Targeting IL-1β and assessing sex-specific molecular differences in mouse models of non-alcoholic steatohepatitis

PhD thesis

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1. Introduction

1.1. Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is a chronic, progressive liver disease. The first stage, steatosis, develops due to metabolic and energy misbalance in the liver. Hepatic lipid influx and decreased lipid export and/or metabolism (β-oxidation of fatty acids) results in accumulation of fats in hepatocytes. This stage is reversible, if the patient ceases the unhealthy behavior (western diet, sedentary lifestyle). If not, the patient may progress into the stage called non-alcoholic steatohepatitis (NASH). During NASH, complex inflammatory and fibrotic events occur. NASH may progress into cirrhosis and, even, hepatocellular carcinoma (HCC).

1.2. Sex- and age-dependent factors in non-alcoholic steatohepatitis

Age and sex are two relevant contributing factors to NAFLD and NASH development. Inflamm-aging, a chronic low-grade systemic inflammation, can negatively affect several organs' function, such as the liver and the heart. Major risk factors and/or co-morbidities of NASH, such as obesity, hypertension, metabolic syndrome, show relevant sexual disparity. Thus, it is no wonder that epidemiological studies of NAFLD itself showed sexual difference in the prevalence of NAFLD: Men and postmenopausal women are at higher risk to develop NAFLD compared to premenopausal women. Similarly, cardiovascular risk of obesity, hypertension and metabolic syndrome is lower in younger females, while it is higher in males and elderly women. However, hepatic lobular

inflammation is more severe in premenopausal women. As females age, their daily dietary requirement of choline increases, thus further increasing the risk of development of steatosis. Choline is an essential nutrient, which is important in VLDL release and fatty acid β-oxidation. PCSK9, the master regulator of cholesterol serum level, is associated with steatosis severity. PCSK9 serum level is lower in males, while in females increases with age.

1.3. Interleukin-1β in non-alcoholic steatohepatitis

During NASH, the chronic stress of steatosis may damage hepatocytes. The wide range of molecules (damage-associated molecular patterns, extracellular vesicles etc.) released from these dying liver cells will stimulate Kupffer cells. This hepatic resident macrophage population will initiate its phagocytic machinery or will start secreting chemokines and pro-inflammatory cytokines, such as IL-1β. This locally secreted IL-1β may promote steatosis, may facilitate insulin resistance, may induce apoptosis, may contribute to the activation of hepatic stellate cells, and to subsequent fibrosis, may activate more resident immune cells and/or stimulate the infiltration of circulating monocytes and neutrophils, resulting more release of pro-inflammatory molecules and finally culminating in a vicious cycle of inflammation, fibrosis and apoptosis. Not only locally released IL-1β is able to contribute to disease progression, but systemic sources of IL-1β, such as inflamm-aging has profound effect on NASH pathophysiology.

2. Objectives

Despite extensive preclinical and clinical studies, there is no drug approved for NASH. Most of the clinical trials aimed to target lipid and glucose metabolism, while trials that attempted to directly affect fibrosis and/or inflammation are little in number. Although epidemiologic data suggest that sex is a major variable in the pathophysiology of NASH, little molecular detail is available. Epidemiologic studies also reveal that prevalence of NAFLD, NASH and NASH-induced HCC increases globally, causing intensive socio-economic burden.

In this work we aimed:

1. to investigate the cardiac and hepatic effects of an interleukin-1β binding monoclonal antibody in an aged mouse model
2. to assess sex-specific expression of genes-related to cholesterol metabolism, inflammation and fibrosis in a middle-aged mouse model of NASH

3. Methods

This work constituted of two projects:

In the first, 24 months old male C57Bl/6J mice were fed with control (CON) or choline deficient (CDAA) diet for 8 weeks. Mice were treated with isotype control (iso CON) or anti-IL-1β monoclonal antibody twice a week. Each mouse received 50 μg of anti-IL-1β monoclonal antibody. On the 7^{th} week conventional and 2D speckle tracking echocardiography was performed.

In the second subproject, 10 months old female and male C57Bl/6J mice were fed with the CON or CDAA diet for 8 weeks.

In both projects, after 8 weeks of diet mice were sacrificed, and organ samples were collected for histologic and molecular analyses.

4. Results

4.1. Targeting IL-1β in NASH

Interleukin-1β is an important mediator in inflammatory processes of both NASH and inflamm-aging. Previous studies and trials of NASH tested the inhibition of inflammasomes and caspases, but direct inhibition of IL-1β has not been tested before. Thus, this cytokine might be considered a viable pharmacologic target.

