REVIEW ARTICLE

Key molecular pathways affected by glaucoma pathology: is predictive diagnosis possible?

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Abstract Prediction and prevention of glaucoma. Neuro-degenerative eye disease glaucoma is the second leading cause of blindness with estimated 67 million patients worldwide. Molecular pathomechanisms of glaucoma demonstrate both a considerable overlap with and remarkable particularities compared to other neurodegenerative disorders e.g. Alzheimer's disease. Identification of pathology-specific biomarker-sets is essential to develop advanced diagnostic approaches and personalised patients' treatment. Subcellular imaging and expression patterns in blood as the reliable platform for early/predictive glaucoma diagnosis. Following key pathways are affected in glaucoma pathology: stress response, apoptosis and DNA-repair, adhesion, blood-brain-barrier-breakdown, tissue remodelling, transcription regulation, multidrug resistance and energy metabolism.

Keywords Personalised medicine · Glaucoma · Blood biomarkers · Specific molecular-profiles · Early/predictive diagnosis · Targeted preventive measures

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Particularities of the neurodegenerative disease glaucoma

Glaucoma is complex neurodegenerative disease the molecular pathomechanisms of which demonstrate both a considerable overlap and remarkable particularities compared to some other neurodegenerative disorders such as Alzheimer's disease. As an example, *neuronal thread protein* (*NTP*) demonstrates enhanced expression levels in glaucoma patients against controls [1], the same observation occurred also for Down Syndrome, Alzheimer's and some other diseases [2], indicating axonal lesions as the common feature but giving no information about the corresponding aetiology. Simultaneous monitoring of expressional rates of further *TAU*-protein enables distinction between the pathologies: in contrast to the accumulation of *TAU*-protein characteristic for Alzheimer's disease and other tauopathies, glaucoma patients demonstrate no increase in the target protein expression *versus* controls [3]. Therefore, the selection of biomarkers specifically affected by glaucoma pathology is of high value creating advanced diagnostic approaches and targeted prevention and individualised treatment.

Glaucoma is a neurodegenerative eye disease and—worldwide—the second leading cause of permanent vision loss with estimated 67 million patients worldwide [4]. Glaucomatous optic neuropathy (GON) is characterised by a combination of a loss of retinal ganglion cells and their axons with disease typical optic nerve head (ONH) atrophy (see Fig. 1). An extensive tissue remodelling accompanying GON is very complex and the molecular pathomechanisms of GON are not yet completely clear.

The aetiology of glaucoma and risk factors are only partially known [6, 7], (see Fig. 2).

IOP (intraocular pressure) is considered the most important and only modifiable risk factor in glaucoma. Although
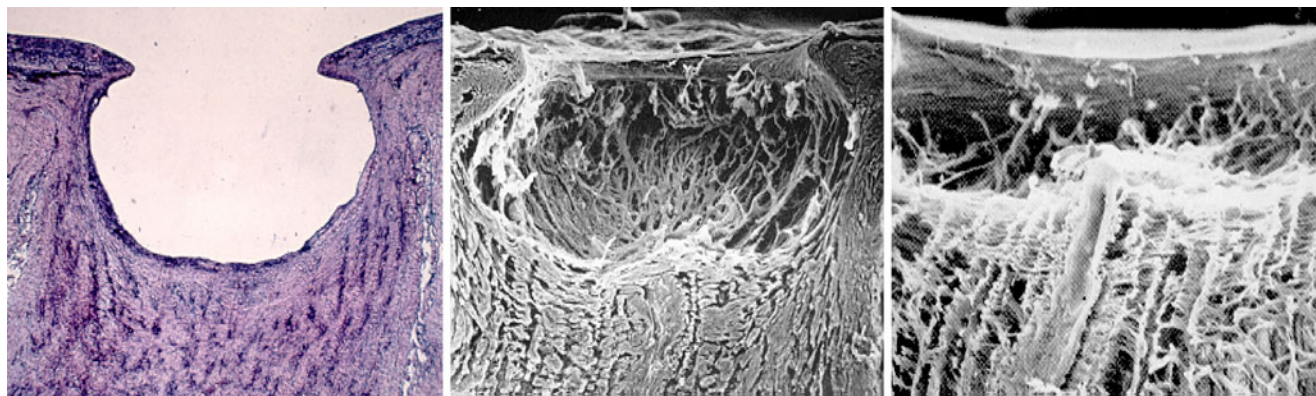
Fig. 1 Characteristic degenerative tissue remodelling by glaucomatous damage [5]

elevated IOP has been shown to be the major risk factor, there is a cohort of patients even at younger ages with normal IOP developing normal-tension glaucoma (NTG) (Fig. 3).

