chicago, IL, USA.

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5. Presentation of significant scientific or artistic activity carried out at more than one university, scientific or cultural institution, especially at foreign institutions

After graduation, as a member of the Team of Barbara Lipinska, PhD, MSc, ProfTit, I was involved in research aimed at characterizing the molecular mechanism of action of the *E. coli* HtrA protease. We made an attempt to solve the HtrA structure by using X-ray crystallography. In the period from January to June 2001, I conducted a series of experiments aiming to obtain monocrystals of the HtrA protein **[Appendix 3, II.11]**. I performed the experiments in the laboratories of the Center of Biocrystallographic Research (CBB) in Poznan, which is affiliated with the Institute of Bioorganic Chemistry of the Polish Academy of Sciences. At that time, I attended the CBB scientific seminars. Although the obtained results were promising, we decided to suspend further studies – in 2002 the *E. coli* HtrA protein structure was solved by another team.

The research aimed at understanding the molecular mechanism of regulation of the HtrA2 protease required a detailed analysis of the structure of this protein. At that time, I established cooperation with Artur Gieldon, PhD (currently: PhD, DSc) from the Team of Jerzy Ciarkowski PhD, DSc, ProfTit from the Department of Theoretical Chemistry of the University of Gdansk, who had experience in the analysis and modeling of the spatial structure of molecules and was involved in our previous studies on the structural characteristics of the *E. coli* HtrA protease carried out by Joanna Skorko-Glonek, PhD, DSc, ProfTit **[Sobiecka-Szkatula *et al.,* 2009 – Appendix 5, II.4, publication no. 9]**. This cooperation was initiated by a discussion on the correlations between the results of the protease activity assays of HtrA2 muteins which I obtained and structural changes caused by substitutions of selected amino acid residues introduced to the PDZ-protease domain interface. This collaboration, lasting several years uninterruptedly, gave the opportunity to confront and discuss the results of *in vitro* experiments, spectroscopic studies and biochemical analyzes in which I participated, with the results of structural analysis and molecular modeling, and contributed to the development of the HtrA2 activation model. These results were published in **[3]**, **[4]**, **[5]**.

During studies on the HtrA2 thermal activation, I established collaboration with Agnieszka Polit, PhD from the Department of Biochemistry, Biophysics and Biotechnology of the Jagiellonian University. We made contact during the 46th Congress of the Polish Biochemical Society in Cracow in 2011. A. Polit, PhD had experience in studying the interactions of macromolecules using fluorescence spectroscopy and labelling molecules with fluorescent markers. I planned experiments using the TriQ method. Moreover, the analysis of the hydrodynamic parameters of the HtrA2 oligomer using the DLS method was carried out. The obtained results confirmed the thesis that thermal activation of HtrA2 is accompanied by relaxation of the protease structure. The above results were published in **[3]**.

In 2012, I started cooperation with Grzegorz Dubin, PhD (currently: PhD, DSc, ProfTit) from the Malopolska Center of Biotechnology of the Jagiellonian University. This contact was triggered by an attempt to explain the increased activity of one of the HtrA2 muteins (HtrA2 V226K). Due to this cooperation, the crystal structure of the mutein was solved and published **[5]**.

Studies on the relationship between human HtrA proteins and pathogenesis of selected neoplasms were conducted in collaboration with Tomasz Stefaniak, MD (currently: PhD, DSc) and

