Univerza v Ljubljani
Fakulteta za farmacijo

KAJA SAJKO

MAGISTRSKA NALOGA

ENOVITI MAGISTRSKI ŠTUDIJ FARMACIJA

Ljubljana, 2017

Univerza v Ljubljani
Fakulteta za farmacijo

KAJA SAJKO

OPTIMIZACIJA IN VREDNOTENJE AKTIVNOSTI TRANSGLUTAMINAZE 2 V ″IN VIVO″ MODELIH

OPTIMISATION AND EVALUATION OF THE TRANSGLUTAMINASE 2 ACTIVITY ON ″IN VIVO″ MODELS

MASTER'S THESIS

UNIFORM MASTER'S STUDY PROGRAMME PHARMACY

Ljubljana, 2017

I performed my master's thesis at the Institute of Translational Immunology, University Medical Center of the Johannes Gutenberg University Mainz, under the mentorship of prof. dr. Borut Božič, mag. pharm., spec. med. biochem. and co-mentorship of prof. dr. dr. Detlef Schuppan, M.D., Ph.D.

Acknowledgment

I would like to thank prof. Borut Božič for professional leading, advice and help with my thesis and prof. Detlef Schuppan for accepting me into his working group and giving me the opportunity to work and learn in his laboratories.

A special thank goes to my supervisor Manuel Encalada for everything that I have learned from him, for doing the treatment and sacrifice of the animals for my project, help with the preparation of the schemes and guiding with my thesis. Thank you for making my research time in Germany unforgettable.

I would also like to thank all my co-workers in AG Schuppan for the good working atmosphere.

My university years would have never been the same without my dear friends and all the moments we have spent together.

Mostly I would like to thank my loving parents and sister Nika for supporting me and all my ideas the whole life, for trusting in me even when I did not.

Declaration

I declare that I performed the master's thesis alone under the mentorship of prof. dr. Borut Božič, mag. pharm., spec. med. biochem. and co-mentorship of prof. dr. dr. Detlef Schuppan, M.D., Ph.D.

Kaja Sajko

Ljubljana, 2017

President of the commission: prof. dr. Marija Bogataj

Member of the commission: doc. dr. Nace Zidar

CONTENTS

ABSTRACT VI
POVZETEK VIII
List of abbreviations XI
1. INTRODUCTION 1
1.1. Celiac disease 1
1.1.1. Genetics 1
1.1.2. Pathogenesis 2
1.1.3. Diagnosis 3
1.1.4. Clinical signs and symptoms 5
1.1.5. Therapy 5
1.2. The role of transglutaminase 2 in CD 9
1.2.1. Localisation and functions of TG2 in the body 9
1.2.2. Measurement of TG2 activity 11
1.2.3. Induction of TG2 activity 12
2. AIM OF THE STUDY 16
3. MATERIALS AND METHODS 17
3.1. Chemicals and solvents 17
3.2. Solutions 18
3.3. Laboratory equipment 19
3.4. Animal experiments 20
3.4.1. Mouse strains 20
3.4.2. Sacrifice procedure and harvesting of the tissue 21
3.5. Transglutaminase extraction 22
3.6. In-house transglutaminase activity assay 22
3.7. Protein assay-BCA protocol 23
3.8. RNA purification 25
3.9. cDNA synthesis 26
3.10. Polymerase chain reaction in real time 26
3.11. Storage conditions of the samples before TG2 extraction 27
3.12. Determination of buffers 28
3.12.1. Buffer used for washing the tissue 29

3.12.2. Triton concentration in lysis buffer 29
3.12.3. Calcium concentration in diluent buffer 29
3.13. Induction of intestinal inflammation 29
4. RESULTS AND DISCUSSION 31
4.1. Optimisation of TG2 activity ″in vivo″ 31
4.1.1. Sectioning and flushing 31
4.1.2. Buffer used for washing the tissue 34
4.1.3. Storage conditions of the samples before TG2 extraction 36
4.1.4. Triton concentration in lysis buffer and dilution of samples 38
4.1.5. Calcium concentration in diluent buffer 39
4.1.6. Handling with the aliquots after the extraction and use 40
4.2. ″In vivo″ evaluation of TG2 activity 41
4.2.1. Mouse strain 42
4.2.2. ″In vivo″ activation of TG2 45
5. CONCLUSION 48
REFERENCES 50
APPENDIX 56