The frequency and incidence of different glaucoma forms vary geographically, depending on race, sex and age [8–10]. A family history of the disease is common among glaucoma patients, suggesting a genetic background for this disorder. The existence of a genetic linkage associated with particular forms of glaucoma is well documented in literature [10–17]. The growing body of evidence suggests the idea that genetic-based approaches in early diagnosis of glaucoma and targeted treatment might yield better results than medical, surgical, and rehabilitation interventions alone [17]. Although mutation research in glaucoma detected some attractive potential targets, the mutated genes found—like *TIGR*—play a limited role in the pathogenesis of glaucoma and do not explain the usual clinical picture [9, 18–22]. Therefore, pharmaco-genetic approaches, if any, would provide only minor success in prediction of glaucoma.

Are there any molecular diagnostic approaches developed to preselect glaucoma-predisposed individuals, to distinguish between glaucoma forms and to estimate a disease progression? We should state a current lack of clinical approaches for early and predictive diagnostics of glaucoma. However, much effort made and recent progress achieved in the identification of disease characteristic molecular patterns

should be clearly acknowledged—the issue which is reviewed in this chapter.

Impact of vascular component and relevance of gene expression patterns in blood for glaucoma pathology

A wealth of literature points to the potential importance of haemodynamics in glaucoma pathology, but randomised controlled trials are not yet available. Healthy individuals with Vasospastic Syndrome demonstrate molecular profiles in blood similar to those of glaucoma patients [23]. Particular predisposition of vasospastic individuals to glaucoma is discussed in the separate review of this journal-issue [24]. Although animal models for glaucoma provided us with useful information about affected molecular pathways and potential targets in the eye, they are barely applicable for non-invasive diagnostics on human being. Since blood is easily accessible, its molecular analysis, in reasonable amounts (few millilitres), may prove practical application in population screening for high-risk subjects. How relevant are gene expression patterns in blood serum and white blood cells for the eye? Glaucoma patients frequently exhibit abnormal T-cell subsets and increased titres of serum