Jaroslaw Kobiela, MD (currently: PhD, DSc) from the Team of Zbigniew Sledzinski, MD, DSc, ProfTit from the Department of General, Endocrine and Transplant Surgery of the Medical University of Gdansk. Thereby, it was possible to plan and carry out biochemical tests on tissue samples originated from patients diagnosed and treated for thyroid tumors. The results of these studies were published in **[2]**. In 2015, I started my research on the HtrA proteins in the pathogenesis of colorectal cancer (CRC). I established collaboration with the Team led by Zbigniew Kmiec, MD, DSc, ProfTit from the Department of Histology of the Medical University of Gdansk, who has extensive experience in research on the pathogenesis of bowel diseases, including CRC. In the period from September 2015 to May 2017, I conducted experimental work in the laboratories of the Department of Histology **[Appendix 3, II.11]**. My activity included evaluation of the *HTRA* genes' expression in tissue samples originated from CRC patients using real-time PCR and determination of the HtrA proteins' location in tissue slides using immunohistochemical staining. In addition, I participated in research on the relationship between *TNFS15* expression and the CRC development. Continuing research on the HtrA proteins in pathogenesis of CRC I established collaboration with Rafal Peksa, MD from the Team of Wojciech Biernat, MD, DSc, ProfTit from the Department of Pathomorphology of the Medical University of Gdansk. As part of the cooperation, tissue slides were subjected to histological and immunohistochemical evaluation. In this way, location of HtrA1-3 proteins in unchanged intestinal mucosa and CRC tissue was determined. These results have been published in publication **[7]** which is a part of the scientific achievement, and publication **[Slebioda *et al.,* 2019 – Appendix 5, II.4, publication no. 18]**, included in the other scientific and research achievements.

Additional literature (except publications that are part of the scientific achievement)

Slebioda T., Stanislawowski M., Cyman M., Wierzbicki P., **Zurawa-Janicka D.**, Kobiela J., Makarewicz W., Guzek M., Kmiec Z. (2019). Distinct expression patterns of two tumor necrosis factor superfamily member 15 gene isoforms in human colon cancer. *Dig Dis Sci* 64(7): 1857-1867.

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6. Presentation of teaching and organizational achievements as well as achievements in popularization of science or art.

a) Teaching achievements

Teaching achievements

1. *Biochemistry – laboratory classes* for second-year students of full-time first cycle studies of Biology, Oceanography and Biotechnology; in the years 1999-2013 – course instructor, participation in elaboration of laboratory experiments, in the years 2010-2013 – course supervisor

2. *Biochemistry – laboratory classes* for second-year students of full-time first cycle studies of Bioinformatics, in the years 2012/2013 – course supervisor

3. *Introduction to biochemistry – auditory classes* for second-year students of full-time first cycle studies of Genetics and Experimental Biology, since 2019 – participation in elaboration of the course, course instructor

4. *Biopolymers – laboratory classes* for second-year students of full-time first cycle studies of Bioinformatics, in the years 2012/2013 – course supervisor

5. *Specialization workshop – laboratory classes* for students of full-time studies of Biology, since 2000 – course instructor

6. *Physics with elements of biophysics – laboratory classes* for second-year students of full-time first cycle studies of Biology and Medical Biology, in the years 2011-2018 – course instructor

7. *Introductory seminar* for third-year students of full-time first cycle studies of Biology, specialization: Molecular Biology; in the years 2011-2013 – course instructor

8. *Techniques for labeling of biological molecules* – lecture for the first-year students of full-time second-cycle studies of Biology, specialization: Molecular Biology, since 2012 – lecturer

9. *Metabolism – clinical aspects* – lecture for the second-year students of full-time first-cycle studies of Medical Biology, since 2015 – elaboration of the course, lecturer

10. *Metabolism – clinical aspects – classes* for second-year students of full-time first cycle studies of Medical Biology, since 2016 – course instructor

11. *Biochemical basis of organisms' function* – lecture for the third-year students of full-time first-cycle studies of Biology, since 2014 – elaboration of the course, lecturer

12. *Seminar* for the third-year students of full-time first cycle studies of Biology and Medical Biology, since 2018 – course instructor

13. *Basis of cellular and molecular immunology* – lecture for the second-year students of full-time first cycle studies of Biology, Medical Biology, Genetics and Experimental Biology, since 2019 – lecturer

Scientific supervision of students

1. Scientific supervision of Master's students (2002 – 2008), University of Gdansk, 5 students

2. Master's thesis supervisor, (2010 – 2021), University of Gdansk, 13 students

Galczyńska N. *Expression of HtrA1, HtrA2 and HtrA3 genes in thyroid neoplasms.* (2010). Biology, specialization: biochemistry.