ABSTRACT

Introduction: Celiac disease is a chronic autoimmune inflammatory disorder affecting approximately 1% of most populations worldwide. Transglutaminase 2 (TG2) is an enzyme that is involved in its main known pathologic mechanism, which makes it an attractive target for the therapy.

Aim: TG2 inhibitors might be a potential therapeutic approach for celiac disease. To test one of them, ZED 1227, the research group at the Institute of Translational Immunology, Mainz, is developing an animal model. As part of this project, we participated in the optimisation and standardisation of the method to measure the activation of TG2 ″in vivo″, which consisted of enhancement of the activity and measurement of activation.

Materials and methods: For the study we used Black 6, Toll-like receptor 4 knockout and Balb/C mice, which were intraperitoneally injected indomethacin, polyinosinic-polycytidylic acid (poly I:C) or only buffers as a control. We evaluated TG2 activity in the tissue using an ELISA-like assay. Protein content was determined by bicinchoninic acid protein assay and for the gene expression we used polymerase chain reaction in real time.

Results and discussion: We standardised the procedure of sectioning and flushing the different sections of the gut to differentiate the parts of the intestine and avoid any influence of the pH on the enzymatic activity. To maintain or preserve the active enzyme, three solutions as washing buffer were tested to confirm that phosphate buffer solution gave optimal results in comparison to KREBS and HEPES buffers. Another way of tissue preservation was sectioning on ice and immediate snap freezing of the sections in liquid nitrogen, before storing them at -80°C. For TG2 extraction, lysis was performed first. The influence of different detergent concentration in lysis buffer on TG2 activity was assessed, for which the optimum was 1% Triton. TG2 is a calcium dependant enzyme, so the impact of its concentration in the diluent buffers used was tested. There was no significant difference between the tested concentrations, so we kept on using the original concentration. Loss of activity occurred after each use of the extracted samples; therefore, fresh extracts were divided into three aliquots. Each could only be used twice, as the activity of a third use was much lower. After optimisation, duodenum, jejunum and terminal ileum of three mouse strains were tested for the TG2 activation, but results did not show significant differences between strains. In the intestinal inflammation model with poly I:C, the TG2 activity and expression were induced mainly in the terminal ileum, but

inhibited in the duodenum. As an alternative model, induction of TG2 activity with indomethacin was evaluated. It resulted in inhibition of both, TG2 activity and gene expression.

Conclusion: Even after optimisation, our models did not show significant difference between treated animals and controls in all tissues, although poly I:C was previously reported to enhance TG2 activity. Since we destroyed the tissue first, we have possibly measured extracellular and intracellular TG2. Thus, we need to look for an alternative method to evaluate in situ TG2 activity that would give us the relevant result for activity of only the extracellular enzyme that is a critical cofactor for gluten induced immune activation in celiac patients.

Key words: celiac disease, transglutaminase 2, animal model, indomethacin, poly I:C

POVZETEK

Uvod: Celiakija je kronična avtoimunska vnetna bolezen tankega črevesja, ki prizadene približno 1% svetovnega prebivalstva. Glutenski peptidi, ki se nahajajo v nekaterih žitih, pri stiku s celicami imunskega sistema bolnikov povzročijo imunski odziv s sprostitvijo vnetnih citokinov in nastajanjem specifičnih protiteles proti glutenu in transglutaminazi 2 (TG2). Le-ta je encim, ki deamidira nevtralne glutenske preostanke in jih pretvori v negativno nabite delce. Ti se močneje vežejo na molekule HLA ter sprožijo imunsko reakcijo, ki povzroči sprostitev vnetnih citokinov in poškodbo tkiva. Trenutno je edini način obvladovanja bolezni stroga brezglutenska dieta, zaradi katere je kvaliteta življenja bolnikov močno zmanjšana. Ker neupoštevanje diete lahko privede do resnejših zdravstvenih zapletov, je pomembno, da se bolezen ustrezno zdravi.