KNOWN RISK FACTORS:
- **IOP**
- **race**
- **female gender**
- **genetics**
- **myopia**
- **corneal thickness**
- **optic disc haemorrhages**
- **haemodynamics, others**
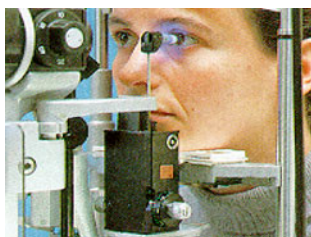
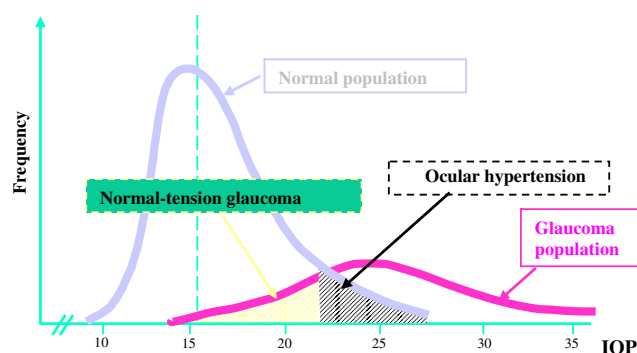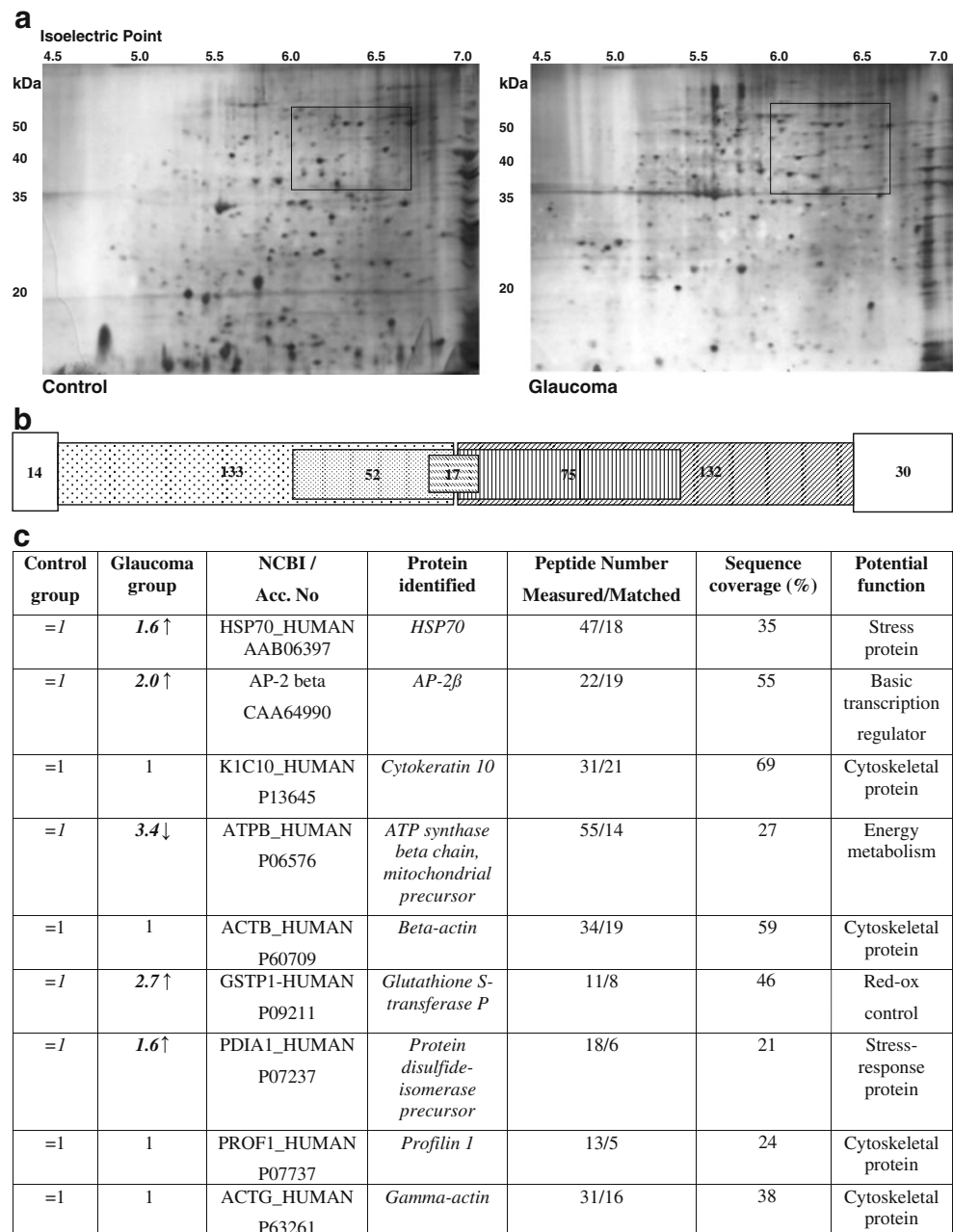Fig. 2 Risk factors in glaucoma and measurement of intraocular pressure (IOP) [5]

