Attachment no 3

Summary of Professional Accomplishments

Elżbieta Wieczorek, PhD

ORCID: 0000-0003-1420-1439

Department of Biochemistry,
Molecular Biology and Biotechnology

Wrocław University of Science
and Technology

Wrocław, 2023

Table of contents

1. Name 3

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 3

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

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

4a. The first achievement submitted for evaluation 4

4a.1. Title of the scientific achievement 4

4a.2. List of monothematic publications documenting scientific achievement 4

4a.3. General characterization of human transthyretin and purpose of the scientific project........ 7

4a.4. Description of the results achieved and their significance 11

4a.4.1. *Pathogenesis-related factors destabilize the structure of transthyretin* 11

4a.4.2*. A new autofluorophore is formed in transthyretin* 16

4a.4.3. *Transthyretin exhibits new activities* 19

4a.4.4. *The structure and properties of transthyretin are associated with the pathogenesis of many diseases* 27

4a.5. Summary of achieved results 29

4a.6. Further research goals 31

4b. The second achievement submitted for evaluation 32

4b.1. Title of the scientific achievement 32

4b.2. List of publications documenting the scientific achievement 32

4b.3. Description of the results achieved and their significance 33

4b.3.1. *Identification of a new type of transcription complexes containing TAF30* 33

4b.3.2. *Study of regulation of human vimentin gene expression* 34

4b.4. Summary of achieved results 35

4.5. Description of other scientific accomplishments and obtained achievements that contribute to science 36

4.5.1. Project: *Study of juvenile hormone binding protein from the hemolymph of the wax moth Galleria mellonella* 36

4.5.2. Project: *Study of proteins regulating gene transcription in insects* 38

5. Presentation of significant scientific or artistic activity carried out at more than one university, scientific or cultural institution, especially at foreign institutions 41

5.1. Summary of scientific achievements 41

5.2. Scientific collaboration with other research institutions 43

5.3. Conference presentations 45

6. Presentation of teaching and organizational achievements as well as achievements in popularization of science or art 46

6.1. Teaching activities 46

6.1.1. Courses taught 46

6.1.2. New courses introduced 46

6.1.3. New courses developed 46

6.1.4. Master's and engineering projects 46

6.2. Popularization and organizational activities 48

7. Additional information concerning professional career 49

7.1. Received awards and distinctions 49

8. Bibliography 50

1. Name

Elżbieta Wieczorek

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 degree in Chemistry, awarded by the Department of Chemistry Technical University of Wrocław (at present Wrocław University of Science and Technology), Poland, on 24.09.1984
Thesis: "Preliminary purification of the juvenile hormone-binding lipoprotein from the haemolymph of the wax moth larvae *Galleria mellonella* (L.)"

Doctor of Philosophy degree in Chemical Sciences, awarded by the Scientific Council of the Institute of Organic Chemistry, Biochemistry and Biotechnology, Wrocław University of Technology (at present Wrocław University of Science and Technology), Poland, on 8.12.1993
Thesis: "Studies on the interaction of juvenile hormone - juvenile hormone binding protein from the haemolymph of the wax moth, *Galleria mellonella*"

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

Division of Biochemistry, Institute of Organic and Physical Chemistry, (at present Department of Biochemistry, Molecular Biology and Biotechnology), Technical University of Wrocław, (at present Wrocław University of Science and Technology), Poland

1.10.1984-30.09.1985	junior assistant
1.10.1985-30.09.1987	assistant
1.10.1987-30.09.1989	senior assistant
1.10.1989-30.11.1993	PhD studies
1.10.1993-31.12.1995	senior assistant

Institute of Genetics and Molecular and Cellular Biology (*Institut de Genetique et de Biologie Moleculaire et Cellulaire*, IGBMC), Strasbourg, France

1.01.1995-31.10.1996	postdoc

Virginia Commonwealth University (VCU), Richmond, Virginia, USA

1.10.1997-31.12.1999	postdoc

Department of Biochemistry, Institute of Organic Chemistry, Biochemistry and Biotechnology (at present Department of Biochemistry, Molecular Biology and Biotechnology), Technical University of Wrocław (at present Wrocław University of Science and Technology), Poland

1.02.2000 to the present	associated professor

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

4a. The first achievement submitted for evaluation

4a.1. Title of the scientific achievement

"Exploring new properties and relationships between the structure and function of human transthyretin in the context of disease pathogenesis and potential therapeutic strategies"

4a.2. List of monothematic publications documenting scientific achievement

The achievement consists of a series of six related scientific articles, which describe results documenting previously unknown properties and activities of human transthyretin (TTR), and their relationship with structure and known functions of TTR. The results of each of the articles submitted for evaluation represent the next step toward achieving the main goal of the project. The articles are described in functional rather than chronological order. The title of each subsection reflects the most important conclusion (achievement), and the subsection contains a synthetic description of the obtained results. A detailed description of the results are presented in the individual publications. All publications can be found in indexed biometric research databases.

The concept of both the project and individual experiments, the performance of most of the experiments, training and scientific supervision of people involved in the research, the processing, and compilation of results, and formulation of conclusions are my original contributions to the realization of the achievements of this scientific project. This is reflected in the first position on the list of authors of the presented publications, as well as the role of correspondence author. The publications are listed below in chronological order and indexed from [P_11] to [P_16]. My detailed contribution is indicated below the title of each publication.

[P_11] **Wieczorek, E.**; Kędracka–Krok, S.; Sołtys, K.; Jankowska, U.; Hołubowicz, R.; Seliga, J.; Ożyhar, A. Is Transthyretin a Regulator of Ubc9 SUMOylation? *PLoS ONE* 2016 Aug 8;11(8). doi: 10.1371/journal.pone.0160536
IF_{2016} = 2.806, $MEiN_{2016}$ = 35

- *Carrying out literature studies concerning TTR and the facilitated sumoylation system, design of the general concept of the project, the choice of the methods and experimental conditions, the detailed analysis and evaluation of the results of all experiments, including the interpretation of the data based on the literature studies, and drawing the final conclusions.*
- *Preparation of the majority of the expression constructs for the facilitated sumoylation system in eukaryotic cells (UFDS) as well as constructs for expression of recombinant TTR in the bacterial system, purification and verification of preparation purity of recombinant TTR, performance of all transfection experiments in HEK 293 cell line and SDS-PAGE and Western blotting experiments.*

- *Preparation of the samples and planning of the experimental conditions and data interpretation of ultracentrifugation and mass analysis experiments.*
- *Writing, editing, and proofreading of the manuscript (including co-editing the sections on mass spectrometry) and preparation of all figures.*

[P_12] **Wieczorek, E.**; Chitruń, A.; Ożyhar, A. Destabilised human transthyretin shapes the morphology of calcium carbonate crystals. Biochim. Biophys. Acta - Gen. Subj. 2019, 1863, 313–324. doi:10.1016/j.bbagen.2018.10.017

IF_{2019} =3.422, $MEiN_{2019-2021}$ = 100

- *Carrying out literature studies concerning the processes of biomineralization, designing the project objectives and methods of its realization, designing the general experimental concept, as well as detailed planning of experimental conditions, analysis and interpretation of the results of each experiment and drawing final conclusions based on the obtained data and literature studies.*
- *Preparation of the expression constructs, the expression in the bacterial system and purification of various aged preparations of recombinant TTR.*
- *Choosing the experimental conditions and participation in the purification of the native TTR, supervision and co-participation in the performance of biomineralization tests, microscopic analyses, including Scanning Electron Microscopy analyses and Raman spectra.*
- *Writing, editing and proofreading of the manuscript and preparing all figures.*

[P_13] **Wieczorek, E.**; Kędracka-Krok, S.; Bystranowska, D.; Ptak, M.; Wiak, K.; Wygralak, Z.; Jankowska, U.; Ożyhar, A. Destabilisation of the structure of transthyretin is driven by Ca^{2+}. Int. J. Biol. Macromol. 2021, 166, 409–423. doi:10.1016/j.ijbiomac.2020.10.199
IF_{2021}= 8.025, $MEiN_{2019-2021}$ = 100

- *Carrying out literature studies on calcium metabolism and the effect of metal ions on protein structure, designing the project objectives and methods of its realization as well as experimental design including detailed planning of the experimental conditions, analysis and interpretation of the results and drawing the conclusions from the obtained results based on the literature studies.*
- *Expression in the bacterial system and purification of a subset of recombinant TTR preparations, performing and processing the results of the following experiments: ANS binding assay, precipitation assay, Ca^{2+} binding assay using fluorescence probe, fluorescence measurements, circular dichroism measurements and the analysis and comparison of the crystallographic structures of TTR.*
- *Designing conditions and performing preliminary molecular filtration and S-trapping experiments, samples preparation and participation in purification of some of the recombinant TTR preparations, participation in planning and interpretation of data obtained in ultracentrifugation, Isothermal Titration Calorimetry, Differential Scanning Calorimetry and Mass Spectrometry experiments.*
- *Writing, editing and proofreading of the manuscript, including the sections of the manuscript concerning the analysis and discussion of the ultracentrifugation and mass spectrometry results, and preparation of figures (Figure 1 was prepared with contribution of Dr. Dominika Bystranowska).*

[P_14] **Wieczorek, E.**; Ożyhar, A. Transthyretin: From structural stability to osteoarticular and cardiovascular diseases. *Cells* 2021 Jul 13;10(7):1768. doi:10.3390/cells10071768
IF_{2021} = 7.666, $MEiN_{2019-2021}$ = 140

- *Designing the concept of the complex, multidimensional model of the relationships linking the structural stability of TTRs and the factors destabilizing the structure of the TTR to the processes of pathogenesis of various diseases and conducting literature studies on the molecular basis of the discussed diseases.*
- *Writing, editing and proofreading the manuscript, preparing the model figure, designing and preparing the tables.*

[P_15] **Wieczorek, E**.; Bezara, P.; Ożyhar, A. Deep blue autofluorescence reveals the instability of human transthyretin. Int. J. Biol. Macromol. 2021, 191, 492–499. doi:10.1016/j.ijbiomac.2021.09.107
IF_{2021}= 8.025, $MEiN_{2019-2021}$ = 100

- *Performance of pilot set of fluorescence measurements of TTR and discovery of a new autofluorophore in TTR.*
- *Carrying out literature studies on deep blue autofluorescence and fluorescent properties of proteins, designing the project objectives and its realization, developing the experimental design, including planning of the experimental conditions, analysis and interpretation the results of all experiments, as well as the overall conclusions of the results based on the literature studies.*
- *Expression in bacterial system and purification of the recombinant TTR, performance of fluorescence spectra, S-trapping experiments, molecular filtration, SDS-PAGE and electrophoresis under native conditions.*
- *Supervision and participation in the performance of aggregation assay and ANS binding assay.*
- *Writing, editing and proofreading of the manuscript and preparation of figures (Figure 2 was prepared with contribution of student Patrycja Bezara).*

[P_16] **Wieczorek, E**.; Wygralak, Z.; Kędracka-Krok. S, Bezara, P.; Bystranowska, D.; Dobryszycki, P.; Ożyhar. A. Deep blue autofluorescence reflects the oxidation state of human transthyretin. Redox Biol. 2022 Aug 9;56:102434. doi: 10.1016/j.redox.2022.102434
IF_{2021} = 10.787, $MEiN_{2019-2021}$ = 140

- *Carrying out literature studies concerning the effects of redox conditions on the structure, function and fluorescence properties of proteins, development of the project objectives and methods of its realization, developing the experimental design including detailed planning of the experiments, analysis and interpretation of the results of all experiments.*
- *Expression in the bacterial system and purification of some of recombinant TTR preparations and performance of the fluorescence spectra (except the spectra presented in Figure 1), TTR light exposure and analysis using SDS-PAGE, elaboration of the conditions and performance of the first series of molecular filtration and S-trapping experiments,*
- *Participation in the purification of the other TTR preparations, supervision in preparation of TTR samples, planning the experimental conditions and data analysis of molecular filtration and mass spectrometry experiments.*
- *Writing, editing and proofreading of the manuscript and preparation of figures including the graphical abstract.*

The cumulative Impact Factor (IF) of the listed publications, according to the **Journal Citation Report (JCR),** taking into account the year of publication, is **40.731**.

The total number of points for the listed publications, according to the lists of scientific journals scored by the **MNiSW/MEiN** (Ministry of Science and Higher Education, pol. *Ministerstwo Nauki i Szkolnictwa Wyższego,* **MNiSW**, presently Ministry of Education and Science, pol. *Ministerstwo Edukacji i Nauki,* **MEiN**), appropriate for the year of publication, is **615**.

4a.3. General characterization of human transthyretin and the purpose of the scientific project

The name transthyretin is derived from its canonical role of transport of thyroid hormones and retinol, where the prefix ***trans*** reflects transporting function, ***thyr*** refers to thyroid, and ***retin*** is derived from retinol. TTR is secreted by the liver cells into the serum and by the cells of *choroid plexus* into cerebrospinal fluid (CSF). However, recent studies show that cells in other tissues also contain or synthesize TTR, albeit in smaller amount. These include cells of the pancreas, kidney, retinal pigment epithelium (RPE), and some types of cells in the brain, such as ependymocytes or neurons [1–3].

It has been shown, that in the early stages of mouse development, TTR is essential to ensure appropriate concentrations of thyroid hormones and retinol to provide the normal development of many organs [4,5]. The function of transport of thyroid hormones is shared by TTR in serum with albumin and thyroxine binding globulin (TBG) [6]. The latter two proteins would be sufficient to ensure the effective transport of thyroid hormones in serum, as confirmed by the results obtained upon silencing the TTR gene in an adult mouse that had a normal distribution of thyroxine [7]. Therefore, it seems interesting whether TTR in serum, apart from the transport of thyroid hormones, may perform other, hitherto unknown functions. In CSF, unlike in serum, TTR is the main transporter of thyroid hormones [8]. In these body fluids, TTR is essential for the transport of retinol bound to retinol-binding protein (RBP) and for protection of RBP from removal by filtration in the glomeruli. In serum, under physiological conditions, almost all holo-RBP molecules are bound to TTR in a 1:1 stoichiometric ratio [9]. Importantly, RBP modulates some properties of TTR, without affecting others. For example, RBP abrogates the binding of TTR to the receptor of Advanced Glycation Endproducts (RAGE) and modulates the interaction of TTR with perlecan, a polysaccharide present in the basement membrane [10,11]. However, RBP does not affect the binding of TTR to apolipoprotein AI in high-density lipoprotein (HDL), which is essential because a certain population of serum TTR molecules (approx. 1-2%) is associated with HDL[12]. Interestingly, this observation also confirms the similarity between TTR and juvenile hormone binding protein in the hemolymph of insects (which is discussed in subsection **4.5.1. and 4.5.2.**).

In addition to the transport of thyroid hormones and retinol, the best known function of TTR is its protective role in the nervous system. TTR not only reduces the formation of amyloid β or protects the brain against neurodegeneration and the consequences of hypoxia or memory impairment, but also promotes neuronal cell regeneration, stimulates neurogenesis and plasticity and regulates metabolism in astrocytes [2,13–15]. Importantly, in astrocytes, it is the free form of TTR, unbound by thyroxine, that is required to stimulate glucose release from glycogen [2]. TTR also plays an essential role in the regulation of glucose levels throughout the body, as it stimulates the secretion of insulin and glucagon by pancreatic cells [3,16]. TTR is a marker of the body's nutritional status and a decreased serum TTR concentrations are associated with the presence of inflammation[17]. Recently, TTR has been found to be an important factor in angiogenesis due to binding of long non-coding RNA molecules [18]. Although the molecular regulatory mechanisms of TTR are still largely unexplored it is known that TTR is involved in signaling pathways regulating gene expression in the processes of apoptosis, autophagy and cell proliferation [13,19–21].

The TTR is a tetramer composed of subunits (A, B, C, D) of 13761 Da (127 amino acid residues) each, which form a dimer of the dimers (A-B and C-D) shown in **Fig. 1**.

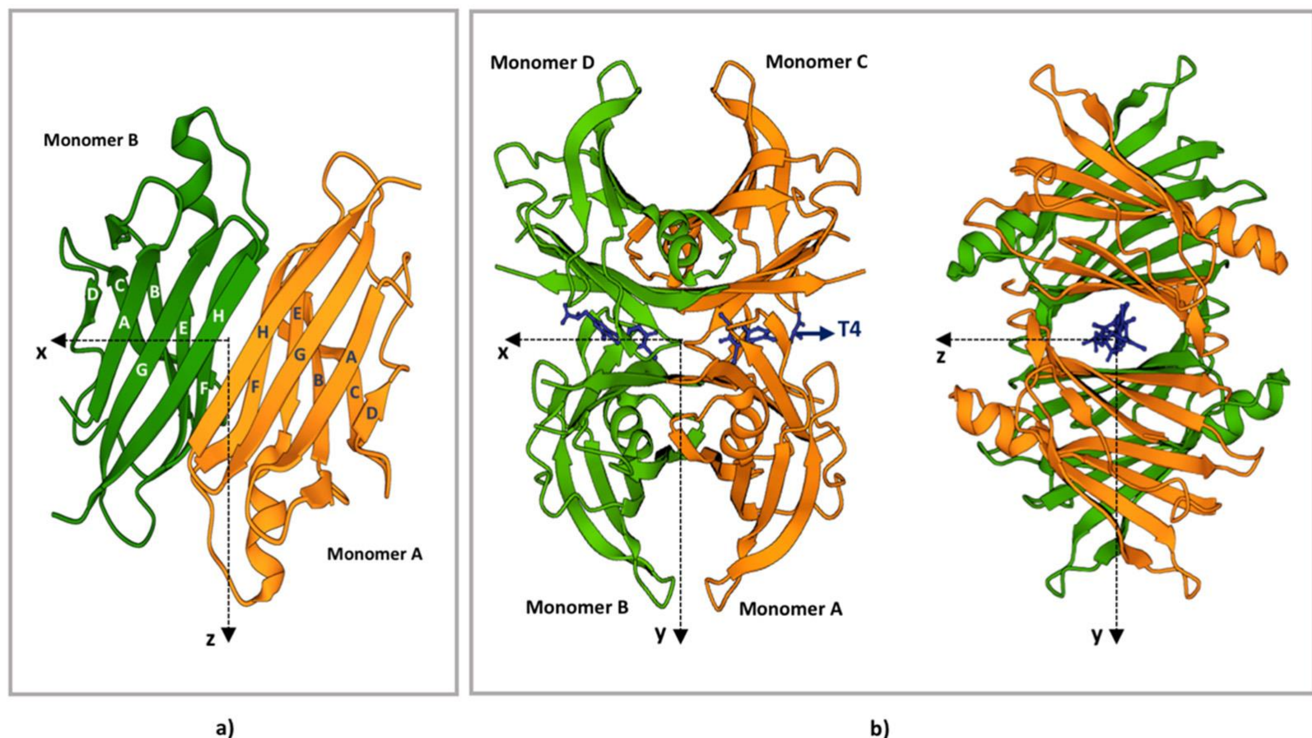


Fig. 1 Structural model of the human TTR

(a) Structural model of a TTR dimer including β-strand markings (b) Structural model of the TTR tetramer shown in two orientations differing in rotation by 90° relative to the y-axis. The figure shows the thyroxine binding sites in the central channel. The model was reproduced from [17].