Chrzanowska E. *Expression of the TGF-β1 gene in thyroid neoplasms*. (2010). Biology, specialization: experimental biology.

Glaza P. *Cloning and expression of the human HtrA3 gene*. (2011). Biology, specialization: molecular biology.

Lubomska A. *Mechanism of action of the human HtrA2 protease. (2011).* Biology, specialization: molecular biology.

Wenta T. *Molecular basis of HtrA2/Omi protease activation.* (2013). Biology, specialization: medical biology.

Augustynowicz M. *HtrA proteins in pancreatic carcinogenesis.* (2014). Biology, specialization: medical biology.

Beutler A. *Mechanism of action of HtrA2 and HtrA3* proteins. (2015). Biology, specialization: medical biology.

Klepacka E. *Identification of physiological substrates of HtrA3 protein isoforms by immunoprecipitation method*. (2016). Biology, specialization: medical biology.

Gos I. *Analysis of the level of anti-Hsp40 antibodies in the serum of children with mastocytosis*. (2017). Biology, specialization: medical biology.

Oleksiak A. *Analysis of the long (L) and short (S) HtrA3 isoforms' levels in patients with colorectal cancer*. (2018). Biology, specialization: molecular biology.

Ciolek M. *Immune response against selected HtrA proteins in patients with rheumatoid arthritis.* (2019). Medical Biology, specialization: molecular and biochemical diagnostics.

Popielarczyk A. *Assessment of the role of bacterial HtrA proteins in the development of rheumatoid arthritis*. (2020). Medical Biology, specialization: molecular and biochemical diagnostics.

Zielonka D. *Analysis of the immune response against the HtrA serine protease from* Helicobacter pylori *in patients with rheumatoid arthritis*. (2021). Medical Biology, specialization: molecular and biochemical diagnostics.

3. Bachelor's thesis supervisor (2011 – 2020), University of Gdansk, Biology and Medical Biology, 13 students

4. Scientific supervision of student traineeships (2008-2009) – 2 students from the University of Gdansk, 1 student from the University of Life Sciences, Lublin

5. Scientific supervisor of one student from Houston-Downtown University participating in workshop on molecular biology organized by Intercollegiate Faculty of Biotechnology of University of Gdansk and Medical University of Gdansk (July, 2008)

6. Scientific supervision of student traineeship under the ERASMUS programme (November – December, 2016) – 1 student from the University of Valencia (Universitat de Valencia, Valencia, Spain)

b) Achievements in popularization of science

1. Elaboration and realization of educational workshop and lecture for high school students *Find the real colors of plant leaves* as part of the project *Meet the work of biologists* conduced at the Faculty of Biology of the University of Gdansk – in the years 2011-2013

2. Baltic Science Festival, Night of Biologists, Open Days of the University of Gdansk's Faculty of Biology – presenting the research projects conducting at the Biochemistry Department for high school students

c) Awards

The National Education Committee Medal for outstanding contribution to education, Warsaw, 2014

d) Organizational achievements

In 2019, I was appointed a member of the Committee of Contained Use of Genetically Modified Organisms at the Faculty of Biology of the University of Gdansk

7. Other information about professional career (apart from information set out in 1-6 above)

a) Description of other scientific achievements

In 1998, I graduated from the Intercollegiate Faculty of Biotechnology of the University of Gdansk and Medical University of Gdansk. The master's thesis entitled: "The interaction of the *Escherichia coli* HtrA protease with membrane phospholipids *in vivo* and *in vitro*" was performed in the Department of Biochemistry under supervision of Barbara Lipinska, PhD, DSc, ProfTit.