4.1.1. IL-1β inhibition improves diastolic function

To evaluate cardiac function, conventional echocardiography and 2D echocardiography with subsequent strain analysis was performed. Strain analysis measures the torsion of myocardial fibers. It is a more sensitive method to identify disturbances in cardiac function. In our aged model of NASH, systolic function was preserved in all groups (**Figure 1**). Strain analysis was able to reveal diastolic deterioration in mice with NASH, which was improved by blocking IL-1β. Lectin histochemistry showed no evidence of change in cardiac morphology (**Figure 2**).

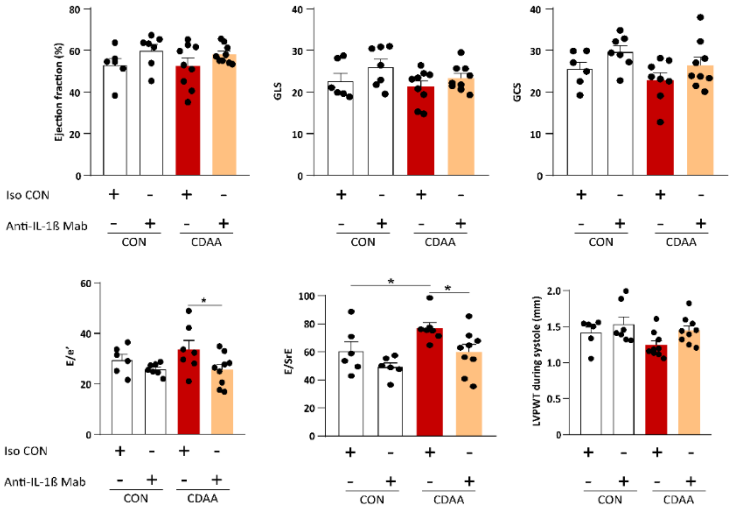

Figure 1 – Cardiac function of mice in an aged mouse model of NASH.
Systolic parameters (ejection fraction, global longitudinal strain, global circumferential strain) are normal. Both E/e' and E/SrE (ratio of early mitral inflow velocity-to- mitral annular early diastolic velocity, and -early diastolic strain rate, respectively) are indirect markers of diastolic function. E/SrE was ameliorated in IL-1β inhibitor treated group. Two-way ANOVA, Fisher's LSD post hoc test, $*P < 0.05$.

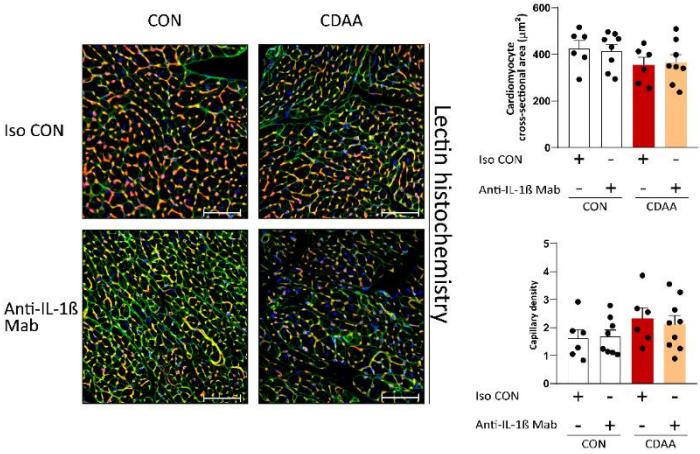

Figure 2 – Assessment of cardiac morphology with lectin histochemistry.
Wheat germ agglutinin (WGA-FITC) shown in green stains myocardial cell membrane. Isolectin B4 (ILB4-DyLight 594) shown in orange stains endothelial cells of cardiac capillaries. Two-way ANOVA, Fisher's LSD post hoc test, $*P < 0.05$.

4.1.2. *IL-1β inhibitor decreases the expression of pro-fibrotic genes, while does not affect overall fibrosis*

Picrosirius red staining was performed to assess hepatic fibrosis, a key characteristic of NASH pathophysiology. CDAA-fed mice had extensive lobular fibrosis, which was improved by the treatment only at transcriptional level (**Figure 3**).