In each TTR monomer, the eight β-strands are arranged in two β-sheets (CBEF and DAGH) forming a β-sandwich structure [17,22,23]. The N- and C-terminal fragments of each monomer are disordered, and the β-strands are connected by flexible loops of various lengths. Within the longest EF loop there is the only short α-helix whose position in the tertiary structure changes when the pH is lowered [17]. The interfaces between monomers A and B and C and D are formed by a network of extensive hydrogen interactions, in which internal water molecules play an important role [24]. The A-B dimer binds to the C-D dimer through hydrophobic point interactions between the loops. The assembly of dimers into a tetramer creates an internal hydrophobic central channel that binds not only thyroid hormones, but a number of other natural TTR ligands [17,22]. In humans, the most strongly bound thyroid hormone is thyroxine, but TTR also binds triiodothyronine and other thyroid hormone analogues [17]. The central channel contains two thyroxine binding sites, but as a result of negative cooperativity only one of these sites is usually occupied [25,26]. Moreover, since serum and CSF concentrations of TTR exceed those of thyroid hormones by several orders of magnitude, the entire pool of thyroid hormones is transported in a protein-bound form, which protects hormone molecules from adsorption on hydrophobic surfaces [6]. It is also worth noting that due to the high molar excess of TTR, almost all TTR molecules in serum and CSF are hormone-free and available for other low-molecular-weight substances [27]. Natural TTR ligands include fatty acids, lutein and other carotenoids, curcumin, quercetin, polyphenols, and many others [17,28,29]. The central channel also binds synthetic compounds that are designed and synthesized to stabilize the TTR structure [29,30].

TTR is characterized by a very high energy barrier separating the native state from the non-native state, therefore it is classified as a kinetically hyperstable protein [31]. The interactions between A and B or C and D monomers, are much stronger than the interactions between A-B and C-D dimers, however, TTR first dissociates slowly into dimers, which quickly dissociate into monomers [17]. The structure of TTR fluctuates and this process in non-mutated, unmodified TTR molecules results in an escape from the native state to a more unfolded non-native state [32]. Upon dissociation, TTR monomers with a non-native structure tend to form heterogeneous oligomers that aggregate to form protofilaments and then mature amyloid fibrils (filaments). The entire, multistep and complex process of amyloid formation is accompanied by structural changes in the TTR [33,34]. The pathway of aggregation process and factors affecting the ability of the TTR to form amyloid deposits, such as mutations, post-translational modifications of the TTR or bound ligands, are extensively studied, as the formation of TTR amyloid is associated with the pathogenesis of many diseases. To date, over 140 mutations have been identified, most of which lead to TTR destabilization and aggregation, resulting in familial amyloidopathy or cardiomyopathy [17]. In addition to familial forms, there are also sporadic forms of diseases in which amyloid is formed by non-mutated TTR in elderly people (usually

after eighty years of age). There were also numerous correlations found between vascular and/or osteoarticular diseases and abnormal concentrations, the presence of oligomeric forms or post-translational modifications of TTR [35].

Amyloid diseases caused by destabilization of the TTR structure are an increasingly recognized, significant medical problem, and the complex process of TTR amyloidogenesis is currently being intensively studied. Various types of therapeutic approaches are being developed, and involve the removal of TTR aggregates or molecules with a changed structure, the stabilization of native TTR tetramers or the correction of TTR mutations by genome editing [36,37]. The first two approaches are now being in general therapeutic use. Interestingly, in sporadic transthyretin amyloidopathy (much less often in the familial form of the disease) TTR fragments are observed in amyloid deposits [38]. The most commonly found TTR fragments are formed by proteolysis occurring near the peptide bond located on the carboxyl side of lysine 48 residue. It has been found that the protease that catalyzes the TTR hydrolysis *in vitro* and leads to the formation of fragments corresponding in length to those found in amyloid *in vivo,* is plasmin [39]. It was also shown that the presence of metal ions (such as Cu^{2+} or Ni^{2+}) leads to proteolytic processing of the N-terminus of the TTR and the formation of short peptide fragments [40,41]. The fact that TTR is proteolytically processed is particularly interesting considering that TTR itself has proteolytic activity. TTR is a Zn^{2+}-dependent protease and its substrates that have been identified so far are amyloid β peptide, apolipoprotein A1 and neuropeptide Y [19,42,43].

The molecular mechanism of ageing-induced amyloid formation is still poorly understood. It is assumed that oxidative stress and related post-translational protein modifications, in particular oxidation of amino acid residues, may underlie the pathogenesis of amyloid and neurodegenerative diseases [44]. Both the identification of post-translational modifications and sensor proteins associated with oxidative stress is therefore a key issue in understanding the mechanisms of ageing and age-related diseases. It is known that the formation of excessive amounts of reactive oxygen species (ROS), disturbances in energy metabolism and dysregulation of calcium metabolism underlie neurodegenerative diseases such as Alzheimer's disease [44–46]. All these processes are closely linked to the functioning of mitochondria. Dysregulation of the normal course of mitochondrial processes and mitochondrial biogenesis results in disruption of homeostasis involving ROS, Ca^{2+} signaling and energy metabolism. The association of TTR with the proper functioning of mitochondria has been observed in a mouse model of Alzheimer's disease [47]. Mitochondrial transcription factor A (TFAM) has been found to positively regulate TTR expression. TTR has been shown to be upregulated in the brain (in the cortex and hippocampus) helping TFAM break the vicious circle of oxidative stress. A co-localization of TTR and TFAM in mitochondria was also found, which indicates the formation of

a complex by these proteins. However, the molecular mechanism that would explain the role of TTR in breaking the vicious circle of mitochondrial stress has not yet been elucidated. So far, there has also been insufficient information on the impact of TTR structural changes caused by environmental factors occurring during oxidative stress on TTR properties and functions provided by the native structure of this protein.

Understanding the mechanism, in which TTR fulfills its protective role against oxidative stress, was the starting point for this project. The protective functions of TTR are inherently linked to its structure. Moreover, the change in the structure of the TTR underlies the process of pathogenesis. Therefore, it seemed crucial to investigate how the structural stability of TTR is affected by environmental factors related to the pathogenesis of amyloid diseases and other diseases in which TTR is involved. Precise knowledge of the mechanism of TTR destabilization, caused by a given factor or combination of factors, allows to determine the role of TTR and/or destabilization of its structure in the pathogenesis of a given disease. Moreover, this knowledge enables the design of effective therapeutic strategies for TTR-related diseases and/or the unveiling of hitherto unknown TTR characteristics. The results of the studies presented below revealed new properties and new relationships between the structure and function of TTR, induced by the factors related to pathogenesis, and showed new activities of this protein.

4a.4. Description of the results achieved and their significance

4a.4.1. Pathogenesis-related factors destabilize the structure of transthyretin

Ca^{2+} ions

[P_13] Wieczorek, E.; Kędracka-Krok, S.; Bystranowska, D.; Ptak, M.; Wiak, K.; Wygralak, Z.; Jankowska, U.; Ożyhar, A. **Destabilisation of the structure of transthyretin is driven by Ca^{2+}**. *Int. J. Biol. Macromol.* 2021, 166, 409–423.

Signaling pathways involving Ca^{2+} ions are an extremely important factor in the regulation of a number of key cellular and extracellular processes, in particular in the functioning and biogenesis of mitochondria. In mitochondrial stress, one of the mechanisms of the regulation of signaling pathways, in which participate Ca^{2+} ions, is the attachment of Sumo to proteins involved in calcium metabolism [48]. Signaling involving Ca^{2+} ions is the basis of tissue homeostasis (especially in the brain and bone tissue) which is critical for the functioning of the whole organism. Dysregulation of calcium metabolism and homeostasis of the Ca^{2+} signaling system underlies the aging process and the

pathogenesis of many diseases. On the other hand, diseases associated with the formation of amyloid lead to an imbalance in the concentrations and fluxes of Ca^{2+} ions [49]. Calcium balance is disturbed in the neurodegeneration process that accompanies the Alzheimer's or Parkinson's disease [50]. It has been known that destabilization of TTR structure, leading to the formation of TTR oligomers and amyloid, results in deregulation of Ca^{2+} signaling and increase in local and transient Ca^{2+} concentrations in cells [51]. However, no studies on the influence of Ca^{2+} ions on the stability and structure of TTR have been conducted before.

The issue, how the non-physiological concentrations of Ca^{2+} ions affect the stability of TTR structure, inspired the work which results were published in the *International Journal of Biological Macromolecules* [41] [P_13]. Studies were performed using recombinant, purified to homogeneity TTR, which was expressed in a bacterial system. The results of isothermal titration calorimetry (ITC) and assay using fluorescence probe showed that, in the studied micromolar range of concentrations, Ca^{2+} ions do not bind to TTR. However, under the influence of Ca^{2+} ions, or Mg^{2+} ions, used as a control of the specificity of effect, a change in the Stokes radius (Rs) of TTR, was observed, in the millimolar range of ions concentrations, as demonstrated by analytical ultracentrifugation and gel filtration experiments. The Rs value decreased with increasing concentration of ions. The change was greater for Ca^{2+} ions than for Mg^{2+} ions, in the lower concentration ranges, but for 200 mM ion concentration, Rs was similar for both ions (equal to about 90% of the value observed in the absence of ions). The influence of Ca^{2+} and Mg^{2+} ions on the TTR structure is of a different nature, because the shape of the curve of Rs value *versus* ion concentration suggests a single-phase process for Mg^{2+} and a two-phase process for Ca^{2+}. No global change (occurring for the entire population of molecules) in TTR conformation was observed, and the content of secondary structures in the presence of ions, determined using circular dichroism (CD) spectroscopy, did not change as well. However, it was found that after long incubation and/or subjecting the TTR to cycles of thermal denaturation and renaturation, carried out in the presence of ions, the sensitivity of TTR to factors destabilizing its structure changes. The results of Fourier-transform infrared spectroscopy (FTIR) experiments, supplemented by CD results, showed that Ca^{2+} ions disrupt the conformational flexibility of TTR molecules. Some factors present in the environment, such as reducing compounds (e.g dithiothreitol, DTT) or the compounds that increase viscosity (glycerol), exert a synergistic effect with respect to the effect induced by Ca^{2+}. This is manifested by a change in the relative absorbance of the amide I band (AI) in the FTIR spectrum of TTR (**Fig.2**).

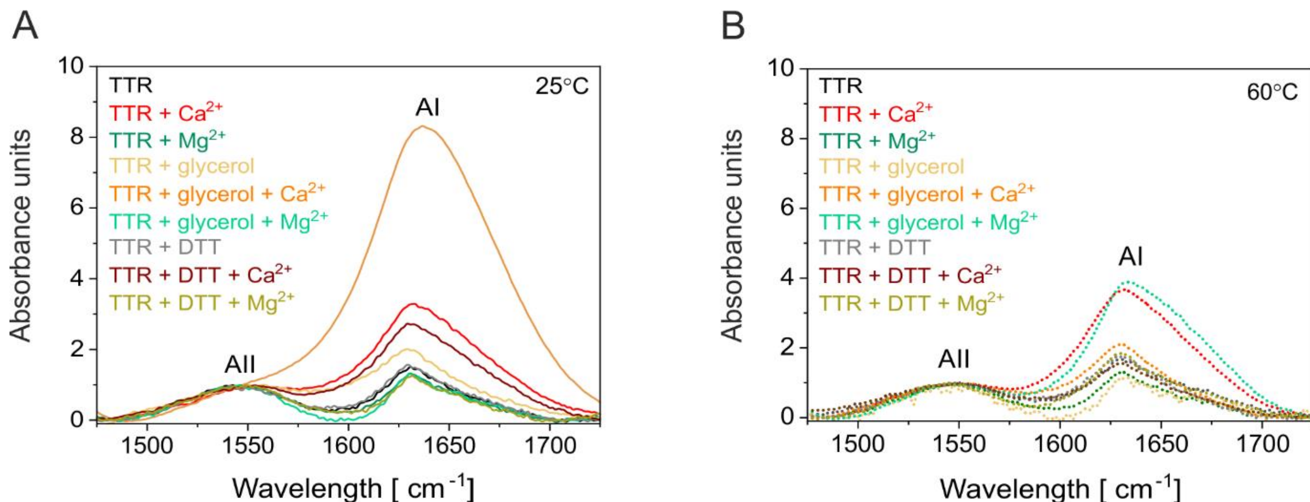


Fig. 2 Environmental factors affect the structure of the TTR

Superimposed FTIR spectra obtained for TTR in the presence of various factors. Protein samples were incubated with the listed agents overnight at room temperature (A) or at 60°C (B) prior to obtaining the spectra. Absorbance units were normalized by setting the absorbance maximum of the AII (amide II) band for TTR without additives to 1. Figure reproduced from the publication [P_13] [41].

Using the SDS-trapping technique, which determines the relative amount of TTR monomers in SDS-PAGE, which is a measure of the extent of protein unfolding, it was shown that in the presence of Ca^{2+} and Mg^{2+} ions, the unfolding of TTR structure is proportional to the concentration of ions. Moreover, in the presence of Ca^{2+} ions, the temperature of thermal transition (determined using Differential Scanning Calorimetry, DSC) from the native form of the correctly folded tetramer to the non-native form of the unfolded monomer decreases by 3°C. In the presence of Ca^{2+} ions, the peak width of the TTR transition from native to non-native form is narrowed, which indicates that Ca^{2+} ions accelerate the two-step process of TTR dissociation (tetramer - dimer - monomer) and make it a truly single-phase process. This work also showed that the destabilizing effect of Ca^{2+} led to the formation of a subpopulation of non-native, unstable TTR conformers, with a high susceptibility to precipitation. Analysis of mass spectrometry (MS) results revealed, that Ca^{2+} ions also increased the proteolytic fragmentation of the TTR. Deconvolution of the MS spectra of TTR, showed that in addition to the predominant population of molecules with a molecular weight of monomer, a population of molecules with a molecular weight lower by about 1000-1200 Da was also observed. This corresponds to a shortening of TTR molecule by a fragment of about 10 amino acid residues. At the same time, a population of molecules with a molecular weight greater than the molecular weight of the TTR monomer by about 1000-1200 Da was observed. These observations suggest that full-length TTR monomers are covalently linked (possibly *via* disulfide bridges) to the short TTR fragments (containing cysteine 10 residue) formed due to proteolysis. In conclusion, the obtained

results showed that Ca^{2+} ions destabilize the structure of TTR by disrupting its interactions with the hydration shell. This influences the conformational fluctuations and facilitates the fragmentation and precipitation of small subpopulations of TTR molecules. The observed increase in proteolysis induced by Ca^{2+}, resulting in the formation of short N-terminal TTR fragments, complements the literature reports on the oxidative proteolysis of the N-terminus of TTR induced by divalent metal ions such as Cu^{2+} or Ni^{2+}, resulting in fragments of the length of ten amino acids residues [40]. Showing that such process also occurs for Ca^{2+} ions is new and very valuable observation. These studies inspired Cantarutti and colleagues, who analyzed in detail the interaction of TTR with Ca^{2+} ions, and their results [52] confirmed the observations presented in the publication [P_13].

In conclusion: The obtained results [P_13] [41] show that Ca^{2+} and Mg^{2+} ions, in millimolar concentrations, exert a destabilizing effect on the structure of TTR. It has been shown that Ca^{2+} and Mg^{2+} ions increase the ability of TTR to proteolytic fragmentation and aggregation. It was also found that Ca^{2+}- mediated changes in the TTR structure were modulated by the reducing agent (DTT) and the process of TTR aggregation was accompanied by the oxidation of the cysteine 10 residue. Therefore, next the combined effects of Ca^{2+} ions and DTT on the TTR structure were investigated. The results of these studies were presented in two papers published in the *International Journal of Biological Macromolecules* [53] [P_15] and *Redox Biology*[54] [P_16].

Redox state

[P_15] Wieczorek, E.; Bezara, P.; Ożyhar, A. **Deep blue autofluorescence reveals the instability of human transthyretin**. *Int. J. Biol. Macromol.* 2021, 191, 492–499.

[P_16] Wieczorek, E.; Wygralak, Z.; Kędracka-Krok, S.; Bezara, P.; Bystranowska, D.; Dobryszycki, P.; Ożyhar, A. **Deep blue autofluorescence reflects the oxidation state of human transthyretin**. *Redox Biol.* 2022 Aug 9;56:102434.

Using the SDS-trapping technique, it was shown in [P_15] that DTT accelerates the unfolding of TTR in a concentration-dependent manner, although to a lesser extent than Ca^{2+} ions (**Fig. 3A**). This leads, due to long-term incubation, to protein precipitation (**Fig. 3B**). The observed amount of TTR precipitates is very similar for the samples analyzed in the absence and presence of Ca^{2+} (or Mg^{2+}), indicating that DTT increases the dissociation rate of TTR tetramer to monomers to a much greater extent than Ca^{2+} (or Mg^{2+}). The process of TTR destabilization occurs in portions, for small subpopulations of molecules and not globally, because in molecular filtration carried out in the presence of Ca^{2+} and DTT, a predominantly tetrameric form of TTR was observed with only a small amount of monomers and oligomers.