The project on the participation of the *Escherichia coli* HtrA protease in response to cellular stress was the first one I participated in. *E. coli* HtrA is a peripheral membrane protein located on the periplasmic side of the inner membrane. HtrA activity is indispensable for the survival of a bacterial cell at temperatures above 42^{o}C **[Lipinska *et al*., 1989]**. HtrA has been shown to degrade improperly folded and denatured proteins, including thermally denatured ones **[Strauch & Beckwith, 1988; Laskowska *et al*., 1996]**. These results indicated that the primary physiological function of HtrA is degradation of aberrant proteins generated in the cell envelope under heat stress conditions. Therefore, it seemed to be logical that HtrA could also be involved in the elimination of damaged proteins generated under oxidative stress. We decided to verify this assumption. It turned out that among the tested compounds, ferrous ions caused growth inhibition of bacterial cells lacking HtrA. Moreover, treatment with ferrous ions caused a larger increase in the level of carbonyl groups – a marker of protein oxidation, specifically in the membrane protein fraction. It is known that expression

of *htrA* gene is induced by an increase of temperature above the physiological value and in response to the presence of abnormal proteins in the periplasmic space **[Strauch & Beckwith, 1988; Lipinska *et al*., 1989]**. The results of the study showed that under oxidative stress the HtrA protein level was also increased. The above results have been published in **[Skorko-Glonek *et al*., 1999 – Appendix 5, II.4, publication no. 1]**. For the first time, the relationship between *E. coli* HtrA and the response against oxidative stress was documented. The obtained results suggested that the HtrA protease plays an important role in the degradation of cellular proteins damaged by oxidizing agents.

Therefore, a question arose about the mechanism of regulation of *E. coli* HtrA activity. In 2002, the crystal structure of HtrA was solved and model of the HtrA hexamer – a functional unit of the protease was published. The hexameric structure is composed of two trimers connected by LA loops, located in the N-terminal parts of the HtrA polypeptides. The LA loops stabilize the hexameric structure with an internal chamber containing the catalytic sites of subunits **[Krojer *et al*., 2002]**. Results of studies on biochemical and functional characteristics of HtrA, conducted by the research team led by Barbara Lipinska, PhD, DSc, ProfTit revealed that under certain conditions the protease undergoes autoproteolysis resulting in the removal of the N-terminal fragment of the HtrA polypeptide **[Skorko-Glonek *et al*., 1995]**. Our studies showed that the autocatalytic cleavage of this protease occurs during degradation of substrates under reducing conditions both *in vitro* and *in vivo*. Cysteine residues present in the HtrA polypeptide form a disulfide bridge, its reduction or removal by amino acid substitution of cysteine residues triggers autoproteolysis even in the absence of a reducing agent. The HtrA polypeptides lacking the N-terminal region were proteolytically active, but they were not able to form hexamers. Based on these results, it can be concluded that the N-terminal region of HtrA is essential for maintaining quaternary structure of this protease. It is possible that the reduction of the disulfide bridge, which is localized in the LA loop, may serve as a trigger for activation of HtrA accompanied by autoproteolysis resulting in conformational changes in the oligomer. We also showed that binding of the proteolytically modified HtrA to membrane phospholipids was weaker and the protein exhibited higher activity. It cannot be excluded that the autocleavage may influence the effectiveness of this protease under physiological conditions. The results described above were published in **[Skorko-Glonek *et al*., 2003 – Appendix 5, II.4, publication no. 2]** and became the starting point for research on the meaning of HtrA reduction in the regulation of activity of this protease and the role of this phenomenon in cell physiology. We found that the protease lacking the disulfide bridge exhibited an increased affinity and elevated activity towards the model substrate, the exposure of the LA loop was increased, and the protein was more easily converted to higher order oligomers. We concluded that the disulfide bridge is important for the stability of the overall oligomeric structure and its reduction promotes mobility of the LA loop, rearrangement of the oligomer and activity **[Koper *et al*., 2015 – Appendix 5, II.4, publication no. 13].** I also participated in the project aimed at the understanding molecular mechanism of HtrA thermal activation. Conformational changes in selected regions of the HtrA protein, including the regulatory loops, triggered by temperature up-shift were tracked using spectroscopic techniques and a set of recombinant HtrA proteins with single Trp substitutions. Analysis of wavelength maxima of Trp fluorescence combined with parameters of Trp fluorescence quenching by acrylamide suggests that under temperature up-shift the HtrA structure appears to open

gradually; a prominent rearrangement occurs in the regulatory LA loop, structural changes in the L2 loop are more subtle **[Sobiecka-Szkatula *et al*., 2009 – Appendix 5, II.4, publication no. 11].**