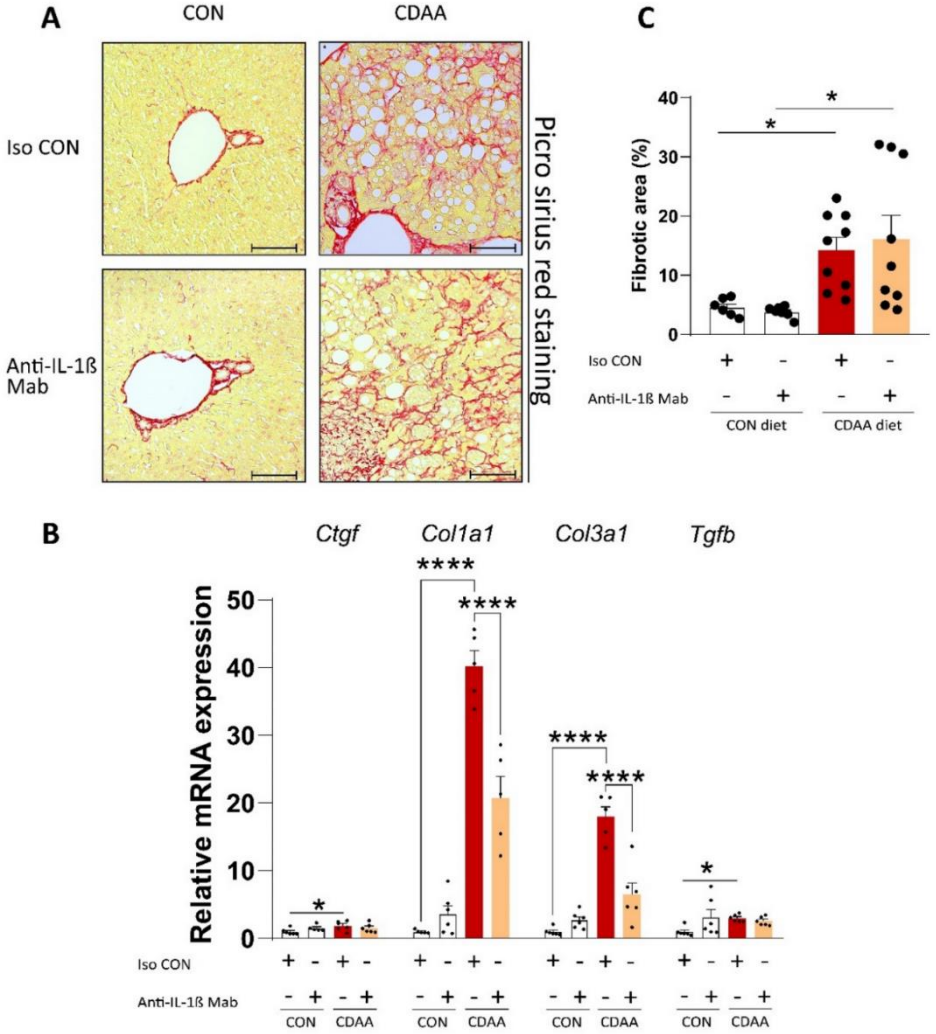

Figure 3 – Evaluation of liver fibrosis with histochemistry and qRT-PCR.
Picrosirius red staining of liver section and qRT-PCR analysis of major pro-fibrotic genes shows profound fibrosis, which was not affected by the treatment at macroscopic level. Two-way ANOVA, Fisher's LSD post hoc test, $*P < 0.05$.

4.1.3. Interleukin-1β inhibition does not improve key features of NASH

CDAA diet induced marked steatosis and facilitated the formation of inflammatory infiltration (**Figure 4A**). NAFLD Activity Score is a wide used comprehensive scoring system, which is comprised of scores of steatosis, hepatocyte ballooning and lobular inflammation. Blockade of IL-1β did not improve these aspects of NASH (**Figure 4B**).