Fig. 3 Although increased IOP is a well-known risk factor, the IOP values of normal-tension glaucoma patients are within statistical norm

antibodies to retina and optic nerve proteins; these alterations in the cellular subset indicate that the immune system plays an important role in the initiation and/or progression of glaucomatous optic neuropathy [25]. Therefore, serum anti-bodies to retina and optic nerve proteins might be considered as potential indicators for the diagnosis of glaucoma pathology [26]. In addition, it has been demonstrated that gene expression patterns of both trabecular meshwork and Schlemm´s canal are similar to those of circulating leucocytes (CL) [27]. Moreover, blood serum is the biggest reservoir of signalling molecules in human beings: many metabolites are simultaneously secreted from different types of cells in blood that represents a universal way of communication between cells. Although this communication is extremely complex, one of the known natural sensors affected by the tremendous number of metabolites present in blood serum is CL. The spectrum of the molecules affecting expression patterns in CL is very broad and partially unknown; their regulation is extremely complex and not yet completely understood. However, it is known that the resulting regulation of CL is triggered by altered gene expression patterns on the levels of transcription and translation. The resulting shift in a gene expression pattern referred to a corresponding physiologic/pathologic condition

Fig. 4 Protein expression patterns characteristic for circulating leucocytes of glaucoma patients [30]. **a.** 2D-PAGE images of differential gene expression patterns in circulating leucocytes of glaucoma patients *versus* controls with particular changes marked. **b.** The scheme represents summarised results from the evaluation of comparative 2D-PAGE images. 132 protein spots were constantly present in all control images, whereby 75 spots were expressed at the same level. 133 protein spots were constantly present in patient images, whereby 52 spots were expressed at the same level. 17 proteins represent the overlap between 75 and 52 protein spots. Thirty spots were present only in the control group whereas 14 spots were present only in the patient group; all spots were detectable only in single images and, therefore, were not included in the above 133 and 132 pools. **c.** Quantitative analysis of 2D-PAGE images: among 75 and 52 conserved protein spots in the control and patient groups, respectively, 17 overlapping protein spots were found to be conserved for members of the same group. Corresponding expression levels in the control group are taken as standard (=1). 9 proteins have been identified as given in the table

Control group	Glaucoma group	NCBI / Acc. No	Protein identified	Peptide Number Measured/Matched	Sequence coverage (%)	Potential function
=1	1.6↑	HSP70_HUMAN AAB06397	HSP70	47/18	35	Stress protein
=1	2.0↑	AP-2 beta CAA64990	AP-2β	22/19	55	Basic transcription regulator
=1	1	K1C10_HUMAN P13645	Cytokeratin 10	31/21	69	Cytoskeletal protein
=1	3.4↓	ATPB_HUMAN P06576	ATP synthase beta chain, mitochondrial precursor	55/14	27	Energy metabolism
=1	1	ACTB_HUMAN P60709	Beta-actin	34/19	59	Cytoskeletal protein
=1	2.7↑	GSTP1-HUMAN P09211	Glutathione S-transferase P	11/8	46	Red-ox control
=1	1.6↑	PDIA1_HUMAN P07237	Protein disulfide-isomerase precursor	18/6	21	Stress-response protein
=1	1	PROF1_HUMAN P07737	Profilin 1	13/5	24	Cytoskeletal protein
=1	1	ACTG_HUMAN P63261	Gamma-actin	31/16	38	Cytoskeletal protein

can be measured on both levels. Both vascular and immune components may play a role in pathomechanisms of glaucoma [28]. Taking the above into consideration, the search for disease specific gene expression patterns in glaucoma is of great value for a better understanding of the molecular pathomechanisms and creation of predictive approaches in glaucoma diagnostics.

Key molecular pathways affected by glaucoma pathology: development of predictive diagnostic strategies

Key molecular pathways affected by glaucoma pathology have been recently summarised in the book specifically dedicated to predictive diagnostics and personalised treatment [29].

The majority of molecules altered in glaucoma belong to well-defined functional gene-groups which, altogether, represent molecular pathways affected by the pathology (see Fig. 4).