I also participated in preliminary studies on the implication of IbpAB – proteins belonging to small HSPs, on activity of enzymes sequestered in inclusion bodies (IBs). IBs appear in the cell as a consequence of accumulation of misfolded proteins. IbpAB bind to polypeptides associated with IBs and act as molecular chaperones; they protect the polypeptides from irreversible aggregation and allow enzymes to maintain their activity **[Kuczynska-Wisnik *et al*., 2004 – Appendix 5, II.4, publication no. 3]**. Issues related to structure and function of bacterial chaperones and proteases in mechanisms of protein aggregation and disaggregation were included in a book chapter **[Skorko-Glonek *et al*., 2011 – Appendix 5, II.2, publication no. 2]** in which I had the opportunity to prepare the part concerning cytoplasmic proteases of *E. coli*.

Being involved in projects regarding the participation of HSPs in stress response, my interests were sparked by the topic of the importance of oxidative stress in the development of pathological conditions. I was involved in the development of a new approach for estimation of the content of oxidized proteins in plasma fractions. The method is a combination of two-stage electrophoresis and immunodetection of carbonyl derivatives of oxidized proteins – a marker of oxidative damage to proteins. The results I obtained with this method indicated that among plasma proteins gamma globulins, followed by albumins, are most modified by oxidative stress in children with juvenile idiopathic arthritis (JIA). Moreover, oxidative modification of plasma proteins correlates with the type of JIA. The developed method can be used in monitoring the level of carbonyl groups as a marker of inflammatory process activity on JIA as well as in other diseases **[Zurawa-Janicka *et al*., 2006 – Appendix 5, II.4, publication no. 4]**. Moreover, due to cooperation with a team of Michal Wozniak, PhD, DSc, ProfTit from the Department of Medical Chemistry of the Medical University of Gdansk I participated in research aimed at studying the dynamics of estradiol-induced oxidative stress. In these studies, an animal model of hormone-dependent breast cancer in which nephrocarcinogenesis in Syrian hamster (*Mesocricetus auratus*) was triggered by prolonged estrogenization had been used **[Li *et al*., 1995]**. The obtained results revealed that upon oxidative stress triggered by short-term exposure to estradiol proteins, closely followed by DNA, were most vulnerable to oxidative damage. These results suggest that oxidative modification of proteins and DNA could be an initial, albeit significant step facilitating neoplasmic transformation **[Kobiela *et al*., 2007 – Appendix 5, II.4, publication no. 5]**, **[Kobiela *et al*., 2008 – Appendix 5, II.4, publication no. 9]**

Because HtrA proteins are induced under stressful conditions, the thread related to the effects of estrogen-induced oxidative stress was continued in a project concerning the implication of eukaryotic HtrA homologues in the development of pathological states. I found that upon short-term estrogen exposition the *HTRA1* expression increased in Syrian hamster kidney. It suggests the involvement of HtrA1 in the response against oxidative stress. Prolonged estrogenization caused a significant decrease in the *HTRA1* gene expression and a significant increase in the expression of *HTRA2*. The obtained results suggested the relationship between HtrA1/2 proteins and the development of estrogen-induced nephrocarcinogenesis in hamster. Using fluorescence *in situ* hybridization (FISH) we localized the *HTRA1* gene at the qb3-4 region of Syrian hamster chromosome 2. Upon prolonged estrogenization this region undergoes a nonrandom deletion **[Papa *et al*., 2003]**. It