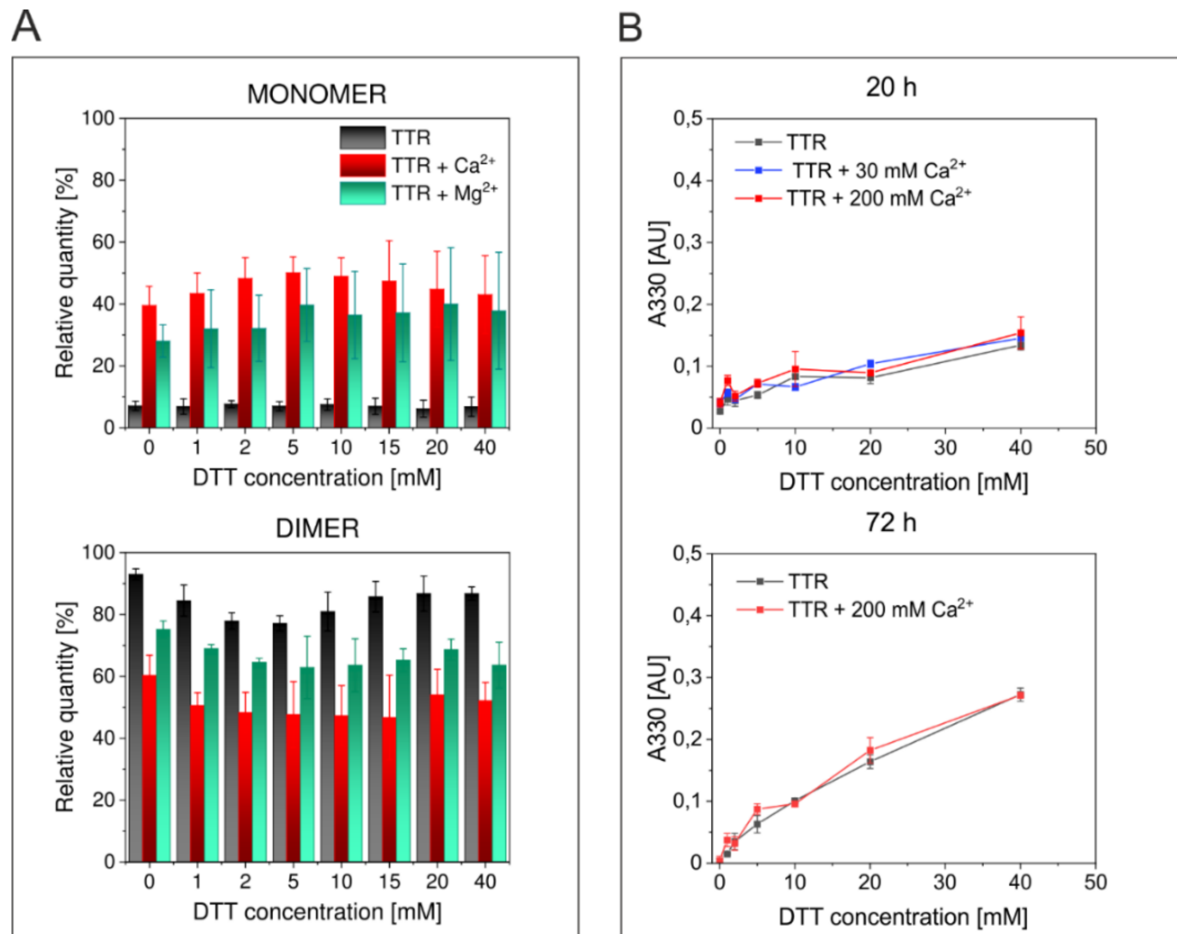


Fig. 3 DTT and Ca^{2+} ions modulate the stability and unfolding of TTR

(A) SDS-trapping assay (the test of unfolding). TTR samples were incubated for at least several hours at room temperature in the absence and presence of 30 mM Ca^{2+} or 30 mM Mg^{2+} and with DTT at the indicated concentrations. The samples were then subjected to thermal unfolding for 60 s at 85°C and separated by SDS-PAGE without routine thermal denaturation. The gels were stained with Blue Stain Sensitive. The normalized amounts of protein, determined for the monomeric and dimeric forms of TTR for all analyzed samples (presented as the average of three separate experiments), were plotted against DTT concentration. **(B) Aggregation assay.** TTR samples in duplicate were incubated at room temperature for 20 or 72 hours in the absence and presence of 30 mM Ca^{2+} or 30 mM Mg^{2+} and with DTT at the indicated concentrations. The absorbance of each sample was then measured at 330 nm. The results represent averaged values. Figure reproduced from [P_15] [53].

Also, no global conformational change or oligomerization of the TTR was observed in electrophoresis performed under native conditions. However, when in SDS-trapping experiments, TTR was unfolded at different DTT concentrations, small subpopulations of TTR conformers and oligomers were observed in silver-stained gels in SDS-PAGE analysis. The presence of Ca^{2+} or Mg^{2+} ions and long incubation of the samples favored the appearance of subpopulations of destabilized molecules of transient TTR conformers observed at specific concentrations of DTT. This was an important observation confirming previous findings on the formation of unstable subpopulations of TTR

conformers induced by Ca^{2+} [P_13]. These observations make a valuable contribution to understanding the destabilization process of TTR, which is a kinetically hyperstable protein. TTR forms amyloid deposits despite having a high energy barrier separating the native from the non-native states. Accumulation of precipitation of small portions of destabilized TTR molecules could be observed both by an increase in turbidity (absorbance at 330 nm, **Fig. 3B)** and by a decrease in the intensity of fluorescence emission of the aromatic residues.

Fluorescence spectroscopy is a technique that allows insight into the structure and conformational rearrangements of a protein thanks to the measurement of changes in the microenvironment of aromatic (mainly tryptophan) residues. TTR has 8 tryptophan residues, 20 tyrosine residues and 20 phenylalanine residues in the tetramer, therefore it is a very good object for fluorescence studies. Measurements of the fluorescence emission spectra of TTR aromatic residues, carried out for protein samples incubated in the presence of DTT and/or Ca^{2+}, confirmed the cumulative nature of the precipitation of the sub-populations of unstable molecules, as the fluorescence intensity gradually decreased during the long incubation of the samples. In addition, DTT slightly affected the microenvironment of the tryptophan residues in the TTR and caused a red shift of the emission maximum. However, the most interesting observation, resulting from the study of TTR fluorescence, was the formation of a unique autofluorophore that emitted light in the visible range and which had not been previously observed for TTR.

In conclusion: Long-term incubation in the presence of DTT and/or Ca^{2+} leads to cumulative precipitation of TTR. Factors destabilizing the structure of the TTR result in the generation of subpopulations of TTR molecules, in which structural changes lead to the formation of a new autofluorophore emitting radiation in the visible range.

4a.4.2. A new autofluorophore is formed in transthyretin

[P_15] Wieczorek, E.; Bezara, P.; Ożyhar, A. **Deep blue autofluorescence reveals the instability of human transthyretin**. *Int. J. Biol. Macromol.* 2021, 191, 492–499.

[P_16] Wieczorek, E.; Wygralak, Z.; Kędracka-Krok, S.; Bezara, P.; Bystranowska, D.; Dobryszycki, P.; Ożyhar, A. **Deep blue autofluorescence reflects the oxidation state of human transthyretin**. *Redox Biol.* 2022 Aug 9;56:102434.

This extremely interesting phenomenon has been detected so fat for a small number of proteins and its nature is not yet fully understood [55]. Due to the characteristics of the emission, this phenomenon has been termed deep blue autofluorescence (dbAF). dbAF phenomenon is believed to be linked to changes in protein structure and may involve the formation of an α-sheet structure in oligomers preceding the formation of amyloid fibrils. dbAF is therefore a valuable tool for monitoring

protein structural changes leading to amyloid formation. The existence of a stable α-sheet structure in proteins was predicted by Pauling and Corey, but its presence has not yet been experimentally definitively proven [56,57]. The recorded autofluorescence emission spectra of TTR, excited by ultraviolet radiation with a wavelength of 360 nm, showed at least three overlapping fluorescence maxima located at 414, 438 and about 450 nm (**Fig. 4**, left). Studies of the emission properties of the autofluorophore formed in the TTR revealed the extremely dynamic nature of this process. In this work [P_15], it was shown that environmental factors such as DTT, long incubation (oxidation) of the TTR and Ca^{2+} ions promote the formation of dbAF in TTR. Therefore, systematic research (based on literature reports on the mechanism of this phenomenon [58–60]) concerning the influence of redox conditions, and more broadly the oxidation state of TTR, on dbAF emission was undertaken. The results of these studies have been published in the *Redox Biology* journal [54] [P_16].

Experiments conducted in conditions from the most reducing to the most oxidizing showed that the oxidation state of TTR affected the properties and dynamics of dbAF formation. Thus, the previously observed [26] destabilizing effect of low-molecular-weight thiols: DTT and reduced glutathione (GSH) on the TTR structure and their synergistic effect with Ca^{2+} was confirmed. This work showed that increasing the exposure of TTR to mild oxidizing conditions (incubation lasting several hours) compensated to some extent the effects of free thiols. However, highly oxidizing conditions led to oxidative modifications of many amino acid residues of TTR, which also reduced TTR stability and resulted in a change in dbAF properties. **Fig. 4** summarizes the effect of environmental conditions on the structure of TTR and emission properties of dbAF. Under reducing conditions, the dbAF emission spectrum, obtained after excitation with 360 nm radiation (**Fig. 4**, left), shows three characteristic overlapping fluorescence maxima at 414, 438 and about 450 nm. The oxidation of TTR results in a unique dbAF emission spectrum (**Fig. 4**, right), possessing one maximum at 457 nm. The obtained results presented in [P_16] also revealed that TTR has the ability to bind riboflavin (vitamin B2) and/or riboflavin photoproducts, which was a new observation. Moreover, the TTR irradiation experiments, performed in the presence of riboflavin used as a photosensitizer, led to an increase in the unfolding rate of the TTR structure. Irradiation using light of a wavelength of 445 nm, both in the presence and absence of riboflavin, resulted in increased TTR fragmentation as well as the formation of covalent bonds between adjacent monomers due to dityrosine formation. The cross-linked dimers showed anomalous mobility in SDS-PAGE. The phenomenon of oxidation of tyrosine residues of TTR, accompanied by the formation of a reduction-insensitive covalent bonds between the subunits, has not been studied so far.

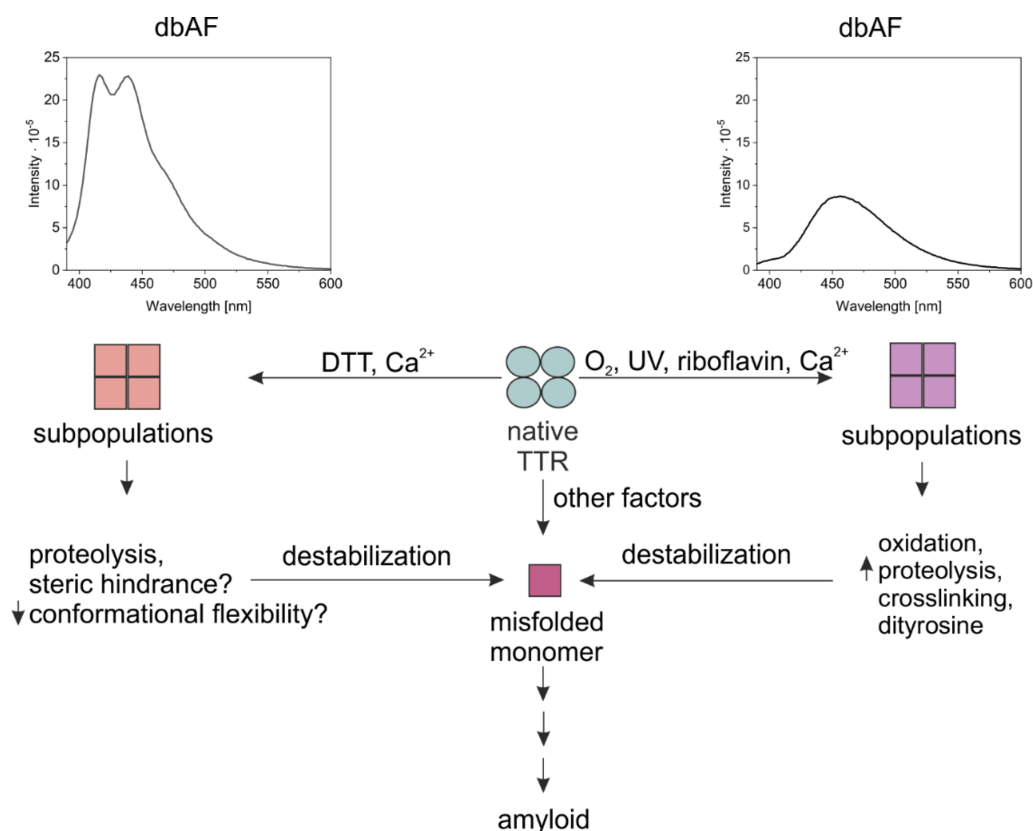


Fig. 4 Differences in dbAF spectra reflect the influence of the environmental factors on the structure of TTR

Strongly reducing conditions destabilize TTR tetramers leading to their dissociation, unfolding and N-terminal proteolysis. Destabilization under oxidative conditions is associated with proteolysis of the polypeptide chain, oxidation of amino acid residues, formation of dityrosine and cross-linking of TTR monomers. The dbAF spectra obtained under reducing and oxidizing conditions are different, suggesting significant structural differences in TTR. Figure reproduced from [54] [P_16].

Using MS to identify the irradiation-induced changes in TTR, which occur in the absence and presence of riboflavin, revealed numerous oxidative modifications of amino acid residues [P_16]. In addition, it was found that riboflavin can change the oxidation profile and protect some amino acid residues of TTR from oxidation. From literature reports it is known that cysteine 10 and methionine 13 residues of TTR are most susceptible to oxidation [61]. Interestingly, the oxidation state of certain amino acid residues of TTR has also previously been shown to be related to the pathogenesis process, particularly in demyelinating diseases such as multiple sclerosis [62,63]. The obtained results presented in the paper [P_16] provide new information on the molecular basis of TTR participation in the pathogenesis of diseases resulting from redox imbalances of cells, tissues or the whole organism. The obtained data presented in publication [P_16] are also in agreement with the reports on the already known mechanisms of the protective effects of vitamin B2 in these diseases and the reports showing the participation of TTR in the process of myelination [64].

In conclusion: It has been shown in the article [P_16] that both strongly reducing or strongly oxidizing conditions exert a destabilizing effects on the TTR structure, leading to the formation and precipitation of subpopulations of its unstable conformers. TTR irradiation results in fragmentation of TTR molecules and oxidative modifications of many amino acid residues. Riboflavin and/or its photoproducts bind to TTR. Irradiation in the presence of riboflavin results in an increase in the rate of monomer unfolding and TTR crosslinking. At the same time, riboflavin changes the sensitivity of individual amino acid residues of TTR to oxidation. This work also shows that the redox state of the environment affects the dynamics of dbAF formation and that the emission spectra of dbAF reflect the TTR oxidation state.

Amyloid formation is a complex, multi-step process. In this process the native structure of protein molecules, under the influence of internal (mutation) or external (environment) factors, becomes destabilized, which leads to oligomerization and then the formation of larger polymer structures (protofilaments and filaments). The aggregation process is associated with dynamic conformational changes occurring in many stages [34,65]. The amyloidogenic properties of TTR, caused by the destabilizing effect of Ca^{2+}, on the TTR structure were presented in the publication [P_13]. These properties, in the context of the regulatory role of TTR in the nervous system and glucose metabolism, are of particular interest because both the nervous system and glucose metabolism are involved in the formation and dynamics of the skeletal system [66]. The hypothesis of the relationship between TTR and the biomineralization process was the starting point for the research presented in publication [P_12] [67]. This hypothesis was also based on 1) reports concerning the relationship between TTR concentration and aggregation and the development, structure, density and pathological states of cartilage and bone tissue [68,69] and 2) the results of studies that showed the presence of TTR in mamillary cones of hen's eggshell, next to proteins whose participation in the biomineralization process was documented by other studies [70].

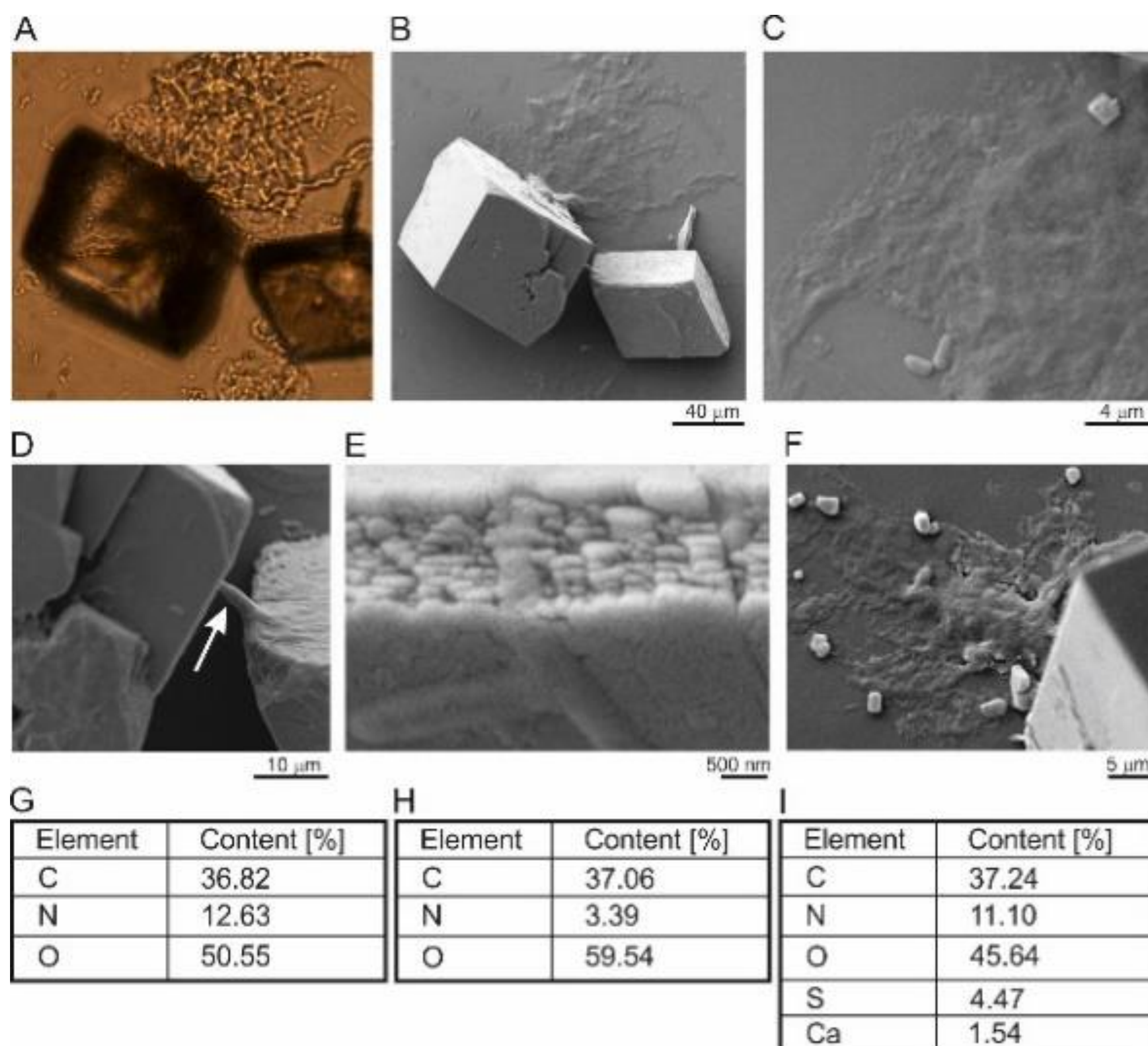
4a.4.3. Transthyretin exhibits new activities

Biomineralization activity

[P_12] Wieczorek, E.; Chitruń, A.; Ożyhar, A. **Destabilised human transthyretin shapes the morphology of calcium carbonate crystals.** *Biochim. Biophys. Acta - Gen. Subj.* 2019, 1863, 313–324.