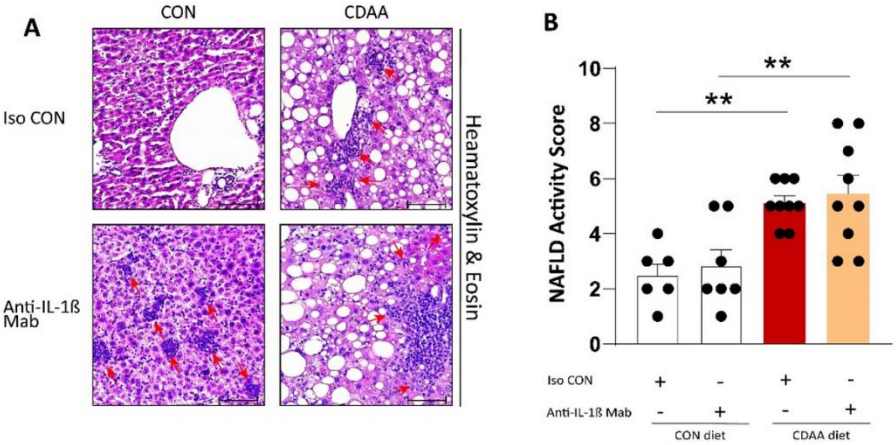

Figure 4 – Evaluation and scoring key features of NASH.
Hematoxylin-eosin staining was performed to investigate the level of steatosis, the extent of inflammatory infiltrations and hepatocellular ballooning, which was followed by a comprehensive scoring. Two-way ANOVA, Fisher's LSD post hoc test, $*P < 0.05$

4.1.4. NASH and inhibition of IL-1β might facilitate a pro-tumorigenic microenvironment

Quantitative RT-PCR revealed that CDAA-diet fed had upregulated four major hepatic proto-oncogenes: *Myc*, *Gpc3*, *Mki67* and *Pcna*.

Furthermore, the three most described immune checkpoints were investigated. In mice with NASH, the expression of *Pd-l1* increased, while the blockade of IL-1β increased the expression of *Pd-1* and *Ctla4* (**Figure 5**).

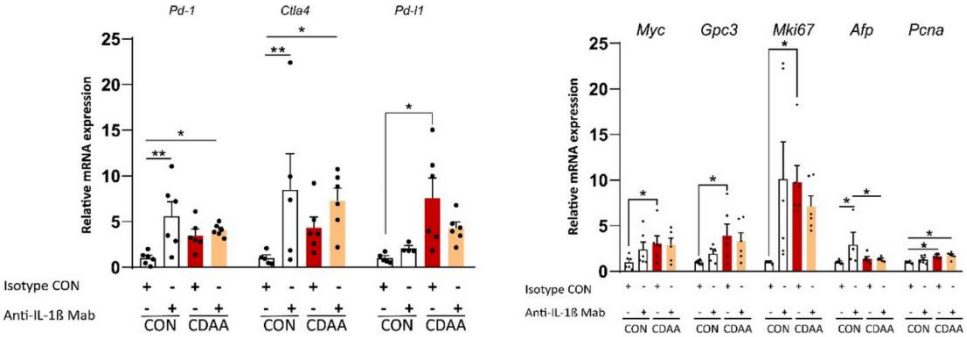

Figure 5 – qRT-PCR analyses of major immune checkpoints and hepatic proto-oncogenes. Both the diet and the treatment affected the microenvironment of the liver, presumably causing an immunosuppressive state. Two-way ANOVA, Fisher's LSD post hoc test. $*P < 0.05$.

4.2. Sexual differences in key features of NASH

Sex is an important disease modifying factor. As such, it is important to investigate molecular mediators of NASH pathophysiology to better understand the sexual disparity of the disease. Sex-specific therapies might improve the success of pharmacotherapies.

4.2.1. Expression of Pcsk9 and its major targets are sex-dependently different

Quantitative RT-PCR analysis showed that female mice have higher gene expression of *Pcsk9* than males, while females with NASH have significantly lower expression (**Figure 6A**). These data were reaffirmed with ELISA measurements (**Figure 6B**). LDL-receptor and CD36 are major targets of PCSK9. Gene expression pattern of *Ldlr* was similar to expression of *Pcsk9*. Females had higher transcriptional level of *Cd36*, while females with NASH had even higher level (**Figure 6A**).

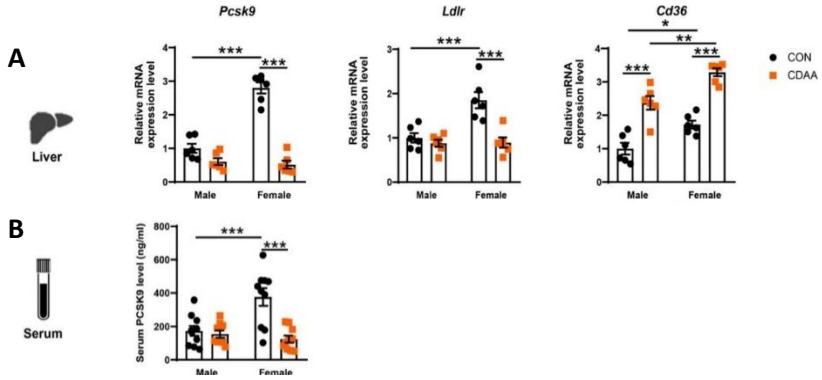

Figure 6 – Sex-dependent expression of Pcsk9, Ldlr and Cd36.
Hepatic expression of major factors of cholesterol metabolism shows sex dependent expression pattern. Two-way ANOVA followed by Tukey's post hoc test, $*P < 0.05$.