is possible that the reduced level of HTRA1 expression is due to this chromosomal aberration. The obtained results were consistent with the results of other authors suggesting that HtrA1 functions as a tumor suppressor and indicated, for the first time, the relationship between the HtrA1 protein and response against oxidative stress. For the first time, we showed the correlation between *HTRA2* expression and tumor development. The coding DNA sequence of *HTRA1* and a fragment of *HTRA2* cDNA of Syrian hamster were cloned and deposited in GenBank (accession numbers EF185310, EF185311). The above results were published **[Zurawa-Janicka *et al*., 2008 – Appendix 5, II.4, publication no. 6]** and served as a basis for my PhD dissertation, which I performed under supervision of Barbara Lipinska, PhD, DSc, ProfTit and successfully defended in November 2008 (title: "Implication of HtrA1 and HtrA2 proteins in defense against estrogen-induced oxidative stress and nephrocarcinogenesis in Syrian hamster (*Mesocricetus auratus*)"), at the Faculty of Biology, Geography and Oceanology of the University of Gdansk. The issues related to characteristics of HtrA proteins were published in a review article **[Zurawa-Janicka *et al*., 2007 – Appendix 5, II.4, publication no. 8]** and a book chapter **[Zurawa-Janicka *et al*., 2008 – Appendix 5, II.2, publication no. 1]**.

Moreover, as part of the cooperation established by Barbara Lipinska, PhD, DSc, ProfTit with the Team of Janusz Emerich, PhD, DSc, ProfTit from the Department and Clinic of Gynecology at the Medical University of Gdansk, I participated in research on the implication of HtrA1-3 in the development of ovarian and endometrial cancers. Due to this cooperation, it was possible to conduct research on human tissue samples. The results of these studies revealed that expression of *HTRA1* and *HTRA3* genes (measured at the transcript and protein levels) was reduced in tumor tissue from various types of ovarian cancer **[Narkiewicz *et al*., 2008 – Appendix 5, II.4, publication no. 7]** and in endometrial cancer tissue **[Narkiewicz *et al*., 2009 – Appendix 5, II.4, publication no. 10]**. In case of endometrial cancer, a negative correlation between the protein levels of HtrA1 and TGF-β was found. These results were consistent with the results of other authors indicating the suppressive function of HtrA1/3 proteases in neoplastic transformation **[Baldi *et al*., 2002; Chien *et al*., 2004, 2006; Bowden *et al*., 2006]** and suggested a link between HtrA proteins and regulation of TGF-β signaling in endometrial tissue.

Issues concerning the physiological function of HtrA proteins and their role in mechanisms of pathogenesis, including cancer development, became the subject of a review article **[Skorko-Glonek *et al*., 2013 – Appendix 5, II.4, publication no. 12],** characteristics of the *HTRA1*, *HTRA2*, *HTRA3* genes were presented in **[Zurawa-Janicka *et al*., 2011 – Appendix 5, II.4, publication no. 22], [Jarząb *et al*., 2012 – Appendix 5, II.4, publication no. 23], [Glaza *et al*., 2012 – Appendix 5, II.4, publication no. 24]**.

I also participated in research concerning the implication of HtrA proteins in pathogenesis of diseases in which mast cells (MCs) play a key role, i.e., mastocytosis and allergic diseases; during degranulation MCs release inflammatory mediators, including proteases, which influence the mechanisms of innate and adaptive immune response and regulate inflammation **[Da Silva *et al*., 2014]**. MCs also produce HtrA1 **[Gilicze *et al*., 2007]**. The first project investigated the role of HtrA proteins in the development of pediatric mastocytosis, a disease caused by pathological accumulation of MCs in skin and/or other organs. It was showed that levels of the serum IgG antibodies against HtrA1 and HtrA3 were elevated in children with cutaneous mastocytosis which suggests a relationship between