The article [P_12] published in *Biochimica et Biophysica Acta* [67] reports the research on the biomineralization properties of TTR and the relationship between the structural stability of TTR and the propensity to form amyloid. This research was carried out. using recombinant, purified TTR, in a biomineralization test involving the formation of calcium carbonate crystals *in vitro*. The obtained

crystals were analyzed by light microscopy and Scanning Electron Microscopy (SEM) using elemental Energy Dispersive X-ray Spectroscopy (EDS) analysis. The data presented in [P_12] show that TTR affects the morphology of the obtained calcium carbonate crystals, leading to the formation of unique crystals different from the control crystals which were obtained in the absence of protein. Crystals obtained in the presence of TTR had rounded edges and corners and exhibited foliated layers, porosity, growth inhibition with respect to selected axes (glide-symmetry). Non-crystalline aggregates that were isolated, or were present on the faces of some crystals, were also observed. These observations suggested that a protein hydrogel may have formed during the mineral's crystallization process. Therefore, the effect of TTR stability on the properties of calcium carbonate crystals obtained in the biomineralization assay was examined and it was found that preparations containing only the stable, tetrameric form of TTR led to obtaining more regular crystals with asymmetric morphology and porous structure, but without visible protein precipitates. When TTR was subjected to controlled thermal destabilization, crystals with irregular morphology and accompanying protein aggregates were obtained. The structure of these aggregates was reminiscent of the amorphous protein-mineral phase of polymer induced liquid precursor (PILP). Similarly, the relationship between stability and the effect of TTR on the biomineralization process was observed by examining preparations obtained using two different purification procedures. TTR, expressed in fusion with glutathione-S-transferase and TTR expressed in fusion with the NusA chaperone protein, were purified and used for biomineralization assays. Although the protein tags were removed by proteolysis in the purification process, TTR expressed in fusion with chaperone protein showed a different effect on the morphology of calcium carbonate crystals than TTR expressed in fusion with glutathione-S-transferase. The crystals obtained in the presence of TTR expressed in fusion with chaperonin had fine porosities and were flat, which suggested that their growth was inhibited in one direction. In experiments using EDS analysis, no protein signatures were observed on the surface of these crystals. Pre-incubation of TTR with Ca^{2+} ions, performed prior to using the protein in the biomineralization assay, changed the crystal morphology, in relation to the crystals obtained in the assay to which TTR was added without prior incubation with Ca^{2+} ions. Pre-incubation with Ca^{2+} also led to the reduction of pore size in the crystals, which additionally showed growth inhibition along the [001] axis and contained pits on one of the faces. Moreover, a protein signal was observed on the surfaces of these crystals by EDS analysis. When a TTR preparation, that contained monomers and oligomers in addition to tetramers, and which was prone to amyloid formation, because it had been subjected to ageing by incubation for many months, was used in the biomineralization assay, the extensive protein precipitates (deposits) were observed (**Fig. 5**).



G

Element	Content [%]
C	36.82
N	12.63
O	50.55

H

Element	Content [%]
C	37.06
N	3.39
O	59.54

I

Element	Content [%]
C	37.24
N	11.10
O	45.64
S	4.47
Ca	1.54

Fig. 5 The unstable TTR results in the formation of calcium carbonate crystals covered with amorphous deposits

Bright-field (A) and SEM images of crystals (B, D, E and F) and amorphous precipitates (A,B,C,E,F) obtained in the presence of an aged TTR. (A, B) and (D) show the same pair of crystals. (D) 4-fold magnification of (B), (C) 10-fold magnification of accompanying precipitate, (E) 50-fold magnification of the upper edge of the smaller crystal from the pair shown in (B), (G, H, I) EDS analysis of the following: (G) bridge, (H) precipitate shown in (C) and (I) precipitate shown in (F). The arrow in (D) indicates a bridge between the two crystals. Figure reproduced from [67] [P_12].

The precipitates were located on the surface, inside the pores, between the layers and next to the crystals, which showed all the morphological features observed for crystals obtained using other TTR preparations. It was interesting to find, by EDS analysis, a high chloride content in the absence of sodium in the amorphous precipitates. Since TTR binds many chloride ions (12 per monomer) [71] this observation supported the fact that TTR was present in the form of a protein gel. In addition, EDS

analysis showed co-localization of calcium and protein. This observation suggested that the crystal formation process, occurring in the presence of TTR, proceeds through the pre-crystalline phase of PILP. which is formed in the natural biomineralization process. Large crystals, especially within the protein gel surrounding the crystal, were accompanied by microcrystals (**Fig. 5F**), suggesting a nucleating role of TTR amyloid in the mineralization process.

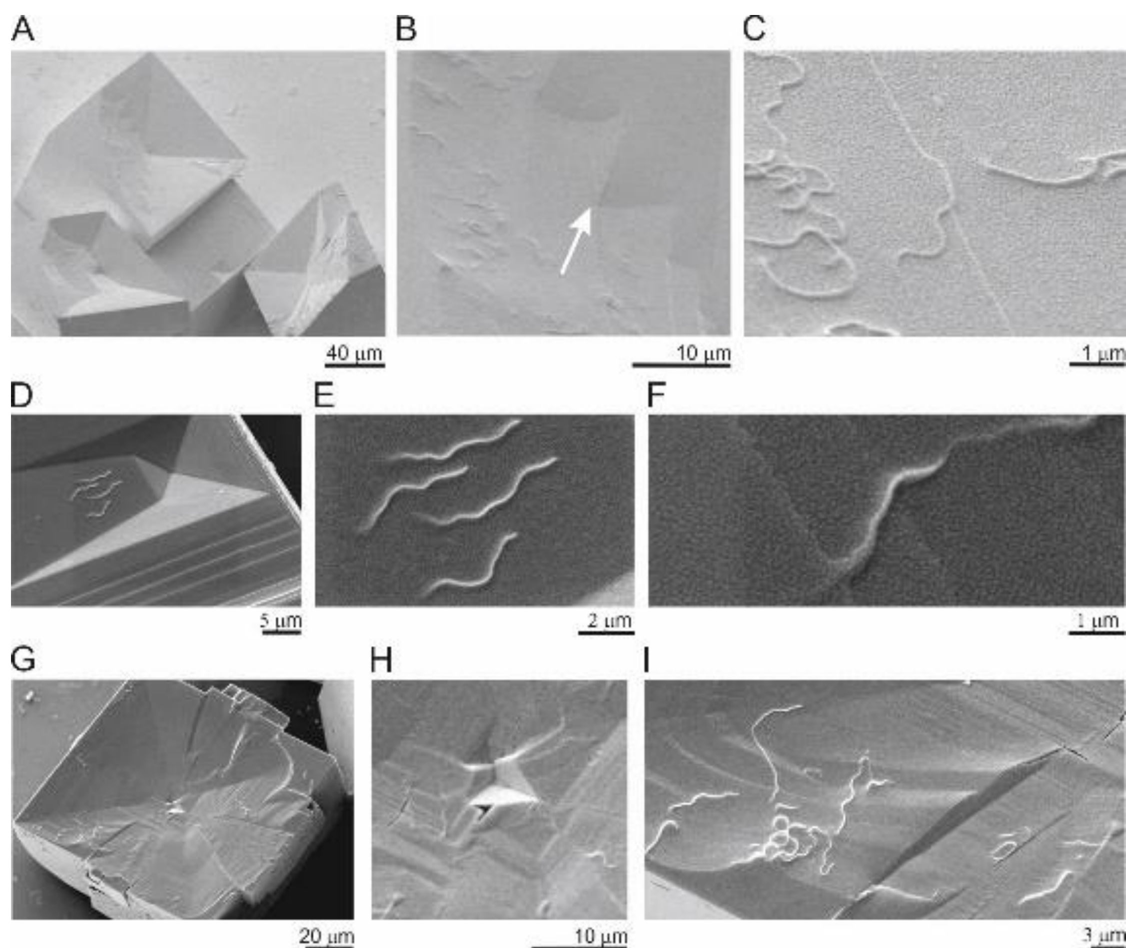


Fig. 6 The crystals obtained in the presence of aged TTR have pits and fibrous structures on their surfaces

(A, D and G) SEM images of crystals, (B, C, E, F, I) Different magnifications of fibrous structures present on the crystal surfaces: 6.4-fold (B) and 36-fold (C) magnification of the surface of the largest crystal from (A), 3.75-fold (E) and 7.5-fold (F) magnification of the crystal surface from (D), 4-fold (H) and 5-fold (I) magnification of the crystal surface shown in (G). The arrow on the panel (B) indicates the triangular hill. Figure reproduced from [P_12] [67].

The most interesting morphology was observed for flat crystals with a characteristic deep etch (**Fig. 6**, **A,D,G,H,I**), which confirmed the inhibitory effect of TTR on crystal growth in certain directions. On the surfaces of such crystals the fibrous structures with dimensions corresponding to amyloid fibrils were present (**Fig. 6**, **C-I**). Spheroids - circular aggregates composed of fibrils (not

shown) were also found on the faces of some crystals. In the Raman spectrum, obtained after cutting the spheroid, a band at 512 cm^{-1} was observed, which suggested the presence of disulfide bridges in the protein fibrils forming the spheroid, indicating that the aggregation process is accompanied by the oxidation of the cysteine 10 residue in the TTR.

The results presented in publication [P_12] confirm the hypothesis of TTR involvement in the process of biomineralization, occurring naturally in skeletal tissue or occurring during pathological processes (calcification) in various tissues. Observations concerning the relationship between the biomineralization activity and the stability of the TTR structure suggest that TTR regulates the formation of the mineral phase in a complex way. Aggregated forms of TTR participate in the formation of the early mineral-protein phase of PILP, accelerating the crystal nucleation process, but also inhibiting crystal growth in certain directions. In addition, the studies described in paper [P_12] confirmed the destabilizing effect of Ca^{2+} ions (in the range of their millimolar concentrations) on TTR stability. The high concentration of Ca^{2+} ions occurs not only during the biomineralization process, but is also the result of mitochondrial stress. when Ca^{2+} is released from the mitochondrion into the cytoplasm. This, in turn, results in a local increase in the concentration of these ions. Disturbance of mitochondrial homeostasis is also accompanied by a disturbance of redox potential caused by the uncoupling of the respiratory chain, which correlates with the results described in publications [P_15] and [P_16].

In conclusion: TTR was found to affect the growth and morphology of calcium carbonate crystals formed *in vitro*, leading to selective inhibition of crystal growth and asymmetry, multiple porosities of variable density, unfinished layers and rounded edges and corners. The strongest effect was observed for unstable TTR preparations subjected to aging, which resulted in the presence of TTR precipitates located next, on and inside the crystals. SEM and EDS analyses showed the existence of a PILP-like mineral-protein phase that naturally occurs in the biomineralization process. TTR aggregates penetrated into the inner mineral layers, which led to complex morphological effects of the crystals. Fibrillar incrustations and protein spheroids, containing disulfide bridges, were also observed. TTR has one N-terminal cysteine residue at position 10 in each monomer. The oxidation state and post-translational modifications of this residue, significantly affect the structure of the TTR and are associated with the pathological processes of TTR-related diseases [26,61,62]. These observations correlate with the data presented in publication [P_15] and [P_16].

Post-translational modifications, directly or indirectly, significantly affect the structure, localization, function, and degradation of proteins. Literature studies undertaken before the experimental approach and *in silico* analyses, conducted in order to gain the knowledge on the human

TTR aggregation, allowed to conclude that there is a close relationship between the functions, post-translational modifications and the structure of the TTR. This assumption was the starting point for the study of the potential involvement of TTR in the process of protein sumoylation, which is associated with the cell's response to stress.

Regulation of Ubc9 autosumoylation

[P_11] **Wieczorek, E.**; Kędracka–Krok, S.; Sołtys, K.; Jankowska, U.; Hołubowicz, R.; Seliga, J.; Ożyhar, A. **Is Transthyretin a Regulator of Ubc9 SUMOylation?** *PLoS ONE* 2016 Aug 8;11(8).

Sumoylation is a complex, reversible, dynamic post-translational modification of proteins, critical for mitochondrial homeostasis and the well-being of the whole organism, that is activated in response to mitochondrial stress [72]. Extracellular and intracellular stimulators of mitochondrial stress, such as nutritional status, ROS and deregulation of calcium signaling may regulate the sumoylation process [72,73]. Sumo, a small ubiquitin-like protein, is attached to lysine residues of protein substrates by the E2-conjugating enzyme (Ubc9) after exposure of Sumo’s C-terminal diglycine motif by a specific endoprotease (SENP) and activation of Sumo by the E1 enzyme. Specific E3 ligases may participate in Sumo transfer to the substrate proteins, but they are not strictly required. Five Sumo paralogues have been identified so far, although only Sumo paralogues 1, 2 and 3 have been studied more extensively [74]. Sumo 2 and 3 paralogues differ from each other by only three N-terminal amino acids and share about 50% homology with Sumo 1. Sumo molecules can be detached from the substrates in the process of desumoylation by the action of specific proteases. Proteins that influence the number and positions of Sumo molecules attached to protein substrates regulate the cell's response to stress [72].

Since TTR is involved in the response to mitochondrial stress and, moreover, each TTR monomer contains eight lysine residues, it was reasonable to verify whether and which TTR lysine residues undergo sumoylation. For this purpose, the analytical system of facilitated sumoylation - UFDS (Ubc9 Fusion Directed Sumoylation) was used [75]. In the UFDS system, to study protein sumoylation, a fusion of the Sumo conjugating enzyme (Ubc9) with a potential substrate (in this case, TTR) is used. By creating such a fusion protein (substrate_Ubc9 or Ubc9_substrate), the sumoylation of the substrate is enhanced and thus more easily detected. The analyzed fusion proteins and Sumo covalently linked to the green fluorescent protein were expressed in human HEK293 cells. The presence of sumoylated forms of the analyzed protein was detected by Western blotting. Fusion proteins: Ubc9_TTR, TTR_Ubc9, their mutants as well as TTR non-fused to Ubc9 and sumoylation

products of these proteins were detected using antibodies against TTR and/or Ubc9. The results of the study have been published in the article [P_11] in the *Plos One* journal [76]. In this work it was shown that the fusion proteins composed of TTR and Ubc9, undergo sumoylation at multiple sites, which leads to the formation of Sumo-modified forms with different molecular weight and of different stoichiometry. Interestingly, when examining the TTR and Ubc9 fusion proteins in the UFDS, not only TTR sumoylation was observed, but most of all, a very strong sumoylation of the Ubc9 enzyme (by auto-sumoylation or cross-sumoylation) was detected. This proved that TTR is not only a substrate but also a regulator of the sumoylation process. The diagram of possible ways of transferring activated Sumo molecules in the UFDS system used for TTR is presented in **Fig.7**.

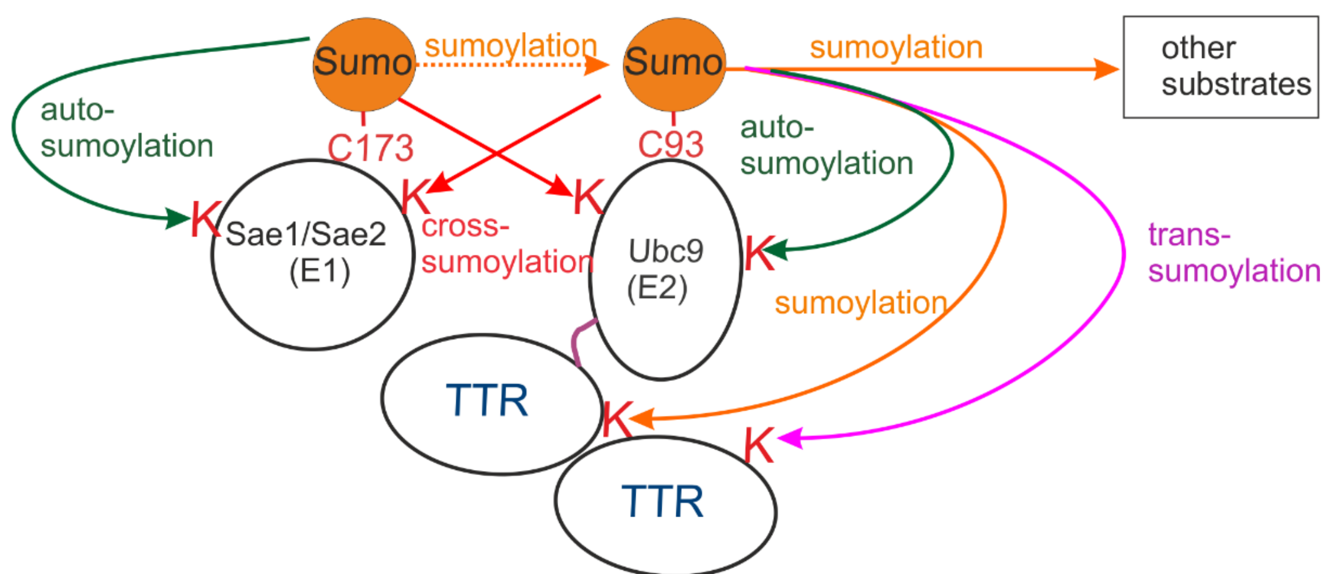


Fig. 7. SUMO transfer pathways between SAE1/SAE2 (E1), Ubc9 (E2), TTR and other potential substrate proteins in the UFDS system

The Sumo molecule, activated by a thioester bond, can be transferred from catalytic cysteine 173 residue (C173) of E1 to cysteine 93 (C93) residue of Ubc9 (first step of substrate sumoylation), to one of its own acceptor lysine residues (E1 auto-sumoylation) or to acceptor lysine of Ubc9 (cross-sumoylation of E2). Ubc9 can distribute activated Sumo from its catalytic cysteine residue 93 to TTR lysine residues in the Ubc9_TTR fusion protein or lysine residues in any other substrate protein (second step of substrate sumoylation).Alternatively, activated Sumo can be transferred from the catalytic cysteine 93 residue of Ubc9 to lysine residues of Ubc9 (auto-sumoylation) or lysine residues of proteins associated with the TTR_Ubc9 fusion protein, e.g. TTR (trans-sumoylation), or to a lysine residue of the E1 enzyme (cross-sumoylation of E1).

The results of MS analysis showed that if TTR is fused to Ubc9 then K154 residue of Ubc9 is the main sumoylation site. The paper [P_11] is the first report documenting the attachment of Sumo to the K154 residue of Ubc9. Moreover, in the presence of TTR, Sumo attachment to the K14 residue of Ubc9, which is the most frequently observed sumoylation site for the free enzyme [77], was not

observed. However, sumoylation of residues K49, K18 and K65 of Ubc9 did occur in the presence of TTR. Interestingly, literature reports show that the K18 residue in Ubc9 undergoes ubiquitylation and is involved in the recognition of Sumo molecules attached to substrates. In addition, the K65 residue of Ubc9 is modified by acetylation in the free enzyme, and sumoylation of the K49 residue promotes the localization of Ubc9 in Promyelocytic leukemia (PML) nuclear bodies – membraneless nuclear organelles involved in various cellular processes, for example, in response to cellular stress [78]. The above data demonstrate that TTR can affect, and even change, the types of post-translational modifications occurring in Ubc9, thus changing the activity, localization or substrate specificity of this key enzyme for the sumoylation process. In the paper [P_11] it was also shown that *in vitro*, under the conditions used, only small subpopulations of TTR molecules were sumoylated by attaching Sumo to its C-terminal K126 residue. TTR sumoylation occurred both when TTR was covalently bound to Ubc9 (or $Ubc9^{mut}$ - Ubc9 mutant lacking the three lysine residues: K14, K153 and K154) and by trans-sumoylation when TTR was unbound to Ubc9. Interestingly, the number and positions of Sumo molecules attached to Ubc9 (Ubc9 Sumo-load) affected TTR sumoylation, because $Ubc9^{mut}$ was found to be more effective in TTR sumoylation than non-mutated Ubc9. Analysis of the sumoylation of fusion proteins composed of TTR and $Ubc9^{C93A}$ - mutant of Ubc9 devoid of the catalytic cysteine residue showed that the attachment of multiple Sumo molecules to the substrate requires an active catalytic residue (C93) of Ubc9. The paper [P_11] also showed that both fused and not fused Ubc9 and TTR stimulated global sumoylation (sumoylation of other substrate proteins) in HEK293 cells. The observed effect of TTR was stronger for the Sumo 1 paralogue than for the Sumo 3 paralogue. In other report, an increase in global sumoylation was observed preferentially for the Sumo 2/3 paralogue (but also for Sumo 1) in response to hypoxia and cerebral glucose deprivation (ischemia) [79]. However, no increase in Ubc9 concentration was observed, which would indicate that a factor other than Ubc9 (TTR?) would be responsible for increasing sumoylation in response to oxidative stress.