Stress response, apoptosis and DNA-repair

An increased oxidative stress has been well documented under glaucoma pathology. Many oxidatively modified proteins were demonstrated in retinal protein lysates from ocular hypertensive eyes of rat-model of glaucoma [31]. Consequently, altered gene expression patterns, particularly for stress response factors, can be expected. Thus, the alteration of *heat shock 27 kDa protein 1* expression was stable in the retina of rat hypertensive eyes [32]. "Gene hunting" technology of "Subtractive Hybridisation" followed by quantification of target transcripts (see Fig. 5) performed in CL of glaucoma patients revealed an upregulation of both the pro-apoptotic factor *p53* and proteolytic enzyme *20S proteasome subunit XAPC7* [1], activity of which is usually increased during reperfusion [33]. In agreement with this, both an apoptotic inhibitor *survivin* and DNA-repair *Xeroderma pigmentosum gene C* have been found to be down-regulated in CL of glaucoma patients [1]. Moreover, *ex vivo* subcellular imaging by "Comet Assay"-analysis demonstrates significantly increased (un-repaired) DNA-damage in circulating leucocytes of both normal- and high-tension glaucoma patients [29, 35].

Adhesion, blood-brain-barrier (BBB)-breakdown and tissue remodelling

Gene expression profiles characteristic for an activated adherent function have been identified in CL of glaucoma patients: up-regulated transcripts of *lymphocyte-IgE-receptor* (*Fc-epsilon-RII/CD23*), *T cell-specific tyrosine kinase (ITK)*,
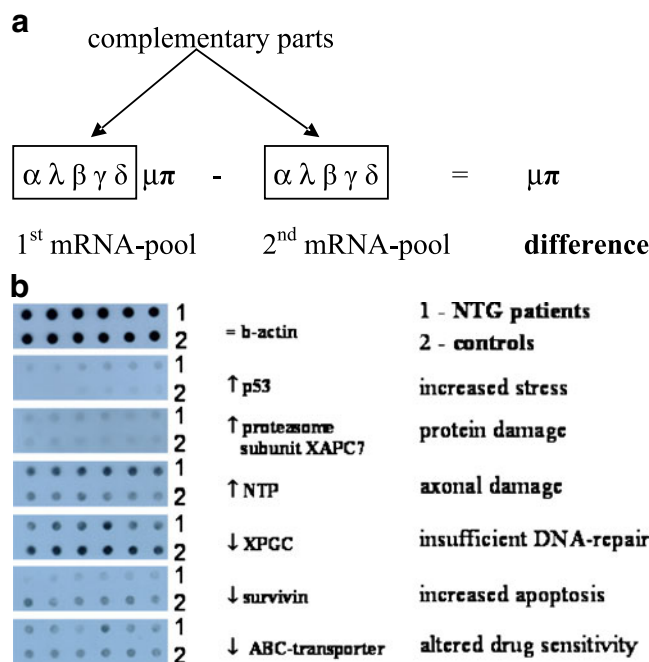
Fig. 5 **a.** Principle of "gene hunting" by the "Subtractive Hybridisation" [1, 34]. Each mRNA-pool consists of individual gene transcripts, which undergo a comparison by hybridisation of identical (complementary) parts. The subtraction results in identification of non-complementary transcripts differentially expressed in two pools of comparison; the technology enables a consideration of both qualitative (present or absent) and quantitative (amount of copies per unit weight of mRNA in the corresponding pool) differences **b.** Consequent quantification of the target transcripts (mRNA "Dot-Blot"-analysis), further provided a strong indication for increased stress, apoptosis, protein damage and axonal damage as well as insufficient DNA-repair and altered drug-sensitivity in patients with glaucoma compared to the control group

thromboxan-A2-receptor, and *alkaline-phosphatase* have been detected by "subtractive hybridisation" [36]. Adherent circulating leucocytes (CL) could be an important contributor to BBB-breakdown observed in glaucoma pathology [37]. The subtraction of metalloproteinase *MMP-9* and *MT1-MMP* transcripts highly increased in CL of glaucoma patients provides further evidence for this functional link and indicates pathways involved in the extensive tissue remodelling observed in glaucoma [38]. Significantly increased protein expression rates of both latent and active forms of *MMP-9* and *MT1-MMP* in CL correlated well with the enhanced levels of transcription and with glaucoma [38]. Once activated, both hydrolases necessarily contribute to remodelling or even degeneration of the tissue whereto they are secreted by CL. This up-regulation might be a consequence of repeated mild ischemia/reperfusion postulated for glaucoma patients. However, the question as to whether or not there is a correlation between an increased *MMPs* activity and glaucoma severity should be further clarified. Recently performed comparative gene transcription profiling in trabecular mesh-