the immune response against self-HtrAs and the development of the disease; IgG autoantibodies can form immune complexes with the antigen and promote inflammatory response involving MCs **[Renke *et al*., 2018 – Appendix 5, II.4, publication 16]**. Further studies focused on the implication of HtrA proteins in the development of allergic diseases in children. Levels of HtrA1-3 proteins were increased in the serum of allergic patients, while levels of autoantibodies against these proteins were reduced. It is known that in the course of IgE-mediated allergy, MCs become inevitably activated and chronic inflammation may promote carcinogenesis. Taking into account the suppressor function of HtrA1 and HtrA3 in oncogenesis it can be assumed that these proteases may have antitumor activity in allergic patients **[Renke *et al*., 2018 – Appendix 5, II.4, publication 21]**.

Apart from the studies regarding the implication of HtrAs in pathogenesis of diseases, I participated in projects aimed at biochemical and functional characterization of HtrA3 and HtrA4 proteases. Due to the cooperation established by Barbara Lipinska, PhD, DSc, ProfTit with Jerzy Osipiuk, PhD (Structural Biology Center Argonne National Laboratory, USA) the crystal structure of HtrA3L was solved; the protease forms a trimer in which PDZ domains place in a position intermediate between that in the pyramid-like HtrA2 structure and the flat disc-like structure of HtrA1. Results of biochemical analyses revealed that the PDZ domain is dispensable for HtrA3 activity; in this respect HtrA3 resembles HtrA1. Unlike in HtrA2, the PDZ domain does not act as a negative regulator of HtrA3 activity. On the other hand, the PDZ domain is essential to maintain the trimeric structure; HtrA3S and HtrA3L lacking the PDZ domain are monomeric, although their activity is retained. HtrA3 isoforms exhibit similar temperature-dependent proteolytic activity towards the tested substrates and similar substrate specificity. The obtained results indicated that HtrA3 seems to have a unique combination of features among human HtrA proteins **[Glaza *et al*., 2015 – Appendix 5, II.4, publication 14]**.

Subsequent studies on the properties of the HtrA3 structure were focused on the explanation of the meaning of the LB loop – PDZ domain interaction. This feature is unique for HtrA3L, unprecedented so far among the HtrA proteins whose crystal structures have been solved. It turned out that substitutions of amino acid residues belonging to the PDZ domain that impair hydrogen interactions in this region caused partial dissociation of trimers into monomers. On the other hand, removal of the unique amino acid residues of the LB loop resulted in dramatic changes in HtrA3 structure towards hexameric forms accompanied by a significant reduction of proteolytic activity. The obtained results allowed to formulate the conclusion that the LB loop participates in stabilization of the native structure of the HtrA3 oligomer and thus promotes proteases activity **[Wenta *et al*., 2017 – Appendix 5, II.4, publication 15].** The results of research on the structural and biochemical characteristics of HtrA3 opened the way to understanding the physiological role of this protease. The results of *in vitro* studies demonstrated that HtrA3L and HtrA3S form complexes with numerous proteins, including cytoskeleton proteins, apoptosis regulating proteins, chaperones and DNA repair proteins. Using immunoprecipitation and western blotting, the *in vivo* formation of HtrA3 complexes with actin, β-tubulin, vimentin as well as TCP1α – a chaperone protein associated with organization of cytoskeleton, and the anti-apoptotic XIAP (X-linked inhibitor of apoptosis protein) was confirmed. This result was supported by the results of confocal fluorescence microscopy demonstrating co-localization of these proteins with endogenous HtrA3. Moreover, an intriguing question arose about

the meaning of HtrA3 interactions with these proteins. The cytoskeleton proteins and XIAP turned out to be substrates for HtrA3 *in vitro*. Furthermore, HtrA3 protease was found to act as a regulator of tubulin polymerization *in vitro*; HtrA3 facilitates formation of microtubules but, on the other hand, it degrades β-tubulin. Thus, the protease may play an important role in the reorganization of the cytoskeleton by digestion of cytoskeleton proteins and participation in microtubule polymerization. Subsequent studies on cell line cultures showed that HtrA3 promotes apoptosis through XIAP digestion. The above results have been published in two original articles **[Wenta *et al*., 2018 – Appendix 5, II.4, publication 17]** and **[Wenta *et al*., 2019 – Appendix 5, II.4, publication 20]**.