Namen dela: Znanstveniki proučujejo različne načine obvladovanja celiakije, pri čemer je velik poudarek na inhibitorjih transglutaminaze 2, ki bi preprečili glavni mehanizem imunskega odziva na gluten. Eden izmed njih je ZED 1227, ki ga je sintetiziralo podjetje Zedira. Za ″in vivo″ testiranje učinkovine in določitev odmerka sta potrebna ustrezen živalski model aktivacije encima in metoda za vrednotenje njegove aktivnosti. Namen magistrske naloge je optimizacija metode za ″in vivo″ aktivacijo in ″ex vivo″ določitev aktivnosti transglutaminaze 2 v tkivih žrtvovanih miši.

Materiali in metode: Pri izvedbi smo uporabili 6-8 tednov stare laboratorijske miši, in sicer divji tip C57BL/6 ter gensko spremenjeni liniji B6 TLR4 -/- in BalbC. 12 ur pred žrtvovanjem je bil testnim živalim intraperitonealno apliciran indometacin ali spojina poly I:C, pripadajočim kontrolnim skupinam pa le pufer, v katerem je bila spojina raztopljena. Hkrati jim je bila tudi odvzeta hrana. Po 12 urah so bile žrtvovane. Odvzeli smo tkivo duodenuma, jejunuma, terminalnega ileuma, kolona in jeter, ga očistili, sekcionirali in ustrezno shranili za kasnejše analize. V sklopu optimizacije metode smo določali način sekcioniranja tkiva, pri katerem smo dobili najbolj ponovljive in stabilne rezultate. Primerjali smo vpliv pogojev dela in shranjevanja tkiva pred ekstrakcijo encima na ohranitev aktivnosti.

Aktivnost TG2 smo določali z biokemijskim postopkom, ki temelji na kolorimetrični reakciji. Gre za določitev celokupnih transglutaminaz, a je dokazano, da predstavlja TG2 največji delež. Substratu dimetilkazeinu smo dodali razredčen vzorec tkiva, ga inkubirali s hrenovo peroksidazo označeno s streptavidinom, nato dodali kromogeni substrat

tetrametilbenzidin in reakcijo po inkubaciji ustavili z žveplovo kislino. Absorbanco vzorcev smo izmerili spektrofotometrično in glede na maso posameznega vzorca izračunali relativno aktivnost encima v tkivu. Na nekaterih vzorcih smo izvedli tudi test na vsebnost proteinov, s katerim smo ovrednotili prisotnost ekstrakta v testni plošči, ter s tem uspešnost ekstrakcije. Pri določitvi najustreznejše snovi za indukcijo encima smo poleg meritve aktivnosti izvedli še verižno reakcijo s polimerazo (PCR) za analizo izražanja gena za TG2, ki smo ga izračunali glede na hišni gen GAPDH.

V sklopu več poskusov smo spreminjali pogoje in izmed treh raztopin izbirali najbolj optimalen pufer za spiranje tkiva. Zaradi hitre izgube aktivnosti encima po odvzetju tkiva iz telesa živali, smo primerjali vpliv shranitve sekcij pred ekstrakcijo: v tekočem dušiku, pri -80°C ali takojšnjo ekstrakcijo brez vmesne shranitve. Nato smo preverili vpliv spremembe koncentracije kalcija v razredčevalnem pufru, saj je TG2 encim, katerega aktivnost je odvisna od prisotnosti kalcija. Izvedli smo tudi poskus z dvema koncentracijama detergenta v pufru za lizo, ter izbrali optimalno za nadaljnje poskuse. S serijo testov aktivnosti TG2 z istimi alikvoti ob različnih dnevih smo ugotavljali izgubo aktivnosti po večkratni uporabi istega alikvota.