work isolated from post-mortem glaucomatous eyes revealed a significant up-regulation of several cell adhesion molecules including *platelet endothelial cells adhesion molecule-1* and *P-selectin* [39]. An enhanced deposition of the cell aggregation protein *cochlin* in trabecular meshwork and Schlemm's canal of glaucomatous eye supports the crucial role of cellular adhesion in glaucoma pathology and favours cell adhesion molecules as potential disease biomarkers [40].

Transcription regulation

Dramatically altered transcription patterns in glaucoma were reported by several research groups [1, 30, 32, 38]. A reason for this extensive shift in transcription regulation was unclear for a very long time till a significant up-regulation of the basic transcriptional regulator *AP-2ß* was attributed to glaucoma pathology: *AP-2ß* has been identified in CL using the smart technology of disease proteomics [30]. Protein mapping is demonstrated in Fig. 4a. *AP-2* proteins play a decisive role, particularly in eye morphogenesis. While the activation of *AP-1* leads to an increased stromelysin (metalloproteinase-3) production in trabecular meshwork *in vitro* [41, 42], the expression and activity of *AP-2* controls the activity of the *gelatinase B* (*MMP-9*) [43]. In consensus, *MMP-9* was demonstrated to be significantly up-regulated in leucocytes of glaucoma patients and to play an important role in tissue remodelling as a part of glaucomatous degeneration [38]. Therefore, the concerted up-regulation of *AP-2ß* and *MMP-9* in leucocytes

of glaucoma patients has been proposed to be an important part of the molecular mechanisms in glaucoma pathology and a reliable biomarker for diagnostic purposes.

Multidrug resistance

An extensive deregulation of *ABC-transporters* has been demonstrated in glaucoma pathology [1, 44, 45]. *ABC-transporters* (*ATP-binding cassette transporter*) usually translocate a wide variety of structurally unrelated lipophilic compounds being responsible for drug efflux, and therefore, for multidrug resistance. *ABC 1* has been shown to be stably up-regulated in CL of glaucoma patients [45]. Joyce et al. demonstrated a crucial role for *ABC 1* in protection against atherosclerosis [46]. An activity of *ABC 1* has been shown to have a regulating effect on endothelial function and stimulate nitric oxide bioactivity in arterial walls [47]. The up-regulation of *ABC1* in CL of glaucoma patients might indicate the involvement of this gene in chronic vascular deregulation frequently observed in glaucoma pathology, and has been suggested as a potential marker for early diagnostics of glaucoma [45].

Energy metabolism

"Gene hunting" in CL of glaucoma patients revealed down-regulated transcripts of Na^+/K^+-ATPase [36]. An identification of the subtracted transcripts is demonstrated in Fig. 2. Further, abnormal sodium handling has been

Table 1 Types of glaucoma-specific molecular alterations that can be potentially used for development of advanced tools for early and predictive diagnosis [60]

Type of molecule	Possible type of alteration	Detection technology
Chromosomal DNA, mitochondrial DNA	1. (Oxidative) damage[a]	- *Comet assay*
	2. Mutations[a]	- *Disease genomics*
	3. Polymorphism[a]	- *PCR, Restriction analysis*, etc.
	4. Methylation status of CpG islands[b]	- *Methylation-specific PCR*
mRNA	1. Multiple alterations in expression patterns[a]	- *Disease transcriptomics*: Subtractive hybridisation, Expression array, Reverse-Transcriptase-PCR, Real-Time-PCR, etc.
	2. Reduced mRNA editing	- *Reverse-Transcriptase-PCR*
Proteins	1. Multiple alterations in expression patterns[a]	- *Disease Proteomics*: 2D-PAGE, MALDI-TOF, Western-blot, etc.
	2. Posttranslational modification[a]	- *Western-blot, Activity tests* (e.g. Zymography for gelatinases)
	3. Phosphorylation status[a]	- *Activity tests*
	4. Protein misfolding[a]	- *Activity tests*
Metabolites (signalling molecules, amino acids, plasma hormones, etc.)	Altered profiles[a]	*Disease Metabolomics*: Comparative blood plasma metabolites profiling, HPLC, Activity tests, etc.