I also participated in research on the biochemical and functional characteristics of HtrA4, the results of which were published in **[Wenta *et al*., 2019 – Appendix 5, II.4, publication 19].** HtrA4 has been shown to form complexes with proapoptotic proteins and cytoskeleton proteins, and most of these proteins were identified as HtrA4 substrates *in vitro*. This leads to the conclusion that this protease may regulate many physiological processes, including mechanisms related to cell death.

Apart from the main research concerning the participation of HtrA proteases in pathogenesis of colorectal cancer, I participated in the research on the relationship between expression of *TNFSF15* (tumor necrosis factor superfamiliy 15) gene and development of this type of cancer. Results of this study have been published in **[Slebioda *et al*., 2019 – Appendix 5, II.4, publication 18].**

Currently, I have started research on characterization of the immune response against HtrA proteins in pathogenesis of rheumatoid arthritis (RA). I received funding from the National Science Center to conduct the research. RA is an incurable, autoimmune disease. The central site of RA pathogenesis is rheumatoid synovium whose cells produce cytokines and proteases that degrade components of the extracellular matrix (ECM) of cartilage and bone tissue, leading to loss of joint function **[Scott *et al*., 2010]**. Human HtrA proteases are suspected to be involved in pathogenesis of arthritic disorders by participation in the ECM reorganization and regulation of TGF-β signaling **[Zurawa-Janicka *et al*., 2017]**. It has been shown that HtrA proteins of pathogenic bacteria act as important virulence factor, are highly immunogenic, and immunization with these proteins may protect against infection. Therefore, HtrAs are considered as components of polyvalent vaccines **[Skorko-Glonek *et al*., 2017]**. So far, the aspect of the relation of HtrA proteins with pathogenesis of RA in the context of autoimmunity has not been described and safety of vaccines using bacterial HtrA proteins as antigens has not been established. Results of the studies carried out have so far shown that levels of antibodies against human HtrA1 and HtrA3, and *E. coli* HtrA were elevated in the serum of RA patients and correlated with certain clinical and diagnostic parameters. Also the immunological similarity between the *E. coli* HtrA protein and homologous human proteins has been revealed. These results indicate a link between HtrA proteins and the pathogenesis and etiology of RA. These results are included in the original article (which is currently being finalized) and serve as the starting point for further studies aiming at characterization of the innate and adaptive immune response against human HtrAs and HtrAs of pathogenic bacteria (e.g., *E. coli*, *Helicobacter pylori*, *Borelia* spp.) in mechanisms of RA pathogenesis. I expect that these studies will provide answers to the following questions: what is the role of the immune response against autologous HtrA proteins in the development of RA, whether the response against bacterial HtrA proteins may be directed against

own HtrA proteins, and finally, to what extent the use of HtrA of pathogenic bacteria as a component of polyvalent vaccine is safe.

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b) Awards for scientific achievements

1. The first-degree team award for a series of five articles focused on the heat shock proteins, their function and regulation, granted by the Chancellor of the University of Gdansk, 2004.

2. The first-degree team award for a series of articles focused on the HtrA proteins, granted by the Chancellor of the University of Gdansk, 2009.

3. The third-degree team award for a series of articles focused on the role of stress-induced proteins (proteases and chaperone proteins) in physiology of prokaryotic and eukaryotic organisms, granted by the Chancellor of the University of Gdansk, 2019.

4. The first-degree team award for a series of articles focused on the mechanisms involved in the maintenance of protein homeostasis (proteostasis) in prokaryotic and eukaryotic organisms, granted by the Chancellor of the University of Gdansk, 2020.

Dorota Żurawa-Janicka

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(Applicant's signature)