4.2.2. *Males with NASH have more extensive fibrosis than females*

Quantification of picrosirius red staining of liver section showed that male mice with NASH had higher level of fibrosis. Accordingly, gene expression of *Ctgf* was also higher in males with NASH. The transcriptional level of collagen type I and III showed no sex-dependent difference (**Figure 7**).

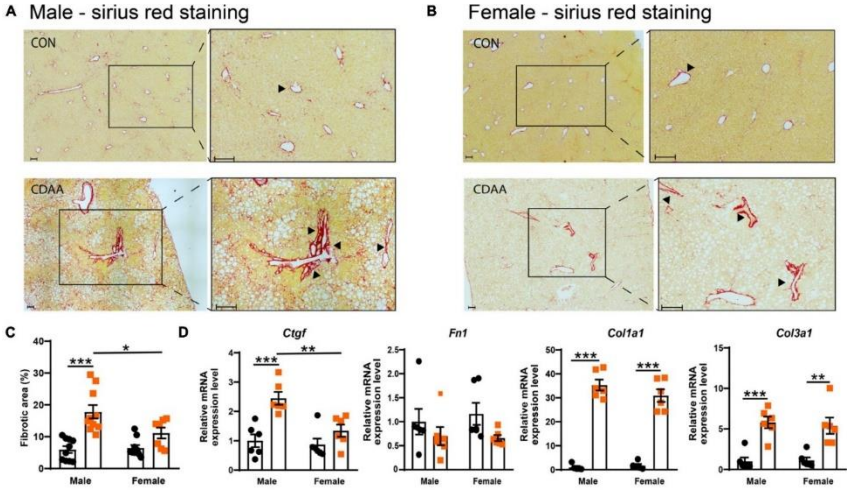

Figure 7 – Sex differences in hepatic fibrosis.
Quantification of overall fibrosis, and qRT-PCR analysis of major pro-fibrotic genes. Two-way ANOVA followed by Tukey's post hoc test, $*P < 0.05$

4.2.3. *Females with NASH have a more pro-inflammatory phenotype*

Quantitative RT-PCR revealed increased expression of *Tnfa*, *Cd68*, *Ccl2 and Ccr1* in both sexes. In contrast, expression of *Il1b* and *Ifng* was higher in females with NASH (**Figure 8A**). Next, we performed western blot analyses to identify that inflammasome that is responsible for the activation of IL-1β. NLRC4 and AIM2 were not active in our model, but NLRP3 was. Although the premature form of IL-1β was elevated in both

sexes, only in females with NASH was cleaved into the active mature form (**Figure 8B, C**).

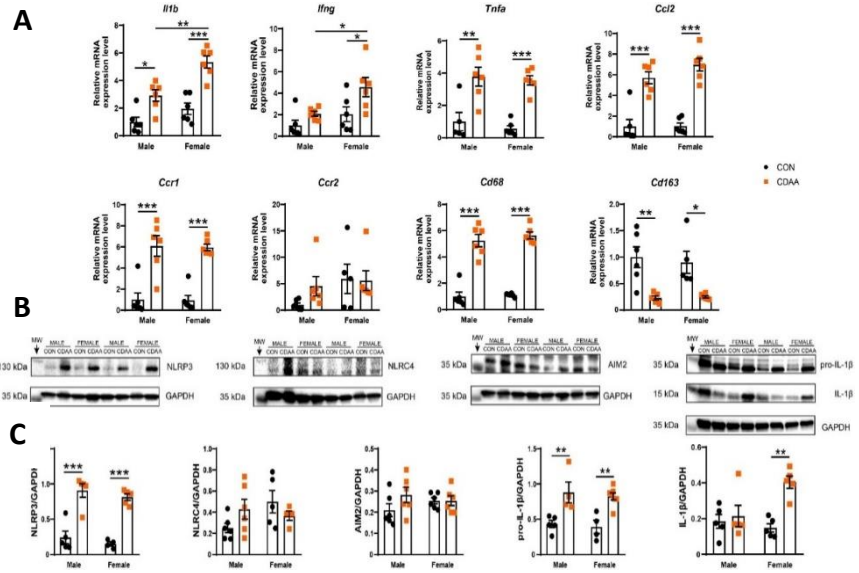

Figure 8 – Sexual differences in liver inflammation.
PCR analysis of major pro-inflammatory factors and western blot of major inflammasome sensors reveal a more intensive inflammation in female mice with NASH. Two-way ANOVA followed by Tukey's post hoc test. *P < 0.05

