Study of the Toll-like receptor 4 gene polymorphisms in diseases presenting with subclinical and chronic inflammation (diabetes mellitus, ischemic stroke, periodontitis)

Ph.D. thesis

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I. Introduction:

The innate immune system uses a number of soluble and cellular sensing mechanisms to recognise potentially harmful substances. Stressors deranging the tissue homeostasis induce acute phase reactions mediated by the innate immune response. Signals produced by innate receptors cause effector response designed to neutralise the stressor and to restore the homeostasis. Cells of the innate immunity include broad-specificity proteins encoded in the germ-line which have been called pattern recognition receptors (PRR) as they are not specific for a particular antigen, but sense conserved structures and molecules characteristic of harmful agents (PAMP: pathogen associated molecular pattern). Toll-like receptor, a central mediator of the innate immunity, is also a PRR. Ten members of TLRs are reported. The best known TLR is TLR4. TLR4 is the receptor for lipopolysaccharides (LPS) from Gram-negative bacteria. It has been reported that hsp60 and 70, oxidized low density lipoprotein, fibronectin and taxol are also putative ligands of the TLR4. Activation of TLR4 mediates the expression of proinflammatory cytokines

via Nf-κB transcription factor. Two common co-segregated single nucleotide polymorphisms (SNP) on the human TLR4 gene were reported. One SNP is an Adenin (A) to Guanin (G) substitution at nucleotid position 896 from the start codon of the TLR4 cDNA. The single nucleotide exchange results in replacement of a conserved aspartic acid residue with glycine at amino acid position 299 (Asp299Gly) (dbSNP databank: rs4986790). The second missense polymorphism results in a change of citosin (C) to timin (T) at nucleotid position 1196 from the start codon (dbSNP databank: rs4986791), which causes replacement of a non-conserved threonine with an isoleucine at amino acid position 399 (Thr399Ile) in the extracellular domain of the TLR4 receptor. An average incidence of these polymorphisms is about 10% in the Caucasian population.

It has been demonstrated that subjects carrying these polymorphisms are hyporesponsive to inhaled LPS, furthermore, the polymorphisms are associated with an increased incidence of gram-negative infections and inflammatory bowel diseases.

The prospective, population-based Brunneck study reported that carriers of the Asp299Gly TLR4 allele appeared to be more susceptible to bacterial infections, but they have a decreased risk of carotid artery atherosclerosis, and a reduced intima-media thickness of the carotid artery compared to subjects carrying the wild genotype. The REGRESS study demonstrated that the efficacy of pravastatin therapy in preventing cardiovascular events was significantly higher in Asp299Gly carriers than in wild-type carriers.

These findings indicate that the importance of TLR4 may well beyond antimicrobal defense against other inflammatory processes inherently related to atherosclerosis and its complication such as ischemic stroke, diabetes mellitus and its microvascular complications.

II. Aims:

The aim of the investigation was to study the association between polymorphisms of the TLR 4 gene (Asp299Gly and Thr399Ile) and three frequent diseases of the modern

population. Therefore, three different studies were designed.

II.1. The background and aim of study I.:

The innate immune response may have a role in both types of diabetes mellitus and their late complications. The role of the TLR4 mediated pathways of innate immunity has never been studied in the pathophysiology of diabetes mellitus and its late complications. Therefore, the aim of study I. was to investigate the associations between the functional polymorphisms of the TLR4 gene and diabetic nephropathy and neuropathy in type 1 and type 2 diabetes mellitus.

II.2. The background and aim of study II.:

The Asp299Gly polymorphism of the TLR4 gene is associated with amelioration of proinflammatory cytokine profile, a reduced risk of carotid atherosclerosis, and a reduced intima-media thickness of the carotid artery. The atherosclerosis of the carotid artery is a classical risk factor of ischemic stroke. However, it is not known, whether the TLR4 gene polymorphism can influence the risk of ischemic stroke. The aim of the study II. was to investigate the association between

polymorphisms of TLR4 gene and the risk of cerebral ischemia.

II.3. The background and aim of study III.:

There is a central role of Gram-negative bacteria induced inflammation in the pathogenesis of periodontitis. The TLR4 gene polymorphisms can reduce the defending system of the host against Gram-negative bacteria. The influence of TLR4 gene polymorphisms on the risk of periodontitis is obscure. The aim of study III. was to investigate the association between TLR4 polymorphisms and the risk of chronic periodontitis.

III. Methods:

Genomic DNA was prepared from peripheral blood mononuclear cells. The polymorphic alleles of TLR4 gene were detected using polymerase chain reaction (PCR) and subsequent cleavage by restriction endonucleases. Patients were recruited from the outpatient clinic of the Department of Endocrinology and the Neurology Department of University Clinic.

IV. Results:

Results of study I.: In the type 1 diabetes group 27 of the 246 patients while in the group with type 2 diabetes 65 of the 530 patients were heterozygous for both polymorphisms. Only one patient with type 2 diabetes was homozygous carrier for both polymorphic genotypes. The prevalence of different genotypes in groups with and without nephropathy and neuropathy showed no significant difference in patients with type 1 diabetes. In patients with type 2 diabetes, the prevalence of different genotypes in groups with and without nephropathy was also not significantly different. However, patients with type 2 diabetes demonstrated a strong association between the Asp299Gly and Thr399Ile polymorphisms and diabetic neuropathy ($p = 0.0002$; OR = 0.35 [95% CI: 0.19-0,61]).