[a] Types of molecular alterations reported for glaucoma pathology. [b] Data indicating this kind of alteration collected in our laboratory, which, however, have not been published until now

proposed to be associated with ocular hypertensivity and to contribute to a progression of the optic nerve damage in both normal-tension and high-tension glaucoma [48, 49]. Na^+/K^+-$ATPase$ is known to be down-regulated in lymphocytes of patients with acute myocardial infarction [50], and may be one of the reasons for ventricular arrhythmias and coronary artery spasms. A decrease in intracellular potassium concentration and an increase in intracellular calcium concentration may play a major role in the pathomechanism of coronary artery spasms. Inhibition of Na^+/K^+-$ATPase$ is one of functional consequences of oxidative membrane damage caused by an increased oxidative stress [51], which is triggered e.g. under ischemia/reperfusion. This affects central thermogenic mechanisms, since Na^+/K^+-$ATPase$ has been shown to play a key role in cellular energy balance and thermogenesis [52, 53]. A decreased activity of Na^+/K^+-$ATPase$ triggers vasospasm [54]. Being an important co-factor of cellular $ATPases$, magnesium can reverse delayed vasospasm and reduce the extent of acute ischemic lesions [55]. Magnesium therapy demonstrates beneficial effects in vasospastic syndrome and glaucoma [9].

Effect of glaucoma medication on the evaluation of biomarkers

Evaluation of glaucoma biomarkers under treatment conditions is a particular issue in glaucoma research. Indeed, can we use the markers proposed for diagnostics of untreated glaucoma also under glaucoma medication? Are there any reliable markers which can predict the outcome of glaucoma treatment? There are some studies clearly demonstrating that glaucoma medication may definitely mask alterations in crucial biomarkers such as those of the nitric oxide pathway [56]. Additionally, toxic side effects of anti-glaucoma drugs on conjunctiva have been reported: histological studies of conjunctival tissues in patients who underwent a long-term glaucoma treatment demonstrated an abnormal infiltration of inflammatory cells. Consequently, an abnormal expression of inflammatory markers under various anti-glaucomatous treatments has been shown [57].

Much progress has been achieved in the development of markers that can potentially predict the outcome of glaucoma surgery. In the research of Souchier et al., surgery success was defined as reduced IOP ≤ 15 mm Hg without any IOP-lowering drugs at 6 months. Conjunctival expression levels of *trefoil factor family 1* (*TFF1*) and *MUC5AC* as well as *HLA-DR* in leucocytes have been measured [58] and an increased expression of all three molecular markers has been proposed as a potential prediction factor for successful glaucoma surgery. However, the issue of glaucoma medication markers is a multifarious one and requires more research effort.

Perspectives for application of most potent biotechnological tools

High-accuracy proteome maps of human body fluids open new perspectives for early molecular diagnostics of chronic disorders. Abundant scientific evidence from disease proteomics suggests that disease specific protein expression patterns in blood are indicative of many—if not most—human disorders, and might be a powerful tool for early molecular diagnostics of glaucomatous damage. A non-invasive molecular diagnostic approach based on disease specific gene expression patterns in CL has been suggested for glaucoma [59]. The test foresees a precise expression profiling of selected genes in CL isolated from fresh blood samples. These genes (gene products) have been proposed to play a role in glaucoma pathology and belong to following pathways: an apoptosis, stress response, DNA-repair, transcription regulation, multidrug resistance, tissue remodelling and degeneration. Currently a clinical application of the test is under consideration, and a nanotechnology which should provide a possibly easy and cheap routine application of the test is under development. Possible types of molecular alterations that can be considered for predictive glaucoma diagnosis and corresponding detection technologies are summarised in Table 1.

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