In conclusion: The results described in paper [P_11] showed that subpopulations of TTR molecules are post-translationally modified by sumoylation, and that TTR significantly alters sumoylation and other post-translational modifications of Ubc9, the sole Sumo-conjugating enzyme in humans, afecting its activity and substrate specificity. Thus, TTR in human cells can potentially (by changing activity and specificity of Ubc9) regulate the level and the character of the cell's response to stress, including oxidative stress.

The assumption that TTR is a factor associated with the response to oxidative stress is supported also by literature reports documenting that TTR is one of the few proteins whose expression increases during adaptation to conditions of oxygen deprivation [80,81]. On the other hand, it is known

that the process of aggregation of proteins in general (including TTR) leads to changes in of ubiquitylation and sumoylation of proteins by an increase in ROS concentration. Therefore, destabilization of the TTR structure changes the pathways of protein refolding and degradation, ultimately affecting cell metabolism [82,83]. Moreover, the formation of TTR aggregates results in an increase in Ca^{2+} concentration and deregulation of calcium signaling, which is one of the mechanisms of pathogenesis [51,84]. From this reason, the sensitivity of the TTR structure to a significant increase in the concentration of Ca^{2+} (which was shown in publications [P_12], [P_13], [P_15] and [P_16]) is the cause of the vicious circle mechanism. The above observations, to which the results presented in publications [P_12], [P_13], [P_15] and [P_16] significantly contribute, reveal that TTR is a protective factor against oxidative stress only in a certain range of stress intensity. Exceeding this range results in TTR aggregation and loss of TTR protective properties or even intensification of the pathogenesis processess.

Concluding: the results presented in papers [P_11], [P_12], [P_13], [P_15] and [P_16], in the light of other literature reports, document that there is a relationship between 1) structural stability, concentration and post-translational modifications of TTR, 2) the presence of unfavorable environmental factors and 3) the pathogenesis of many diseases.

The properties of TTR and the relationships between its structure and function, with regard to the (broadly understood) links between the TTR and diseases with the TTR involvement, have been described in a review published in *Cells*[35] [P_14].

4a.4.4. The structure and properties of transthyretin are associated with the pathogenesis of many diseases

---

[P_14] Wieczorek, E.; Ożyhar, A. **Transthyretin: From structural stability to osteoarticular and cardiovascular diseases**. *Cells* 2021 Jul 13;10(7):1768.

In a review paper [P_14], based on literature reports and the results of own research, a comprehensive model of TTR connections with the pathogenesis processes of many diseases was proposed. The article begins with a description of the structure and known characteristics and functions of TTR. Next, the factors determining the structural stability and the process of TTR amyloid formation are discussed in detail. Particular attention was paid to the relationships between the environmental factors, especially the oxidative stress and Ca^{2+} ions, and the pathogenesis of diseases in which TTR is involved. The arguments, that the proper/native structure of the TTR is sensitive to the factors associated with oxidative stress and may act as an oxidative stress sensor, have been presented. A general model of the relationship between native and non-native forms of TTR was

proposed (**Fig. 8**). In this model, the tetrameric (native) form and the aggregated (non-native) forms of TTR are linked to each other under certain environmental conditions, such as the level of oxidative stress, the presence of Ca^{2+} ions, lipids, and inflammatory factors. Such association leads to the formation of vicious circles, in which the destabilized forms of TTR participate in accelerating destabilization process of native TTR molecules.

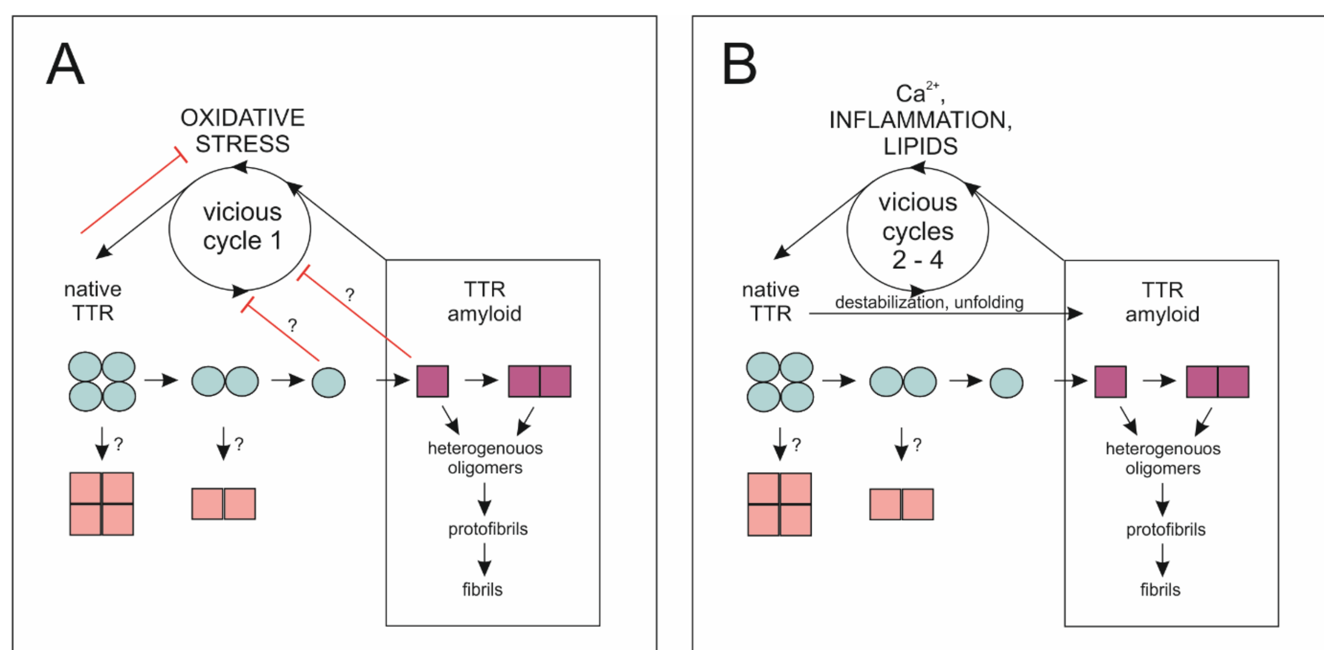


Fig. 8 Vicious circles mechanisms connecting native and non-native forms of TTR

The models show amyloid formation steps and correlations between TTR stability and the presence of factors that reduce TTR stability, which results in vicious circles of interactions. Native and non-native forms of TTR are represented by circles and squares, respectively. Native TTR tetramers, under the influence of destabilizing factors, dissociate into unstable dimers and then to monomers. The aggregation-prone monomers, dimerize and then form heterogeneous populations of oligomers that assemble into protofibrils and fibrils. Vicious circle 1 (A) differs from other vicious cycles (B) by the protective role of TTR against oxidative stress. The question marks indicate the direct unfolding of properly folded TTR tetramers and dimers (without dissociation to monomers), as well as the contribution of monomeric forms of TTR to the protection against oxidative stress, which require further study. Figure reproduced from [P_14] [35].

The results of many studies, which were described and discussed in the article [P_14], indicate the existence of a relationship between the forms and concentration of serum TTR and calcification of tendons and blood vessels. TTR is also involved in other diseases, the pathogenesis of which may be related to the negative impact of Ca^{2+} ions and redox imbalances on the TTR structure[35]. For this reason, the influence of TTR structure on the role of TTR in biomineralization and calcification processes was extensively described. The association of TTR with skeletal diseases was also discussed. In particular, reports showing links between TTR and diseases such as arthritis,

osteoporosis, osteoarthritis and juvenile idiopathic arthritis were presented. The association of TTR with reorganization of extracellular matrix was described and the literature concerning the connections of TTR with cardiovascular disease and vascular calcification was reviewed in detail. Publications showing the association of TTR with inflammation and pathogenesis of atherosclerosis as well as the reports on the role of TTR in angiogenesis were presented. In addition, based on publications, the hypothesis, regarding the involvement of TTR and its aggregated forms in the regulation of coagulation and fibrinolysis processes, was proposed. The imbalance of coagulation and fibrinolysis processes is the basis of the pathogenesis of thrombosis. The hypothesis of TTR involvement in thromboembolic disease is supported by the correlation of abnormal serum TTR levels with the risk of future thromboembolism [85] . The decrease in concentrations of TTR and three protein factors related to coagulation, observed in the pathogenesis of biliary tract cancer (*cholangiocarcinoma*), also indicate a link between TTR and blood coagulation and fibrinolysis [86].

Presented in publications [P_11] to [P_16] newly discovered relationships between the structural stability and the properties and/or activities of TTR allow to understand some of the mechanisms of TTR involvement in pathogenesis processes and explain why TTR functions at the intersection of biomineralization/calcification, blood coagulation/fibrinolysis and the body's immune response. They also explain some puzzling properties of TTR and TTR involvement in the pathogenesis of certain diseases.

4a.5. Summary of achieved results

The result of this original and independent project are the following new observations and discoveries:

1. Ca^{2+} ions in high concentration (both in the presence and absence of other factors) destabilize the structure of the TTR, which leads to the fragmentation of its molecules, formation of disulfide bridge and the precipitation of the TTR by the formation of the subpopulations of unstable conformers. [P_12], [P_13], [P_15] and [P_16]

2. The TTR structure is sensitive to the redox state of the environment. At high concentrations of ROS, TTR undergoes oxidative modifications and cross-linking with formation of dityrosine. TTR binds riboflavin and its photoproducts. These observations have important implications for understanding the pathogenesis of multiple sclerosis and formation of TTR amyloid in UV - exposed tissues. [P_13], [P_15] and [P_16]

3. The process of TTR transition from native to non-native state occurs *in vitro* in the presence of factors such as Ca^{2+}, DTT or ROS by destabilizating small portions (subpopulations) of TTR molecules. [P_11], [P_13], [P_15] and [P_16]

4. A new dynamic visible-light emitting autofluorophore (dbAF) is formed in TTR. The presence of free thiols favors the formation of dbAF. The conditions of formation and the redox state of the environment affect the properties of dbAF. This allows dbAF to be used as a tool to study the aggregation process of TTR. [P_15] and [P_16]

5. TTR shows biomineralization activity and affects the morphology of calcium carbonate crystals by creating a protein-mineral phase and selectively inhibiting crystal growth. There is a feedback between structure stability and biomineralization activity of TTR: the more unstable the structure of the TTR, the stronger the effect of TTR on calcium carbonate crystal morphology. Since Ca^{2+} ions destabilize the TTR structure, a mechanism of a vicious circle is created. [P_12] and [P_13]

6. TTR regulates the autosumoylation and thus the substrate specificity and sumoylation activity of Ubc9 (the only human Sumo conjugating enzyme) which affects the cell's response to stress. The lysine 126 residue in a small subpopulation of TTR molecules is sumoylated. [P_11]

Publications from [P_11] to [P_16] are the first to document the relationships between structure, and activities/properties of TTR, which are influenced by Ca^{2+} and ROS – factors, whose perturbations occur in the pathogenesis of many diseases. This works show the mechanisms of sensitivity of the TTR structure to the pathological increase in Ca^{2+} and oxidative stress and their potential consequences in the diseases involving TTR. Moreover, presented results imply the involvement of TTR in the pathogenesis of diseases, in which the involvement of TTR has not been recognized so far, and whose pathogenesis mechanism is associated with dysregulation of calcium and/or redox state homeostasis. This is of fundamental importance for a precise and complete understanding of both the newly discovered functions/activities (regulation of Ubc9 autosumoylation and the influence on the mineralization process) and the known functions performed by TTR, as well as for the processes of TTR aggregation and amyloid formation. The newly discovered relationships between TTR structure and function also explain why and how TTR links together the processes of mineralization/calcification, blood coagulation/fibrinolysis, and the body's immune response. Such associations or their combinations occur in the pathogenesis of diseases involving TTR. In addition, papers [P_15] and [P_16] document the formation of a new autofluorophore in the TTR as a result of structural changes leading to the formation of TTR amyloid.

4a.6. Future research goals

The results of the presented articles (from [P_11] to [P_16]) document a close relationship between the TTR structure and its properties and functions. The protective properties of TTR demonstrated so far mostly require a native, tetrameric structure. Non-native forms of TTR are predominantly associated with pathological processes leading to numerous diseases. Understanding the exact impact of many/all (?) factors, accompanying the pathogenesis processes, on the TTR structure will allow to answer many important issues, including the question whether the destabilization of the TTR structure is the cause or the effect of the pathogenesis process in a given disease. The knowledge already acquired allows to pose new questions:

- How destabilization of the TTR structure affects the metabolism of the cell?
- How do other factors (lipids) affect the structure of TTR, are they synergistic or antagonistic to Ca^{2+}, DTT or oxidizing agents?
- What is the precise molecular mechanism of destabilization of the TTR structure by Ca^{2+}?
- What is the mechanism by which non-native/oxidized forms of TTR affect eukaryotic cells?
- How does the change in TTR concentration, the presence of its non-native forms or specific (post-translational and/or oxidative) modifications affect the pathogenesis of a given disease?
- Does the biomineralization activity of TTR involve interactions with other proteins?
- What is the molecular mechanism of dbAF formation and what are the structural properties of this fluorophore?
- Does TTR proteolysis affect the fluorescent properties of dbAF?

These questions (and many others) define new research directions regarding the relationship between structure and function of TTR. During the completion of this project, in addition to the presented achievements, other results were obtained, which already provide partial answers. These results have not yet been published as they require further research. Therefore, it is planned to submit a grant application and start new engineering and master's projects. New topics will be implemented sequentially in cooperation with centers with which cooperation has already been established and with other centers, including medical ones. Research will be conducted in the main directions presented below.

- Influence of TTR structure on selected processes of pathogenesis
- Influence of TTR and its non-native forms on nerve cells
- Understanding the properties of dbAF and the molecular mechanism of its formation

- Influence of factors destabilizing TTR structure on formation and properties of amyloid fibrils

For example, current reports indicate that TTR and its abnormal forms are involved in the pathogenesis of multiple sclerosis [62,63] and lung cancer [87]. However, experimental data, concerning the relationship between these diseases and TTR, are scarce and, in the case of non-small cell lung cancer, even contradictory [87–89]. Further research goals will be focused on a detailed understanding of the role of TTR in the onset and/or protection against the development of these diseases. It is planned to establish collaborations with research centers work on the medical aspects of lung cancer and/or multiple sclerosis and to initiate joint research allowing to develop the diagnostic and/or therapeutic tools for the treatment of these diseases.

4b. The second achievement submitted for evaluation

4b.1. Title of the scientific achievement

"Study of the regulation of gene transcription in *Homo sapiens*"

I started research on this subject after obtaining my PhD (the rationale for undertaking research is described in subsection **4.5.1.**) and I conducted it in two foreign institutions:

- ***Institut de Genetique et de Biologie Moleculaire et Cellulaire*, Strasbourg, France (1995-1996)**
- ***Virginia Commonwealth University, Richmond*, Virginia, USA (1997-1999)**

4b.2. List of publications documenting the scientific achievement

The achievements obtained in this research topic are documented in the following publications and my detailed contributions are listed under the title of each article.

[P_4] **Wieczorek, E.;** Brand, M.; Jacq, X.; Tora, L. Function of TAF(II)-containing complex without TBP in transcription by RNA polymerase II. Nature. 1998 May 14;393(6681):187-91. doi: 10.1038/30283
IF_{1998} = 28.833

- *Designing purification procedure and immunoprecipitation conditions, especially elution conditions, of complexes containing TAF30, identification of protein components of complexes by SDS-PAGE and Western blotting analysis, discovery of TBP-free complexes containing TAFs, performing the first in vitro transcription assays, expression and purification of recombinant*

TAF30 in a bacterial system, preparation of complexes containing TAF30 for structural analyses (which were not included in this publication), co-mentoring of PhD student Majorie Brandt.

- *Planning the conditions of individual experiments, interpreting the results and participating in the discussion of the results with the principal investigator (prof. Laszlo Tora).*

[P_5] Izmailova, E.S.; **Wieczorek, E.;** Perkins, E.B.; Zehner, Z.E. A GC-box is required for expression of the human vimentin gene. Gene. 1999 Jul 22;235(1-2):69-75. doi: 10.1016/s0378-1119(99)00209-7
IF_{1999} = 2.258

- *Performing the first transient transfection assays and finding that the GC-box element in promotor region of human vimentin gene is required for vimentin expression.*
- *Preparing nuclear extract from HeLa cells and performing EMSA (Electromobility Shift Assay).*

[EW_6] **Wieczorek, E.**; Lin, Z.; Perkins, E.B.; Law, D.J.; Merchant, J.L.; Zehner, Z.E. The zinc finger repressor, ZBP-89, binds to the silencer element of the human vimentin gene and complexes with the transcriptional activator, Sp1. J Biol Chem. 2000 Apr 28;275(17):12879-88. doi: 10.1074/jbc.275.17.12879
IF_{2000} = 7.368

- *Expression in the bacterial system and purification of recombinant ZBP-89, performing all EMSA assays, performing crosslinking, Southwestern blotting, coimmunoprecipitation and affinity chromatography experiments. Contribution to the identification of two regulatory elements in the human vimentin promoter (performing transient transfection assays).*
- *Planning the conditions of individual experiments, interpreting the results and the discussion of the results with the principal investigator (prof. Zendra Zehner).*
- *Writing the version of the manuscript which was corrected by prof, Zendra Zehner and drawing the figures.*

The cumulative IF of the listed publications, according to JCR, taking into account the year of publication is equal to **38.459**.

4b.3. Description of the results achieved and their significance

The research concerned two separate research projects, which are described in separate subsections, according to the order of their accomplishment.