Po optimizaciji metode smo določali aktivnost transglutaminase 2 v ″in vivo″ modelih treh mišjih linij, tako da smo primerjali odziv kontrolne skupine s skupino, ki smo ji aplicirali poly I:C. Na koncu smo na liniji B6 izvedli poskus z uporabo indometacina, oziroma poly I:C, ter s hkratno PCR analizo določali vpliv na aktivnost in izražanje transglutaminaze 2.

Rezultati in razprava: Najprej smo s pomočjo predhodnih rezultatov oddelka, znanstvenih člankov in anatomskih podatkov o miših pripravili načrt sekcioniranja, ki je vključeval natančno dolžino in predel posameznih delov tkiva, ki smo ga odvzeli. Standardizacija odvzema vzorcev je bila pomembna zaradi variiranja aktivnosti TG2 v različnih delih posameznega tkiva. Da bi preprečili vpliv pH lumna prebavnega trakta na aktivnost encima, smo določili spiranje notranjosti odvzetih delov prebavnega trakta s pufrom v smeri, ki smo jo izbrali glede na čimmanjši učinek na tkivo. Za duodenum in terminalni ileum je to bilo v smeri proti želodcu, jejunum in kolon pa distalno od želodca.

Pri določitvi optimalnega pufra za spiranje smo izbrali PBS, saj rezultati s KREBS-ovim ali HEPES-ovim pufrom niso izkazali izboljšane zaznave. Izbrani način shranitve tkiva odvzetih sekcij pred ekstrakcijo je bil takojšnja zamrznitev v tekočem dušiku in nato prenos v zamrzovalnik pri -80°C. Kljub majhni izgubi aktivnosti v primerjavi s takojšnjo ekstrakcijo po odvzetju ali ekstrakcijo po kratkotrajni shranitvi v tekočem dušiku, smo se

odločili za ta način, saj bi bilo pri večjem številu vzorcev za enega izmed ostalih dveh načinov potrebnih več raziskovalcev, ki bi hkrati izvedli poskus, kar ni vedno izvedljivo. Prav tako smo ugotovili, da je rezultat še vedno relevanten za študijo. Glede ne območje rezultatov meritev smo kot najustreznejšo koncentracijo tritona v lizirnem pufru določili 1% raztopino, pipetirano v merilno ploščo pri razredčitvi 1:50. Višja koncentracija kalcija v razredčevalnem pufru ni doprinesla k višji, oziroma bolje ohranjeni aktivnosti encima, zato je v nadaljevanju nismo spreminjali, ampak smo pri poskusih vedno uporabljali 5 mM raztopino. Z rezultati določitev aktivnosti istih alikvotov po več uporabah smo ugotovili, da lahko isti alikvot za meritev aktivnosti uporabimo le dvakrat, če želimo, da so rezultati še primerljivi, saj je pri tretji uporabi aktivnost zaradi večkratnega odtajanja in zamrzovanja močno padla, ter rezultati niso bili več reprezentativni.

V okviru ″in vivo″ določitve aktivnosti transglutaminase 2 ni bilo razlik med linijami miši, na podlagi katerih bi lahko eno izmed njih določili kot ustreznejšo. Uporaba indometacina in poly I:C je privedla do zanimivih rezultatov, saj so živali, ki so prejele indometacin, imele nižjo ekspresijo gena za TG2, prav tako pa tudi nižjo aktivnost encima, kar pomeni, da je prišlo do nasprotnega rezultata, kot smo pričakovali. Glede na to, da je indometacin protivnetna učinkovina, je lahko bil ta učinek v miših močnejši kot destruktivni učinek na črevesje. Za natančnejšo določitev bi bilo smiselno narediti še histološko analizo, česar pa nam časovni okvir moje magistrske naloge ni dopuščal in bo narejena kasneje. Pri poly I:C je bil rezultat aktivnosti TG2 in izraženosti gena zanjo v duodenumu tretiranih živali nekoliko nižji od kontrolnih, v terminalnem ileumu pa je aktivnost pri tretiranih živalih malo, izražanje gena pa močno narastlo.