Results of study II:

The data of three different subgroups were analysed: a high-risk population, subjects with diabetes mellitus (n_d= 776), and two case-control groups (n_1= 267 and n_2=375). Neither in the high risk group, nor in the case-control groups we were able to detect any association between the TLR4 polymorphisms and cerebral ischemia (p_d =

0.812 OR_d = 1.13, 95%CI_d:0.45-2.78; p_1 = 0.572; OR_1 = 1.26; 95%CI_1:0.57-2.8; p_2 = 0.106; OR_2 = 1.83; 95%CI_2: 0.88-3.8).

Results of study III.:

We analyzed 129 subjects, of which 85 suffered from periodontitis, and 54 served as control. 12 subjects were heterozygous carriers of the TLR4 gene polymorphisms. Although an increased prevalence of the *Asp299Gly* and *Thr399Ile* genotypes in the periodontitis group was shown, the difference failed to reach the level of statistical significance using the univariate method. In individuals carrying the polymorphism of TLR4 gene statistically significant more severe clinical attachment loss and radiographic bone loss were found.

V. Conclusions:

- There is no difference in the prevalence of the Asp299Gly and Thr399Ile polymorphisms of the TLR 4 gene between pateints with type 1 or type 2 diabetes mellitus and subjects representing the average population.

- There is a significantly decreased prevalence of diabetic polyneuropathy in patients with type 2 diabetes mellitus carrying the Asp299Gly and Thr399Ile polymorphisms of the TLR4 gene.
- The Asp299Gly and Thr399Ile polymorphisms of the TLR4 gene have no influence on the risk of cerebral ischemia.
- The Asp299Gly and Thr399Ile polymorphisms of TLR4 gene are not enough powerful genetic markers for susceptibility for chronic periodontitis in clinical practice.

VI. Publications:

VI. 1. Publications related to the thesis

- Rudofsky G jr*, **Reismann P*,** Witte S, Humpert PM, Isemann B, Chavakis T, Tafel J, Nosikov VV, Hamann A, Nawroth P, Bierhaus A (2004) *Asp299Gly and Thr399Ile genotypes of the TLR4 gene are associated with a reduced prevalence of diabetic neuropathy in patients with type 2 diabetes.* Diabetes Care 27(1): 179-83.

- **Reismann P*,** Lichy C*, Rudofsky G, Humpert PM, Genius J, Si TD, Dörfer C, Grau AJ, Hamann A, Hacke W, Nawroth P, Bierhaus A (2004) *Lack of association between polymorphisms of the toll-like receptor 4 gene and cerebral ischemia.* J Neurol. 251(7):853-8.

- **Reismann P**, Lichy C, Rudofsky G, Meiser H, Nawroth P, Staehle HJ, Bierhaus A, Dörfer C. (2007) TLR4-polymorphism and the risk of chronic periodontitis.Perio 4(1):41-45.

- **Reismann P,** Racz K, Tulassay Zs. (2008) *A Toll-like receptor 4 génpolimorfizmusok valamint lehetséges klinikai szerepük fertőzésekben és krónikus gyulladásos betegségekben.* Orv. Hetil 149 (38): 1791-9.

VI. 2. Other publications:

- Rudofsky Jr G, **Reismann P**, Schiekofer S, Petrov D, Eynatten M, Humpert PM, Isermann B, Muller-Hoff C, Thai TP, Lichtenstein S, Bartsch U, Hamann A, Nawroth P, Bierhaus A. (2004) *Reduction of postprandial hyperglycemia in patients with type 2 diabetes reduces NF-kappaB activation in PBMCs.* Horm Metab Res. 36(9):630-8.

- Rudofsky Jr G, Schlimme M, Schlotterer A, von Eynatten M, **Reismann P**, Tafel J, Grafe I, Morcos M, Nawroth P, Bierhaus A,

Hamann A. (2005) *No association of the 94T/G polymorphism in the adiponectin gene with diabetic complications.* Diabetes Obes Metab. 7(4):455-9.

- Humpert PM, Battista MJ, Lammert A, **Reismann P**, Djuric Z, Rudofsky G Jr, Zorn M, Morcos M, Hammes HP, Nawroth P, Bierhaus A. (2006) *Association of stroma cell-derived factor 1 genotype with diabetic foot syndrome and macrovascular disease in patients with type 2 diabetes.* Clin Chem. 52(6):1206-8.

- Lichy C, Kloss M, **Reismann P**, Genius J, Grau A, Reuner K. (2007) *No evidence for plasminogen activator inhibitor 1 4G/4G genotype as risk factor for cerebral venous thrombosis.* J Neurol. 254(8):1124-5.

- Rudofsky G Jr, **Reismann P**, Grafe IA, Konrade I, Djuric Z, Tafel J, Buchbinder S, Zorn M, Humpert PM, Hamann A, Morcos M, Nawroth PP, Bierhaus A. (2007) *Improved vascular function upon pioglitazone treatment in type 2 diabetes is not associated with changes in mononuclear Nf-kappaB binding activity.* Horm Metab Res 39(9):665-71.

- **Reismann P**, Varga I, Patócs A, Gergics P, Tóth M, Szücs N, Gláz E, Rácz K, Tulassay Zs. (2007) *A hormonvizsgálatok fejlődésének 50 éve, nemzetközi visszatekintés és hazai tapasztalatok.* Magy Belorv Arch 60:129-134.

- **Reismann P**, Rácz K. (2008) *A polycystas ovarium szindóma patogenetika háttere.* Diab Hung. Suppl. 2. 6-13.

- **Reismann P**, Tulassay Zs. (2008) *Lizoszóma tárolási rendellenességek kezelési lehetőségei.* Orv. Hetil 149(25):1171-9.