4b.3.1. Identification of a new type of transcriptional complexes containing TAF30

[P_4] **Wieczorek, E.;** Brand, M.; Jacq, X.; Tora, L. Function of TAF(II)-containing complex without TBP in transcription by RNA polymerase II. Nature. 1998 May 14;393(6681):187-91

Regulation of gene expression is a complex process requiring, in addition to RNA polymerase II, the general transcription factors and proteins that introduce/remove post-translational

modifications, also the gene-specific regulatory proteins such as nuclear receptors, coactivators or repressors. General transcription factors are part of the preinitiation complex that serves to localize RNA polymerase II to the promoter region of the gene and initiate the transcription process. The general transcription factor TFIID is a complex composed of TATA box binding protein (TBP) and TBP-associated proteins (TAFs). As part of the project carried out during the postdoctoral fellowship at IGBMC in Strasbourg (France) in a laboratory headed by prof. Laszlo Tora, from the nuclear extract of HeLa cells, I purified complexes containing TAF30 (which is now known as TAF10) and studied their transcriptional activity. An important achievement was to show that TAF30 was not only bound to TBP, by being a part of TFIID, but was also present in another complex which possessed the transcriptional activity but did not contain TBP. This results suggested a much broader function of TAF proteins than was thought at the time. It is now known that then identified complex, containing TAF30 but lacking TBP, is the human equivalent of the yeast transcription coactivator complex (SAGA). SAGA complexes contain proteins that introduce post-translational modifications (including acetylation) of chromatin histones and associated proteins, which result in, among others, in chromatin remodeling and regulation of transcription processes [90]. The obtained results were very innovative and were published in the journal *Nature* [91] [P_4]. The discovery of transcriptionally active complexes, containing factors (TAFs), which, contrary to their name (TBP Associated Factors) and the postulated functions, were not associated with TBP was so important that an article discussing the results of this work was published in the same issue of *Nature* journal [92]. During my fellowship, I was invited to present my results and give a seminar at IGBMC, which was met with great interest. My work was highly appreciated by prof. Zendra Zehner from VCU Richmond (USA), then staying at IGBMC as part of her sabbatical year. Prof. Zehner invited me to her laboratory to collaborate on a project to identify the repressor of the human vimentin gene. The results of this project are described in the next subsection (**4b.3.2.**).

4b.3.2. Study of regulation of human vimentin gene expression

---

[P_5] Izmailova, E.S.; **Wieczorek, E.;** Perkins, E.B.; Zehner, Z.E. A GC-box is required for expression of the human vimentin gene. Gene. 1999 Jul 22;235(1-2):69-75.

[P_6] **Wieczorek E,** Lin Z, Perkins EB, Law DJ, Merchant JL, Zehner ZE. The zinc finger repressor, ZBP-89, binds to the silencer element of the human vimentin gene and complexes with the transcriptional activator, Sp1. J Biol Chem. 2000 Apr 28;275(17):12879-88.

The project I worked on at VCU, Richmond (USA) in the laboratory headed by prof. Zendra Zehner, was part of the research concerned the regulation of human vimentin gene expression. The aim of my part of this project was to identify and purify the transcription repressor of the vimentin gene. The attempts made previously by other researchers failed as a non-specific DNA binding

protein was identified. At the beginning of the realization of my project, in cooperation with Dr Zhili Lin, I determined the localization of the key regulatory site in the promoter region of the vimentin gene and showed that it contained two regulatory elements located very close to each other; a negative (proximal silencer, PS) and a positive (Δ19). In further studies, I identified the nuclear transcription factor ZBP-89 as a repressor protein for vimentin gene expression and showed in the paper [P_6] that ZBP-89 binds to the PS element [93]. The consequence of this binding was the heterodimerization of ZBP-89 with the general transcription factor SP1 and the formation of a complex with DNA[93] [P_6]. As part of my project, I also performed experiments showing that GC-box element, located in the promoter region of the vimentin gene, close to the transcription start site, binds the general transcription factor SP1, which is necessary for the expression of vimentin [93,94] [P_5], [P_6]. In conclusion, an important contribution of my work was the identification of regulatory elements; PS, Δ19 and GC box as well as two proteins (ZBP-89 repressor and SP1 activator) that bind the regulatory elements of the vimentin gene. It was also important to show that these two proteins, having the opposite activities, heterodimerize in the presence of DNA and cooperate in regulating transcription of one gene. Further studies, performed in other laboratories, confirmed the important role of both proteins, especially ZBP-89, in the complex regulation of vimentin expression/transcription and showed that ZBP-89 recruits the histone deacetylase HDAC1 to the vimentin gene, leading to transcriptional repression [95].

4b.4. Summary of achieved results

- Identification of a new type of transcriptionally active complexes containing TAF30 but do not containing TBP
- Identification of new regulatory elements within the vimentin gene promoter
- Identification and purification of the vimentin gene repressor (ZBP-89)
- Demonstration of the cooperation of the repressor (ZBP-89) and the activator (Sp1) in the regulation of vimentin gene expression

The results obtained in both postdoctoral projects represent a significant achievements in the discipline. Although these results were part of research conducted in the institutions where they were obtained, my participation significantly contributed to their fulfillment. All the key achievements listed in the summary are my original contributions to both projects. Although these results were consulted and extensively discussed with the principal investigators of the projects (prof. Zehner and prof. Tora), within the scope of my research topic, all stages of the work, from the planning and

performance of experiments to the compilation and analysis of results, were the effects of my independent work.

4.5. Description of other scientific accomplishments and obtained achievements that contribute to science

In addition to research carried out in two projects concerning human proteins: 1) research on the structure and novel properties and activities of TTR and 2) research on the regulation of gene transcription in *Homo sapiens*, I also conducted research in two other scientific topics. Both topics concerned the study of insects proteins. The first research topic was devoted to the JHBP - juvenile hormone (JH) binding and transporting protein from the hemolymph of the wax moth *Galleria mellonella*. The second research topic concerned the regulation of gene transcription processes in insects. The subsections bellow successively describe the achievements obtained in both thematic areas related to insect proteins. Their description reflects the evolution of my scientific interest.

4.5.1. Project: ***Study of juvenile hormone binding protein from the hemolymph of the wax moth Galleria mellonella***

My scientific contribution to this topic includes three publications listed below. The first two papers contain the results obtained during my PhD work. The third paper was published after obtaining a PhD degree.

[P_1] Kochman M, **Wieczorek E**. Molecular mechanism of the juvenile hormone action. Acta Biochim Pol. 1991;38(4):393-405. PMID: 1814133.
IF_{1991} = 0.083

[P_2] **Wieczorek E**, Kochman M. Conformational change of the haemolymph juvenile-hormone-binding protein from *Galleria mellonella* (L). Eur J Biochem. 1991 Oct 15;201(2):347-53. doi: 10.1111/j.1432-1033.1991.tb16292.x. PMID: 1935932.
IF_{1991} = 3.171

[P_3] **Wieczorek E**, Parkitna JM, Szkudlarek J, Ożyhar A, Kochman M. Immunoaffinity purification of juvenile hormone-binding protein from *Galleria mellonella* hemolymph. Acta Biochim Pol. 1996;43(4):603-10. PMID: 9104496.
IF_{1996} = 0.321

JH and 20-hydroxyecdysone (20E) are two hormones indispensable for the development and reproduction of insects. Both hormones control many processes related to oogenesis, embryogenesis, morphogenesis, and metamorphosis in insects. JH is bound and transported in the insect blood-

haemolymph by Juvenile Hormone Binding Protein (JHBP). Previous work has shown that in the haemolymph of *Galleria mellonella* , the dominant part of JH pool is bound and transported by low-molecular-weight JHBP of 30 kDa, although the JH-binding ability was also observed for high-molecular-weight proteins [96]. The aim of my MSc thesis was to determine the conditions for the purification of JH-binding lipoprotein from the haemolymph of the wax moth *Galleria mellonella*. I carried out my work under the supervision of prof. Andrzej Ożyhar, who at that time was an assistant at the Department of Biochemistry at the Technical University of Wrocław. During my master's thesis, I isolated lipoprotein fractions of different densities from the haemolymph of *Galleria mellonella*, characterized their protein composition and tested their ability to bind JH. I observed that in HDL fraction that showed JH-binding activity, a low-molecular-weight protein of approximately 30 kDa was present. The obtained results showed that, low-molecular-weight JHBP associates with HDL in the haemolymph of *Galleria mellonella*. This new observation was confirmed in a later report where JHBP from *Galleria mellonella* was shown to interacts with lipoproteins and cell membrane proteins [97].

As part of my PhD thesis carried out under the supervision of prof. Marian Kochman, I studied the influence of JH binding on the structure of low-molecular-weight JHBP. The obtained results showed that JH induces a specific conformational change in JHBP. The conformational change was manifested by an increase in the sedimentation coefficient, a decrease in electrophoretic mobility in non-denaturing conditions and a lower sensitivity of JHBP molecules complexed with JH to protease (subtilisin) digestion. The results of these studies were reported in a paper [P_2] published in the *European Journal of Biochemistry* [98]. The observation of the conformational change of JHBP induced by ligand binding was important because in insects haemolymph the concentration of JH is several orders of magnitude lower than the concentration of JHBP, therefore the entire hormone pool is bound to protein (which is similar to human TTR and thyroid hormones in the blood). Thus, it was hypothesized [96,98] [P_1] and [P_2] that the conformational change of JHBP induced by JH binding have physiological significance. The hypothesis of the functional role of conformational change of JHBP induced by JH was later confirmed for a protein from related species (*Bombyx mori*) in another laboratory. The haemolymph JHBP from *Bombyx mori* was found to undergo a ligand-induced conformational change resulting in the delivery and release of JH to the cell membrane [99]. As part of my work concerning JHBP, in cooperation with graduate student MSc Jan Parkitna Rodriguez, I developed and implemented a new, efficient method for the purification of JHBP from *Galleria mellonella* haemolymph. This method involves size exclusion chromatography and affinity chromatography using monoclonal antibodies directed against JHBP. This procedure has been

published in [P_3] [100] and has been used by many other researchers [101–105] to purify JHBP from *Galleria mellonella* haemolymph.

Summary of achieved results:

- JHBP associates with HDL in the hemolymph of *Galleria mellonella*
- JHBP undergoes a functional conformational change induced by JH binding

An additional achievement was the development of an efficient and rapid procedure for the purification of JHBP from *Galleria mellonella* hemolymph.

The regulation of molecular processes in insects involving JH and 20E is very complex. These hormones, in addition to participating in signaling pathways localized in the cell membrane and/or cytoplasm, exhibit their essential biological activity in the cell nucleus, where they regulate the transcription of many genes [106]. Today, to a much greater extent than before, the complex molecular processes of actions of these hormones and their nuclear receptors, supported by other proteins regulating the process of transcription and chromatin structure, are known. At the time I completed my PhD thesis experiments, the nuclear receptor for 20E (EcR) was known. However, the nuclear receptor for JH was unknown, although one of the candidates proposed in the literature was the ultraspiracle protein (Usp), which heterodimerizes with EcR and has the ability to bind JH [107,108]. Therefore, the mechanisms of the activation of target genes by these hormones, in particular by JH, were unknown. In order to study the JH and 20E signaling pathways, knowledge of techniques for studying transcription processes was essential. For that purpose, in the years 1995-1996 I completed a scientific internship at the IGBMC in Strasbourg in a laboratory headed by prof. Laszlo Tora, where I conducted research concerning the general transcription factor TFIID. Since my work was appreciated by prof. Zendra Zehner from VCU (Richmond, USA) I had the opportunity to work in her laboratory in 1997-1999 on the project of regulation of human vimentin gene expression. Both projects were described in subsection **4b.3.**

4.5.2. Project: *Study of proteins regulating gene transcription in insects*

I conducted research on this subject after returning from fellowships abroad to the Wrocław University of Science and Technology. The results of my work became part of the publications listed below:

[P_7] Nieva C, Gwóźdź T, Dutko-Gwóźdź J, Wiedenmann J, Spindler-Barth M, **Wieczorek E**, Dobrucki J, Duś D, Henrich V, Ożyhar A, Spindler KD. Ultraspiracle promotes the nuclear localization of ecdysteroid receptor in mammalian cells. Biol Chem. 2005 May;386(5):463-70.
IF = 2.577, **MEiN$_{2010}$ = 27**

[P_8] Zoglowek A, Orłowski M, Pakuła S, Dutko-Gwóźdź J, Pajdzik D, Gwóźdź T, Rymarczyk G, **Wieczorek E**, Dobrucki J, Dobryszycki P, Ożyhar A. The composite nature of the interaction between nuclear receptors EcR and DHR38. Biol Chem. 2012 May;393(6):457-71.
IF = 2.683, **MEiN$_{2012}$ = 25**

[P_9] Bielska K, Seliga J, **Wieczorek E**, Kędracka-Krok S, Niedenthal R, Ożyhar A. Alternative sumoylation sites in the Drosophila nuclear receptor Usp. J Steroid Biochem Mol Biol. 2012 Nov;132(3-5):227-38.
IF = 3.984, **MEiN$_{2012}$ = 25**

[P_10] Seliga J, Bielska K, **Wieczorek E**, Orłowski M, Niedenthal R, Ożyhar A. Multidomain sumoylation of the ecdysone receptor (EcR) from Drosophila melanogaster. J Steroid Biochem Mol Biol. 2013 Nov;138:162-73.
IF = 4.049, **MEiN$_{2013}$ = 25**

I started my work with the task of developing an *in vitro* transcription assay that would allow testing the activity of insect nuclear receptors for 20E and JH. For these studies, I prepared probes containing non-mutated and mutated *EcRE* (Ecdysone Regulatory Element) the sequence of which was derived from the promoter region of the *hsp27* gene (*hsp27pal*). I also prepared a number of constructs (in various vectors) that enabled the expression in the bacterial system and purification of EcR and Usp proteins labelled with FLAG and c-Myc tags. I also obtained both proteins in the mammalian system (rabbit reticulocyte lysate). Using the prepared probes and purified recombinant proteins, I performed a series of *in vitro* transcription tests. Unfortunately, the results of the transcriptional activity of the tested recombinant proteins, obtained using the developed *in vitro* assay, did not show statistically significant values that could be functionally relevant. Numerous studies, carried out later in other laboratories, have shown that the nuclear receptor for JH may not be the Usp protein, but the Met protein, and the process of transcriptional regulation of genes under the control of Met and EcR is complex and requires the Tai protein [106]. To date, the identity of the functional receptors for 20E and JH and the mechanisms of regulation of the expression of genes under the control of these two hormones, have not been fully clarified. The role of the GCE protein (a homologue of the Met protein) in the regulation of 20E- and JH-directed transcription is also unclear to this day. The lack of this knowledge was likely the reason for the lack of success in developing an effective *in vitro* transcription assay, that would enable the functional characterization of the insect JH receptor. Therefore, I decided to change the project and continue the research undertaken in my doctoral thesis on the relationship between the structure and function of the protein that binds and transports hormone(s). At the same time, I decided to change the research object from insect to human

protein, in view of the potential significance of the obtained results for therapeutic applications. It is postulated that the insect hormones: 20E and JH are functionally equivalent to the mammalian thyroid hormones [109]. As the object of my research I have chosen TTR, which in complex with RBP seems to be a functional counterpart of insect low-molecular-weight JHBP. JH structurally and functionally corresponds to retinoic acid which, in addition to retinol, is bound by RBP [110]. RBP also binds fatty acids and belongs to the lipocalin family [111], while JHBP has a structural motif specific for proteins transporting lipids and the organization of the JHBP gene is similar to that of the lipocalin gene [101,112]. These similarities suggest that perhaps TTR-RBP complex, which transports thyroid hormones and retinol/retinoids, is a human functional equivalent of JHBP for at least some of the functions performed by insect protein. For this reason, the research concerning human TTR, which was presented in subsection **4a.4.**, is a continuation of research conducted during my MSc thesis and the PhD thesis.

A project dedicated to understanding the relationships between the structure and function of the TTR began with the examination of the potential sumoylation of TTR. The rationale for such an approach is presented in subsection **4a.**, in particular in the part devoted to the regulation of the Ubc9 autosumoylation process by the TTR, which is included in subsection **4a.4.3**. The initiation of these studies required the introduction of a system of sumoylation facilitated by fusion with Ubc9 – the Sumo conjugating enzyme (UFDS) [75]. This system was developed by prof. Rainer Niedenthal, with whom I started collaboration. In addition to the preparation of constructs containing the cDNA of TTR and Ubc9 fusion proteins, I also prepared the control constructs. These constructs eencoded, in fusion with Ubc9, insect nuclear receptors and hormone-binding proteins (EcR, Usp and JHBP), which were highly likely to be substrates for sumoylation. Control experiments gave positive results for all proteins selected for analysis. Detailed studies on the sumoylation of EcR and Usp subsequently became the subject of two PhD theses, and their results were published [P_9] [113]and [P_10] [114]. My participation in this work involved literature studies concerning the UFDS system, setting up the project framework and the preparation of DNA constructs for the analysis of TTR, EcR, Usp and JHBP sumoylation in the UFDS system. I also participated with two PhD students: MSc Katarzyna Bielska, and MSc Justyna Seliga in the implementation of the UFDS system and in the establishment of the tissue culture laboratory in the Department of Biochemistry of the Wrocław University of Science and Technology. Two other publications - [P_7] [115]and [P_8] [116], of which I am a co-author, also fall within the thematic scope of regulation of gene expression in insects. My contribution to their realization was limited and concerned methodological aspects, such as the introduction of Western blotting technique at the Department of Biochemistry of the Wrocław University of Science and Technology.

Summary of achievements with my contribution:

- Detection of sumoylation of EcR, Usp and JHBP proteins involved in hormonal regulation and regulation of gene expression in insects

In addition, the methodological achievement was the introduction of two research techniques: the UFDS system and Western blotting at the Department of Biochemistry of the Wrocław University of Science and Technology.

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

My scientific achievements represent a coherent body of research that has evolved from a master's degree project, through a doctoral project and two international fellowships to a mature, comprehensive project devoted to human TTR. This project was presented as my first scientific achievement and is described in detail in subsection **4a**. Including my doctoral project, each of the stages of my work is documented by publications in the recognized international scientific journals. During my scientific work, I conducted research and obtained significant achievements both at my home institution, which is the Wrocław University of Science and Technology, and at two international institutions: IGBMC in Strasbourg (France) and VCU, in Richmond (USA). Especially, the former is known as the leading center of scientific research in the world. The outcome of my work in international research centers were three scientific articles with my participation, which appeared in the *Nature, JBC* and *Gene* journals. The results of each of these papers, which form the basis of the second achievement submitted for evaluation, are discussed in more detail in subsection **4b**. They represent achievements in the discipline and significantly contributed to the developments of projects implemented it the institutions where they were carried out, which was confirmed by the opinions of the group leaders (**Attachment 5**).

5.1. Summary of scientific achievements

The overall scope of my research, in a broad sense, concerns the study of the protein structure and function, but in particular focuses at their interconnections. Although the objects of my research work were different, the functions of the proteins I worked on were related to the transport of hormones or the regulation of gene expression. In my scientific career, I have completed two research projects concerning human proteins and two projects concerning insect proteins. These projects were conducted in the following chronological order:

- **Study of JHBP from the hemolymph of the wax moth *Galleria mellonella*.** The project was carried out as part of my master's and doctoral theses at the Wrocław University of Science and Technology. The greatest achievement of this project is the discovery of the conformational change that occurs in JHBP upon JH binding.

  This project resulted in **3** publications with a total **IF** of **3.575**.

- **Study of the regulation of gene transcription in *Homo sapiens* (the second achievement)** was carried out after obtaining a doctoral degree at two international institutions, IGBMC in France and VCU in the USA. The most important discovery was to show the existence of a transcriptionally active complex without TBP, but with TBP-associated proteins (TAFs). An important achievement was also the identification of a repressor and activator of the human vimentin gene.