5. Conclusion

Interlukin-1β is an important factor to inflammation linked to cardiometabolic diseases, such as obesity, type II diabetes, metabolic syndrome, and non-alcoholic steatohepatitis. We showed that interleukin-1β blockade was able to improve diastolic deterioration of mice with NASH but failed to ameliorate key features of NASH. It is likely that the treatment facilitated the development of an immunosuppressive microenvironment with the potential risk to further deteriorate into a premalignant state.

Additionally, we found that male mice with NASH were characterized by extensive hepatic fibrosis, while females with NASH showed higher level expression of inflammatory molecules.

All in all, we might conclude that interleukin-1β is not a viable pharmaceutical target for NASH. In the future, maybe males might benefit more from anti-fibrotic therapy, while females might require anti-inflammatory treatment.

6. Bibliography of candidate's publications

6.1. Publications related to the candidate's PhD dissertation

I. Dániel Kucsera, Viktória E. Tóth, Nabil V. Sayour, Tamás Kovács, Tamás G. Gergely, Mihály Ruppert, Tamás Radovits, Alexandra Fábián, Attila Kovács, Béla Merkely, Péter Ferdinandy, Zoltán V. Varga (2023). "IL-1β neutralization prevents diastolic dysfunction development, but lacks hepatoprotective effect in an aged mouse model of NASH" *Scientific Reports.* **IF: 4.997**

II. Dániel Kucsera, Viktória E. Tóth, Dorottya Gergő, Imre Vörös, Zsófia Onódi, Anikó Görbe, Péter Ferdinandy, Zoltán V. Varga (2021). "Characterization of the CDAA Diet-Induced Non-alcoholic Steatohepatitis Model: Sex-Specific Differences in Inflammation, Fibrosis, and Cholesterol Metabolism in Middle-Aged Mice" *Frontiers in Physiology*. **IF: 4.755**

Sum of impact factors of dissertation-related publications: 9.752

6.2. Publications not related to the candidate' PhD dissertation

III. Tamás G. Gergely, Dániel Kucsera, Viktória E. Tóth, Tamás Kovács, Nabil V. Sayour, Zsófia D. Drobni, Mihály Ruppert, Balázs Petrovich, Bence Ágg, Zsófia Onódi, Nóra Fekete, Éva Pállinger, Edit I. Buzás, Laura I. Yousif, Wouter C. Meijers, Tamás Radovits, Béla Merkely, Péter Ferdinandy, Zoltán V. Varga (2022). "Characterization of immune checkpoint inhibitor-induced cardiotoxicity reveals interleukin-17A as a driver of cardiac

dysfunction after anti-PD-1 treatment" *British Journal of Pharmacology*. **IF: 9.473**

IV. Zsófia Onódi, Mihály Ruppert, Dániel Kucsera, Alex Ali Sayour, Viktória E. Tóth, Gábor Koncsos, Julianna Novák, Gábor B. Brenner, András Makkos, Tamás Baranyai, Zoltán Giricz, Anikó Görbe, Przemyslaw Leszek, Mariann Gyöngyösi, Iván G. Horváth, Rainer Schulz, Béla Merkely, Péter Ferdinandy, Tamás Radovits, Zoltán V. Varga (2021). "AIM2-driven inflammasome activation in heart failure" *Cardiovascular research.* **IF: 14,239**

V. Judit Szepesy, Gabriella Miklós, János Farkas, Dániel Kucsera, Zoltán Giricz, Anita Gáborján, Gábor Polony, Ágnes Szirma, László Tamás, László Köles, Zoltán V. Varga, Tibor Zelles (2020). "Anti-PD-1 Therapy Does Not Influence Hearing Ability in the Most Sensitive Frequency Range, but Mitigates Outer Hair Cell Loss in the Basal Cochlear Region" *International Journal of Molecular Sciences.* **IF: 5.924**

Sum of all impact factor: 39.388