  This project resulted in **3** publications with a total **IF** of **38.453**.

- **Study of proteins regulating gene transcription in insects** was carried out at the Wrocław University of Science and Technology. The most important achievement with my participation was the detection of sumoylation of EcR, USP and JHBP proteins. The result of my work was also the establishment of tissue culture laboratory and the implementation of two experimental techniques at the Department of Biochemistry of the Wrocław University of Science and Technology.

  This project resulted in **4** publications with a total **IF** of **13.263**.

- **Study on the relationship between the structure and function/activity of human TTR (the first achievement)** constitute my original, comprehensive research project carried out at the Wrocław University of Science and Technology, partially conducted in cooperation with polish and foreign centers (as is described in section **5.2.**). The greatest achievements of this project are the following observations/discoveries:

1. Destabilization of the TTR structure evoked by factors related to pathogenesis: Ca^{2+}, ROS and low molecular weight thiols (DTT, GSH)
2. Discovery of a new autofluorophore (dbAF) associated with structural changes in TTR
3. Demonstration of the biomineralization activity of TTR
4. Demonstration of the effect of TTR on the autosumoylation of the Sumo conjugating enzyme (Ubc9) and detection of the sumoylation of lysine residue 126 of the TTR

This project resulted in **6** publications with total **IF** of **40.731**.

In total, my scientific achievements include **16** articles published in peer-reviewed journals, of which **14** articles were published after receiving my PhD degree. I am the first author of **10** papers, including all **6** papers constituting a thematically related series of articles concerning TTR (first achievement). and **2** papers concerning the regulation of gene transcription in *Homo sapiens* (second achievement). I am the corresponding author of all **6** papers composing a series of articles concerning TTR. My participation in the realization of the majority of work constituting my scientific achievements is comprehensive because it involves all stages of work, including literature studies, designing an experimental approach, performing the vast majority of experiments, as well as the data processing, analysis and interpretation of the results and their discussion based on literature studies.

The total IF for all publications, according to the **JCR** list, considering the year of publication, is **96.058**.

The total number of points according to the lists of **MNiSW/MEiN** for **10** publications that appeared after the year 2000, according to the year of a given publication, is **717**, and refers to post-doctoral works.

As of 21.08.2023 the total number of citations of my publications according to the **Web of Science** database is **427 (411** excluding self-citations) and according to the **Scopus** database is equal to **452** (**436** excluding self-citations).

The **Hirsch index** of my works, according to the **Web of Science** and **Scopus** databases, is equal to **9**.

Full scientometric data are provided in **Attachment 4.**

5.2. Scientific collaboration with other research institutions

The realization of my research projects involved cooperation with other research centers, including the following three international institutions:

- *Institut de Genetique et de Biologie Moleculaire et Cellulaire (IGBMC),* Strasbourg, France
- *Virginia Commonwealth University*, Richmond, Virginia, USA
- *Hannover Medical School*, Federal Republic of Germany

Of particular note is the Institute of Genetics and Molecular and Cellular Biology in Strasbourg, which is one of the leading biomedical research centers in Europe. It is a scientific center affiliated with the CNRS (National Center for Scientific Research, *Centre national de la recherche scientifique*), INSERM (National Institute for Health and Medical Research*, L'Institut national de la santé et de la recherche médicale*) and the University of Strasbourg. At IGBMC most scientific projects are carried out in international research groups. In addition to cooperation with foreign centers, I have also established cooperation with recognized scientific centers in Poland. Within the scope of cooperation, numerous personal scientific meetings took place in Poland, the Federal Republic of Germany, France and the USA.

Below I have listed all the centers with which I cooperated with a short description of the scope of the cooperation.

- ***Institut de Genetique et de Biologie Moleculaire et Cellulaire,* Strasbourg, France**
  The collaboration included a post-doctoral fellowship in the years 1995-1996 in the team of prof. Laszlo Tora. As part of the cooperation, HeLa cells were obtained for the tissue culture laboratory of the Department of Biochemistry, Wrocław University of Science and Technology. The results obtained as part of this cooperation were also presented at the VII workshop "Transcription Regulation in Eukaryotes" in Strasbourg in 1998.

- ***Virginia Commonwealth University*, Richmond, Virginia, USA**
  This collaboration involved a postdoctoral fellowship which I completed in the years 1997 – 1999 and a short, three-month-stay in a team led by prof. Zendra Zehner.

- ***Hannover Medical School*, Hannover, Federal Republic of Germany**
  The cooperation was initiated in 2007 and concerned the implementation of the UFDS system at the Department of Biochemistry of the Wrocław University of Science and Technology. As part of the collaboration, in a laboratory of Dr. Rainer Niedenthal, preliminary experiments were performed on sumoylation of the TTR, EcR, USP and JHBP proteins.

- ***Department of Biochemistry, Biophysics and Biotechnology*. Jagiellonian University, Krakow, Poland**
  Long-term collaboration with Dr. Sylwia-Kędracka-Krok was initiated in 2013. The collaboration concerned the calorimetric and mass spectrometry analyses of TTR.

- ***Institute of Low Temperatures and Structural Research*, Polish Academy of Sciences. Wrocław, Poland**
  The collaboration with Dr. Maciej Ptak concerned FTIR analysis of TTR samples. It was initiated in 2017.

- ***Electron Microscopy Laboratory*, University of Life Sciences, Wrocław, Poland**
  Collaboration with Dr. Krzysztof Marycz concerned the analysis of the morphology and elemental composition of samples obtained in TTR biomineralization assays using SEM and EDS techniques. The research was conducted under the KNOW grant obtained in 2016.

5.3. Conference presentations

In addition to the publication of the results of my work in international scientific journals, part of my contacts with the international scientific community was also the participation in international conferences where I presented my achievements in graphic or oral form.

The full list of conference reports is included in **Attachment 4**. Here, I would like to highlight two conference presentations:

Wieczorek E. and Kochman M. (1990) The effect of juvenile hormone on molecular properties of juvenile hormone binding protein from the haemolymph of *Galleria mellonella (L)*. Presented at the 15th Conference of European Comparative Endocrinologists, Leuven, Belgium. Abstracts published in Gen. Comp. Endocrinol. 82(2).

Wieczorek E. Kędracka-Krok S., Jankowska U., Bystranowska D. i Ożyhar A "Transthyretin binds riboflavin photoproducts and undergoes structural changes upon irradiation" Poster at the Gordon Research Conference on Thiol based Redox regulation and Signalling, July 15th, 2018, Barcelona, Spain

At the 15th Conference of European Comparative Endocrinologists in Leuven (Belgium) I presented in an oral report, the data documenting the conformational change of JHBP which occurred upon JH binding. The JH-induced change of JHBP structure was the first such report on the hemolymph JH-binding protein in insects, and my presentation was met with great interest.

The second report was presented as a poster at the prestigious Gordon International Conference on Redox Regulation and Signaling involving Thiol Groups. The conference was held near Barcelona (Spain) and gathered a group of scientists whose research work is dedicated to the broadly understood oxidative and reducing processes occurring with the participation of thiol groups in living organisms. A series of excellent lectures, conference speeches, posters and personal conversations allowed not only to present my research but also to complete the knowledge, which later led to new interesting results on the effect of oxidizing and reducing conditions on the structure and properties of TTR. These results were presented in two papers (described in subsections **4a.4.1.** and **4a.4.2.**), one of which was published in the journal *Redox Biology* (**IF 10.787**).

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

---

6.1. Teaching activities

6.1.1. Courses taught

- Since 1985 (excluding the periods of my fellowships), I have been teaching practical courses on “**Biochemistry**” and/or “**Enzymology**” every year.
- Since 2005 I have been teaching the original course “**Tissue Culture**” every year.
- Since 2012 I have been teaching laboratory courses: “**Genetic engineering in analytics and diagnostics**” and “**Modern research methods and tools in Biochemistry and Molecular biology**”.
- From 2019 I give two-hour lecture "**Fluorescence, diamonds and coronavirus**" as part of the course “**Trends in Biotechnology Development**” twice a year.

6.1.2. New courses introduced

I designed, prepared and implemented the following courses for students of Biotechnology at the Wrocław University of Science and Technology:

- Author’s course "**Tissue culture**" (mandatory course), which includes a series of 15 lectures (30 hours) and a series of 15 two-hour seminars. As part of the course a database of questions and an electronic form of students examination were also developed.
- Exercise "**Haplotyping of human mitochondrial DNA**" within the course: "**Genetic engineering in analytics and diagnostics**"
- Exercise "**The use of denaturing electrophoresis (SDS-PAGE) and Western blotting technique for protein detection and identification**" as part of the POWER course "**Modern research methods and tools in biochemistry and molecular biology**"
- Two-hour lecture "**Fluorescence, diamonds and coronavirus**" as part of the course “**Trends in Biotechnology Development**”

6.1.3. New courses developed

I have also prepared two courses that are currently awaiting implementation:

- Course: "**Tissue culture**" – laboratory, for students of Biotechnology (together with Dr. Katarzyna Sołtys)
- Course: " **Tissue culture**" – practical exercises, for students of the newly created Faculty of Medicine at the Wrocław University of Science and Technology

6.1.4. Master’s and engineering projects

I supervised a total of **10** master's degree projects and **14** engineering projects and also participated in **2** students projects during my research stays in France and USA. One of the students who worked under my supervision (MSc Marjorie Brandt) is the co-author of a publication [P_4]

resulting from my internship in Strasbourg. I also conducted **4** research projects under the Erasmus program with students from different countries. All the student projects were designed to contribute, at least in part, to the realization of the major research project or to explore alternative experimental approaches. The results of the master's degree projects of students whose names and surnames are marked in blue contributed directly to the publications and the students became co-authors of these papers. The work of students whose names and surnames are marked in green indirectly contributed to the mail research project (e.g. by participating in the preparation of DNA constructs or the development of a protein purification procedure).

Master's projects

1. *Development of an in vitro transcription assay for the study of ecdysteroid receptor activity.* Grażyna Andruszewska (2003)
2. *Preparation of an expression system for EcR and Usp proteins labeled with peptides recognized by antibodies.* Agnieszka Słaba (2005)
3. *Designing and obtaining DNA constructs allowing investigation of TTR phosphorylation in vitro.* Magdalena Kozlowska (2012)
4. *Obtaining the TTR mutants which allow investigation of the influence of phosphorylation on TTR sumoylation in eukaryotic cells.* Malgorzata Kaminska (2012)
5. *Obtaining DNA constructs of TTR mutants and elaborating the preliminary purification conditions of GST-TTR-His protein for investigating TTR sumoylation in in vivo and in vitro systems.* Magdalena Jaskot (2013)
6. *Obtaining DNA constructs of TTR mutants and elaborating the preliminary purification conditions of Nus-TTR-His protein for investigating TTR sumoylation in in vivo and in vitro systems.* Dagmara Sagan/Hryckiewicz (2013)
7. *Study of the effect of calcium ions on the properties of the recombinant transthyretin. Part 2* Karolina Kościk (2017)
8. *Investigation of the effect of human transthyretin on calcium carbonate mineralisation.* Anna Chitruń (2018)
9. *The investigation of changes of human transthyretin induced by UV-VIS irradiation in the presence of riboflavin.* Zofia Wygralak (2019)
10. *The investigation of the stability of human transthyretin in the reducing conditions.* Patrycja Bezara (2021)

Engineering projects

1. *The investigation of Toll-Like Receptor function in elucidation of the Systemic Inflammatory Response Syndrome.* Magdalena Kozlowska (2011)

2. *Explaining the function of transcription factor NF-kB in etiology of allergy.* Malgorzata Kaminska (2011)
3. *Preparation of DNA construct coding mutant fusion protein: transthyretin - Ubc9.* Magdalena Jaskot (2012)
4. *Preparation of DNA construct coding mutant fusion protein: Ubc9 – transtyretin.* Dagmara Sagan (2014)
5. *The application of microarray techniques to biomarker analysis.* Ewa Szymków (2012)
6. *Optimization of the purification procedure of the recombinant transthyretin after expression of Nus_TTR_His in Escherichia coli.* Aneta Peszko (2015)
7. *Study of the effect of calcium ions on the properties of recombinant transthyretin obtained after expression of GST_TTR in Escherichia coli.* Agata Wierzbinska (2015)
8. *Sumoylation of proteins involved in neurodegenerative diseases.* Blaszczyk Maria (2015)
9. *The preparation of DNA construct whicj allows the expression of human transthyretin, with primary structure containing no extra amino acids at the C-terminus, in a bacterial system.* Piotr Dindorf (2017)
10. *The influence of the serum proteins on the process of biomineralization.* Zofia Wygralak (2018)
11. *The detection methods of proteins fluorescence in microfluidics.* Paulina Powilan (2019)
12. *Cellular mechanisms of biomineralization processes in humans.* Patryk Cierpisz (2019)
13. *Posttranslational modifications of human transthyretin.* Katarzyna Kolodzinska (2020)
14. *Three-dimensional cell cultures as the research models of neurodegenerative diseases.* Gabriela Chrobot (2020)

6.2. Popularization and organizational activities

- I participated in the Vth Student School of Biotechnology, Szklarska Poręba, 1992, where I gave a lecture: "Juvenile hormone: molecular mechanism of action".
- In the years 2003-2004 I was a study advisor at the Department of Chemistry at the Wrocław University of Science and Technology.
- Since 2006, I have been the head of seminars at the Department of Biochemistry (currently Department of Biochemistry, Molecular Biology and Biotechnology) at the Wrocław University of Science and Technology.
- I participated in the establishment and since 2006 I have been involved in running the tissue culture laboratory at the Department of Biochemistry, Molecular Biology and Biotechnology of the Wrocław University of Science and Technology.
- In 2012, I conducted a training on the principles of running a tissue culture laboratory in the Wrocław Biotechnology Park.
- In 2017, I obtained funds (KNOW grant) for computer equipment for students carrying out research projects at the Department of Biochemistry, Molecular Biology and Biotechnology of the Wrocław University of Science and Technology.

7. Additional information concerning professional career

7.1. Received awards and distinctions

- Award of Włodzimierz Mozołowski for the poster at the XXV conference of Polish Biochemical Society, 1989, Toruń Poland
- Award of the Rector of the Wrocław University of Science and Technology for outstanding performance of professional duties, December 1992
- Award of the Rector of Wrocław University of Science and Technology in recognition of outstanding contribution to the university, 2014
- National Decoration; Gold Medal for Long Service, 2020

8. Bibliography

1. Buxbaum, J. N. & Reixach, N. Transthyretin: The servant of many masters. *Cellular and Molecular Life Sciences* vol. 66 3095–3101 Preprint at https://doi.org/10.1007/s00018-009-0109-0 (2009).

2. Zawiślak, A., Jakimowicz, P., McCubrey, J. A. & Rakus, D. Neuron-derived transthyretin modulates astrocytic glycolysis in hormone-independent manner. *Oncotarget* **8**, 106625–106638 (2017).

3. Refai, E. *et al.* Transthyretin constitutes a functional component in pancreatic β-cell stimulus-secretion coupling. *Proc Natl Acad Sci U S A* **102**, 17020–17025 (2005).

4. Monk, J. A. *et al.* Delayed development of specific thyroid hormone-regulated events in transthyretin null mice. *American Journal of Physiology-Endocrinology and Metabolism* **304**, E23–E31 (2013).

5. Episkopou, V. *et al.* Disruption of the transthyretin gene results in mice with depressed levels of plasma retinol and thyroid hormone. *Proceedings of the National Academy of Sciences* **90**, 2375–2379 (1993).

6. Rabah, S. A., Gowan, I. L., Pagnin, M. & Osman, N. Thyroid Hormone Distributor Proteins During Development in Vertebrates. *Frontiers in Edocrimology* **10**, 1–8 (2019).

7. Palha, J. A. *et al.* Transthyretin is not essential for thyroxine to reach the brain and other tissues in transthyretin-null mice. *American Journal of Physiology-Endocrinology and Metabolism* **272**, E485–E493 (1997).

8. Schreiber, G. *et al.* Thyroxine transport from blood to brain via transthyretin synthesis in choroid plexus. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **258**, R338–R345 (1990).

9. Monaco, H.J., Rizzi, M., Coda, A. Structure of a complex of two plasma proteins: transthyretin and retinol-binding protein. *Science (1979)* **268**, 1039–1041 (1995).

10. Sousa, M. M., Yan, S. du, Stern, D. & Saraiva, M. J. Interaction of the receptor for advanced glycation end products (RAGE) with transthyretin triggers nuclear transcription factor kB (NF-kB) activation. *Laboratory Investigation* **80**, 1101–1110 (2000).

11. Smeland, S., Kolset, S. O., Lyon, M., Norum, K. R. & Blomhoff, R. Binding of perlecan to transthyretin in vitro. *Biochemical Journal* **326**, 829–836 (1997).

12. Sousa, M. M., Berglund, L. & Saraiva, M. J. Transthyretin in high density lipoproteins: Association with apolipoprotein A-I. *J Lipid Res* **41**, 58–65 (2000).

13. Gomes, J. R. *et al.* Transthyretin provides trophic support via megalin by promoting neurite outgrowth and neuroprotection in cerebral ischemia. *Cell Death Differ* **23**, 1749–1764 (2016).

14. Schwarzman, A. L. *et al.* Transthyretin sequesters amyloid β protein and prevents amyloid formation. *Proc Natl Acad Sci U S A* **91**, 8368–8372 (1994).

15. Santos, S. D. *et al.* CSF transthyretin neuroprotection in a mouse model of brain ischemia. *J Neurochem* **115**, 1434–1444 (2010).

16. Su, Y. *et al.* Novel function of transthyretin in pancreatic alpha cells. *FEBS Lett* **586**, 4215–4222 (2012).

17. Sanguinetti, C. *et al.* The Journey of Human Transthyretin: Synthesis, Structure Stability, and Catabolism. *Biomedicines* vol. 10 Preprint at https://doi.org/10.3390/biomedicines10081906 (2022).

18. Shao, J. *et al.* A novel transthyretin/STAT4/miR-223-3p/FBXW7 signaling pathway affects neovascularization in diabetic retinopathy. *Mol Cell Endocrinol* **498**, (2019).

19. Magalhães, J., Eira, J. & Liz, M. A. The role of transthyretin in cell biology: impact on human pathophysiology. *Cellular and Molecular Life Sciences* vol. 78 6105–6117 Preprint at https://doi.org/10.1007/s00018-021-03899-3 (2021).

20. Shao, J., Zhang, Y., Fan, G., Xin, Y. & Yao, Y. Transcriptome analysis identified a novel 3-LncRNA regulatory network of transthyretin attenuating glucose induced hRECs dysfunction in diabetic retinopathy. *BMC Med Genomics* **12**, 1–18 (2019).

21. Chu, Y.-P. *et al.* Transthyretin attenuates TDP-43 proteinopathy by 1 autophagy activation via ATF4 in FTLD-TDP. *Brain* (2022) doi:10.1093/brain/awac412/6820967.

22. Blake, C.C.F, Burridge, J.M. and Oatley, S. J. X-Ray Analysis of Thyroid Hormone Binding to Prealbumin. *Biochem Soc Trans* **6**, 1114–1118 (1978).

23. Duarte, A. G. *et al.* A Brief Journey Through Protein misfolding in transthyretin amyloidosis (ATTR amyloidosis). *Int. J. Mol. Sci* **22**, (2021).

24. Yokoyama, T. *et al.* Hydrogen-bond network and pH sensitivity in transthyretin: Neutron crystal structure of human transthyretin. *J Struct Biol* **177**, 283–290 (2012).

25. Ferguson, R.F. Edelhoch, H.Saroff, H., A. Robins, J. Cahnmann, H. J. Negative cooperativity in the binding of thyroxine to human serum prealbumin. *Biochemistry* **14**, 282–289 (1875).

26. Gales, L., Saraiva, M. J. & Damas, A. M. Structural basis for the protective role of sulfite against transthyretin amyloid formation ☆. *Biochimica at Biophysica Acta* **1774**, 59–64 (2007).

27. HAGEN, G. A. & ELLIOTT, W. J. Transport of Thyroid Hormones in Serum and Cerebrospinal Fluid1. *J Clin Endocrinol Metab* **37**, 415–422 (1973).

28. Liz, M. A., Mar, F. M., Franquinho, F. & Sousa, M. M. Aboard transthyretin: From transport to cleavage. *IUBMB Life* **62**, 429–435 (2010).

29. Saponaro, F., Kim, J. H. & Chiellini, G. Transthyretin stabilization: An emerging strategy for the treatment of alzheimer's disease? *Int J Mol Sci* **21**, 1–13 (2020).

30. Morais-de-Sá, E., Pereira, P. J. B., Saraiva, M. J. & Damas, A. M. The crystal structure of transthyretin in complex with diethylstilbestrol: A promising template for the design of amyloid inhibitors. *Journal of Biological Chemistry* **279**, 53483–53490 (2004).

31. Xia, K. *et al.* Quantifying the kinetic stability of hyperstable proteins via time-dependent SDS trapping. *Biochemistry* **51**, 100–107 (2012).

32. Lim, K. H., Dyson, H. J., Kelly, J. W. & Wright, P. E. Localized structural fluctuations promote amyloidogenic conformations in transthyretin. *J Mol Biol* **425**, 977–988 (2013).

33. Brito, R., Damas, A. & Saraiva, M. Amyloid Formation by Transthyretin: From Protein Stability to Protein Aggregation. *Current Medicinal Chemistry-Immunology, Endocrine & Metabolic Agents* **3**, 349–360 (2005).

34. Pires, R. H., Karsai, Á., Saraiva, M. J., Damas, A. M. & Kellermayer, M. S. Z. Distinct Annular Oligomers Captured along the Assembly and Disassembly Pathways of Transthyretin Amyloid Protofibrils. *PLoS One* **7**, (2012).

35. Wieczorek, E. & Ożyhar, A. Transthyretin: From structural stability to osteoarticular and cardiovascular diseases. *Cells* **10**, (2021).

36. Park, G. Y., Jamerlan, A., Shim, K. H. & An, S. S. A. Diagnostic and treatment approaches involving transthyretin in amyloidogenic diseases. *Int J Mol Sci* **20**, (2019).

37. Gião, T. *et al.* Undiscovered roles for transthyretin: From a transporter protein to a new therapeutic target for Alzheimer's disease. *Int J Mol Sci* **21**, (2020).

38. Bergström, J. *et al.* Amyloid deposits in transthyretin-derived amyloidosis: Cleaved transthyretin is associated with distinct amyloid morphology. *Journal of Pathology* **206**, 224–232 (2005).

39. Mangione, P. P. *et al.* Plasminogen activation triggers transthyretin amyloidogenesis in vitro. *Journal of Biological Chemistry* **293**, 14192–14199 (2018).

40. Poltash, M. L. *et al.* New insights into the metal-induced oxidative degradation pathways of transthyretin. *Chemical Communications* **55**, 4091–4094 (2019).

41. Wieczorek, E. *et al.* Destabilisation of the structure of transthyretin is driven by Ca2+. *Int J Biol Macromol* **166**, 409–423 (2021).

42. Liz, M. A., Faro, C. J., Saraiva, M. J. & Sousa, M. M. Transthyretin, a new cryptic protease. *Journal of Biological Chemistry* **279**, 21431–21438 (2004).

43. Liz, M. A. *et al.* Transthyretin is a metallopeptidase with an inducible active site. *Biochemical Journal* **443**, 769–778 (2012).

44. Squier, T. C. Oxidative stress and protein aggregation during biological aging. *Exp Gerontol* **36**, 1539–1550 (2001).

45. Gupta, R. *et al.* Post-translational modifications: Regulators of neurodegenerative proteinopathies. *Ageing Research Reviews* vol. 68 Preprint at https://doi.org/10.1016/j.arr.2021.101336 (2021).

46. Shinbo, Y. *et al.* Proper SUMO-1 conjugation is essential to DJ-1 to exert its full activities. *Cell Death Differ* **13**, 96–108 (2006).

47. Oka, S. *et al.* Human mitochondrial transcriptional factor A breaks the mitochondria-mediated vicious cycle in Alzheimer's disease. *Sci Rep* **6**, (2016).

48. Coelho-Silva, L., Stephens, G. J. & Cimarosti, H. SUMOylation and calcium signalling: potential roles in the brain and beyond. *Neuronal Signal* **1**, (2017).

49. Carafoli, E. & Krebs, J. Why calcium? How calcium became the best communicator. *Journal of Biological Chemistry* **291**, 20849–20857 (2016).

50. Kumar, A., Bodhinathan, K. & Foster, T. C. Susceptibility to calcium dysregulation during brain aging. *Front Aging Neurosci* **1**, 1–13 (2009).

51. Hou, X. *et al.* Transthyretin oligomers induce calcium influx via voltage-gated calcium channels. *J Neurochem* **100**, 446–457 (2007).

52. Cantarutti, C. *et al.* Calcium Binds to Transthyretin with Low Affinity. *Biomolecules* **12**, (2022).

53. Wieczorek, E., Bezara, P. & Ożyhar, A. Deep blue autofluorescence reveals the instability of human transthyretin. *Int J Biol Macromol* **191**, 492–499 (2021).

54. Wieczorek, E. *et al.* Deep blue autofluorescence reflects the oxidation state of human transthyretin. *Redox Biol* **56**, (2022).

55. Balasco, N. *et al.* A Comprehensive Analysis of the Intrinsic Visible Fluorescence Emitted by Peptide/Protein Amyloid-like Assemblies. *International Journal of Molecular Sciences* vol. 24 Preprint at https://doi.org/10.3390/ijms24098372 (2023).

56. Pauling, L. & Corey, R. B. THE PLEATED SHEET, A NEW LAYER CONFIGURATION OF POL YPEPTIDE CHAINS. *PNAS* **37**, (1951).

57. The role of α-sheet structure in amyloidogenesis: characterization and implications. *Open Biol* **12**, 1–14 (2022).

58. Niyangoda, C., Miti, T., Breydo, L., Uversky, V. & Muschol, M. Carbonyl-based blue autofluorescence of proteins and amino acids. 1–15 (2017) doi:10.1371/journal.pone.0176983.

59. Chan, F. T. S., Pinotsi, D., Kaminski Schierle, G. S. & Kaminski, C. F. Structure-Specific Intrinsic Fluorescence of Protein Amyloids Used to Study their Kinetics of Aggregation. *Bio-nanoimaging: Protein Misfolding and Aggregation* 147–155 (2013) doi:10.1016/B978-0-12-394431-3.00013-4.

60. Grelich-Mucha, M. *et al.* Autofluorescence of Amyloids Determined by Enantiomeric Composition of Peptides. *Journal of Physical Chemistry B* **125**, 5502–5510 (2021).

61. Gales, L. *et al.* X-ray absorption spectroscopy reveals a substantial increase of sulfur oxidation in transthyretin (TTR) upon fibrillization. *Journal of Biological Chemistry* **278**, 11654–11660 (2003).

62. Salazar, I. L. *et al.* Posttranslational modifications of proteins are key features in the identification of CSF biomarkers of multiple sclerosis. *J Neuroinflammation* **19**, (2022).

63. Pieragostino, D. *et al.* Oxidative modifications of cerebral transthyretin are associated with multiple sclerosis. *Proteomics* **13**, 1002–1009 (2013).

64. Alshehri, B., Pagnin, M., Lee, J. Y., Petratos, S. & Richardson, S. J. The Role of Transthyretin in Oligodendrocyte Development. *Sci Rep* **10**, (2020).

65. Si, J.-B., Kim, B. & Kim, J. H. Transthyretin Misfolding, A Fatal Structural Pathogenesis Mechanism. *Int J Mol Sci* **22**, 4429 (2021).

66. Minoia, A., Dalle Carbonare, L., Schwamborn, J. C., Bolognin, S. & Valenti, M. T. Bone Tissue and the Nervous System: What Do They Have in Common? *Cells* vol. 12 Preprint at https://doi.org/10.3390/cells12010051 (2023).

67. Wieczorek, E., Chitruń, A. & Ożyhar, A. Destabilised human transthyretin shapes the morphology of calcium carbonate crystals. *Biochim Biophys Acta Gen Subj* **1863**, 313–324 (2019).

68. Westermark, P., Westermark, G. T., Suhr, O. B. & Berg, S. Transthyretin-derived amyloidosis: Probably a common cause of lumbar spinal stenosis. *Ups J Med Sci* **119**, 223–228 (2014).

69. Nunes, A. F. *et al.* Neuropeptide y expression and function during osteoblast differentiation - Insights from transthyretin knockout mice. *FEBS Journal* **277**, 263–275 (2010).

70. Rose-Martel, M., Smiley, S. & Hincke, M. T. Novel identification of matrix proteins involved in calcitic biomineralization. *J Proteomics* **116**, 81–96 (2015).

71. Hörnberg, A., Hultdin, U. W., Olofsson, A. & Sauer-Eriksson, A. E. The effect of iodide and chloride on transthyretin structure and stability. *Biochemistry* **44**, 9290–9299 (2005).

72. He, J., Cheng, J. & Wang, T. SUMOylation-mediated response to mitochondrial stress. *International Journal of Molecular Sciences* vol. 21 1–15 Preprint at https://doi.org/10.3390/ijms21165657 (2020).

73. Mukherjee, D., Chander, V. & Bandyopadhyay, A. PARIS-DJ-1 Interaction Regulates Mitochondrial Functions in Cardiomyocytes, Which Is Critically Important in Cardiac Hypertrophy. (2020) doi:10.1128/MCB.

74. Acuña, M. L. *et al.* Alternative splicing of the SUMO1/2/3 transcripts affects cellular SUMOylation and produces functionally distinct SUMO protein isoforms. *Sci Rep* **13**, 2309 (2023).

75. Jakobs, A. *et al.* Ubc9 fusion-directed SUMOylation identifies constitutive and inducible SUMOylation. *Nucleic Acids Res* **35**, (2007).

76. Wieczorek, E. *et al.* Is transthyretin a regulator of Ubc9 SUMOylation? *PLoS One* **11**, (2016).

77. Knipscheer, P. *et al.* Ubc9 Sumoylation Regulates SUMO Target Discrimination. *Mol Cell* **31**, 371–382 (2008).

78. Uggè, M., Simoni, M., Fracassi, C. & Bernardi, R. PML isoforms: a molecular basis for PML pleiotropic functions. *Trends in Biochemical Sciences* vol. 47 609–619 Preprint at https://doi.org/10.1016/j.tibs.2022.02.002 (2022).

79. Yang, W., Sheng, H., Warner, D. S. & Paschen, W. Transient global cerebral ischemia induces a massive increase in protein sumoylation. *J Cereb Blood Flow Metab* **28**, 269–79 (2008).

80. Ahmad, Y. *et al.* An Insight into the Changes in Human Plasma Proteome on Adaptation to Hypobaric Hypoxia. *PLoS One* **8**, e67548 (2013).

81. Sharma, M., Khan, S. & Rahman, S. The Extracellular Protein , Transthyretin Is an Oxidative Stress Biomarker. **10**, 1–8 (2019).

82. Bence, N. F., Sampat, R. M. & Kopito, R. R. Impairment of the Ubiquitin-Proteasome System by Protein Aggregation. *Science (1979)* **292**, 1552–1555 (2001).

83. Gomes, R. A. *et al.* The Proteome Response to Amyloid Protein Expression In Vivo. *PLoS One* **7**, e50123 (2012).

84. Sartiani, L. *et al.* Biochemical and Electrophysiological Modification of Amyloid Transthyretin on Cardiomyocytes. *Biophys J* **111**, 2024–2038 (2016).

85. Jensen, S. B. *et al.* Discovery of novel plasma biomarkers for future incident venous thromboembolism by untargeted synchronous precursor selection mass spectrometry proteomics. *Journal of Thrombosis and Haemostasis* **16**, 1763–1774 (2018).

86. Wang, J., Zhu, W., Tu, J. & Zheng, Y. Identification and Validation of Novel Biomarkers and Potential Targeted Drugs in Cholangiocarcinoma: Bioinformatics, Virtual Screening, and Biological Evaluation. *J Microbiol Biotechnol* **32**, 1262–1274 (2022).

87. Shimura, T. *et al.* Prognostic impact of serum transthyretin in patients with non-small cell lung cancer. *Mol Clin Oncol* (2019) doi:10.3892/mco.2019.1837.

88. Wang, D.-B. *et al.* Transthyretin (TTR) Suppressed Tumor Progression in Non-Small Cell Lung Cancer by Inactivating MAPK/ERK Pathway. (2020) doi:10.21203/rs.3.rs-38779/v1.

89. Lee, C.-C. *et al.* Transthyretin Stimulates Tumor Growth through Regulation of Tumor, Immune, and Endothelial Cells. *The Journal of Immunology* **202**, 991–1002 (2019).

90. Bardot, P. *et al.* The TAF10-containing TFIID and SAGA transcriptional complexes are dispensable for early somitogenesis in the mouse embryo. *Development* **144**, (2017).

91. Wieczorek, E., Brand, M., Jacq, X. & Tora, L. Function of TAF(II)-containing complex without TBP in transcription by RNA polymerase II. *Nature* **393**, 187–191 (1998).

92. Apone, L. M. & Green, M. R. Transcription sans TBP. *Nature* **393**, 114–115 (1998).

93. Wieczorek, E. *et al.* The zinc finger repressor, ZBP-89, binds to the silencer element of the human vimentin gene and complexes with the transcriptional activator, Sp1. *Journal of Biological Chemistry* **275**, (2000).

94. Izmailova, E. S., Wieczorek, E., Perkins, E. B. & Zehner, Z. E. A GC-box is required for expression of the human vimentin gene. *Gene* **235**, (1999).

95. Wu, Y., Zhang, X., Salmon, M. & Zehner, Z. E. The zinc finger repressor, ZBP-89, recruits histone deacetylase 1 to repress vimentin gene expression. *Genes to Cells* **12**, 905–918 (2007).

96. Kochman, M. & Wieczorek, E. *Proteins involved in juvenile hormone signal transmission Insects. Chemical, physiological, and environmental aspects. Proceedings of the 1st International Conference on Insects: Chemical, Physiological and Environmental Aspects*. (University Press, 1995).

97. Zalewska, M. *et al.* Juvenile hormone binding protein traffic — Interaction with ATP synthase and lipid transfer proteins. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1788**, 1695–1705 (2009).

98. WIECZOREK, E. & KOCHMAN, M. Conformational change of the haemolymph juvenile-hormone-binding protein from Galleria mellonella (L). *Eur J Biochem* **201**, (1991).

99. Dupas, S. *et al.* Collisional mechanism of ligand release by Bombyx mori JHBP, a member of the TULIP / Takeout family of lipid transporters. *Insect Biochem Mol Biol* **117**, (2020).

100. Wieczorek, E., Rodriguez Parkitna, J. M., Szkudlarek, J., Ozyhar, A. & Kochman, M. Immunoaffinity purification of juvenile hormone-binding protein from Galleria mellonella hemolymph. *Acta Biochim Pol* **43**, (1996).

101. Kolodziejczyk, R. *et al.* Insect Juvenile Hormone Binding Protein Shows Ancestral Fold Present in Human Lipid-Binding Proteins. *J Mol Biol* **377**, 870–881 (2008).

102. Dobryszycki, P. *et al.* Unfolding and Refolding of Juvenile Hormone Binding Protein. *Biophys J* **86**, 1138–1148 (2004).

103. Bystranowska, D. *et al.* Intramolecular cross-linking in the native JHBP molecule. *Arch Biochem Biophys* **517**, 12–19 (2012).

104. Winiarska, B. *et al.* N-linked glycosylation of G. mellonella juvenile hormone binding protein - Comparison of recombinant mutants expressed in P. pastoris cells with native protein. *Biochim Biophys Acta Proteins Proteom* **1814**, 610–621 (2011).

105. Dębski, J. *et al.* Positions of disulfide bonds and N-glycosylation site in juvenile hormone binding protein. *Arch Biochem Biophys* **421**, 260–266 (2004).

106. Jindra, M., Bellés, X. & Shinoda, T. Molecular basis of juvenile hormone signaling. *Curr Opin Insect Sci* **11**, 39–46 (2015).

107. Jones, G. *et al.* Ligand binding pocket function of Drosophila USP is necessary for metamorphosis. *Gen Comp Endocrinol* **182**, 73–82 (2013).

108. Jones, G., Jones, D., Teal, P., Sapa, A. & Wozniak, M. The retinoid-X receptor ortholog. *FEBS J* **273**, 1–14 (2006).

109. Flatt, T., Moroz, L. L., Tatar, M. & Heyland, A. Comparing thyroid and insect hormone signaling. *Integr Comp Biol* **46**, 777–794 (2006).

110. Malpeli, G., Folli, C. & Berni, R. Retinoid binding to retinol-binding protein and the interference with the interaction with transthyretin. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology* **1294**, 48–54 (1996).

111. Steinhoff, J. S., Lass, A. & Schupp, M. Retinoid Homeostasis and Beyond: How Retinol Binding Protein 4 Contributes to Health and Disease. *Nutrients* **14**, 1236 (2022).

112. Sok, A. J., Czajewska, K., Ożyhar, A. & Kochman, M. The structure of the juvenile hormone binding protein gene from Galleria mellonella. *Biol Chem* **386**, 1–10 (2005).

113. Seliga, J. *et al.* Multidomain sumoylation of the ecdysone receptor (EcR) from Drosophila melanogaster. *Journal of Steroid Biochemistry and Molecular Biology* **138**, (2013).

114. Bielska, K. *et al.* Alternative sumoylation sites in the Drosophila nuclear receptor Usp. *Journal of Steroid Biochemistry and Molecular Biology* **132**, (2012).

115. Nieva, C. *et al.* Ultraspiracle promotes the nuclear localization of ecdysteroid receptor in mammalian cells. *Biol Chem* **386**, (2005).

116. Zoglowek, A. *et al.* The composite nature of the interaction between nuclear receptors EcR and DHR38. *Biol Chem* **393**, (2012).