Zaključek: Glede na rezultate, ki smo jih dobili kljub optimizaciji, lahko sklepamo, da uporabljena metoda ni najprimernejša za določitev aktivnosti TG2 v tkivu in evalvacijo inhibitorjev. V prihodnje bi bilo potrebno opraviti histološke analize in preveriti ustreznost ex-vivo metod aktivacije in vrednotenja aktivnosti encima, saj bi zaradi lastnosti transglutaminaze 2 lahko bile te metode bolj uporabne za testiranje inhibitorjev.

Ključne besede: celiakija, transglutaminaza 2, živalski model, indometacin, poly I:C

List of abbreviations

5-BP	5-(biotinamido)pentylamine
Asn	asparagine
Asp	aspartate
β-ME	β-mercaptoethanol
B6	black 6 (mouse strain)
BCA	bicinchoninic acid
C	colon
CD4	cluster of differentiation 4
cDNA	complementary deoxyribonucleic acid
Cys	cysteine
D	duodenum
DGP	deamidated gliadin peptide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsRNA	double stranded ribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMA	anti-endomysial antibodies
GDP	guanosine 5′-diphosphate
GMP-PCP	βγ-methyleneguanosine 5′-triphosphate
GTP	guanosine-5'-triphosphate
GTPγS	guanosine-5'-O-(gamma-thio)triphosphate
H	liver (hepatic tissue)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	histidine
HLA	human leukocyte antigen
HRP	horseradish peroxidase
IFN- γ	interferon γ
IgA, IgG	immunoglobuline A, immunoglobuline G

IKK-ε	IkappaB kinases-epsilon
IL-15	interleukin 15
IRF-3	interferon regulatory factor 3
ISRE	interferon-stimulated regulatory element
J	jejunum
k_{cat}	catalytic constant
k_i	kinetic parameter
K_I	inhibitor-enzyme complex dissociation constant
K_M	Michaelis constant
NF-κB	nuclear factor kappa B
NSAID	non-steroidal anti-inflammatory drugs
PBS	phosphate buffer solution
PCR	polymerase chain reaction
poly I:C	polyinosinic-polycytidylic acid
RIP-1	receptor-interacting serine/threonine-protein kinase 1
RT- PCR	polymerase chain reaction in real time
TBK1	TRAF-family-member-associated NF-κB activator binding kinase 1
TG2	transglutaminase 2
Th1, Th2	T-helper cell 1, T-helper cell 2
TI	terminal ileum
TIR	toll/interleukin-1 receptor
TLR	Toll-like receptor
TNF-α	tumor necrosis factor α
TRAF	tumor necrosis factor receptor-associated factor
TRIF	TIR-domain-containing adapter-inducing interferon-β
TRIS	tris(hydroxymethyl)aminomethane

1. INTRODUCTION

1.1. Celiac disease

Celiac disease, also known as celiac sprue, gluten-sensitive enteropathy, or nontropical sprue, is a chronic autoimmune inflammatory disorder with prevalence around 1% in the general population of most countries and ethnicities. The disease is more common in women than in men, since women represent 60%–70% of all clinically diagnosed patients (1). In former years celiac disease was diagnosed mostly in children and young adults, but lately the incidence in adults, especially the elderly, is growing. This is mainly due to the increasing use of serological screening, since in many adult and juvenile patients the disease shows atypical symptoms or even no overt symptoms at all, which makes it hard to diagnose (2, 3).

1.1.1. Genetics

Genetics play an important role in the development of celiac disease. This was confirmed by studies that found a 75% concordance rate in monozygotic twins and a 10-15% prevalence rate between first-degree relatives of patients with celiac disease (4). The main genetic factors are specific major histocompatibility complex class II alleles. They are located on the human leukocyte antigen (HLA)-DQ locus, specifically 6p21.3, which is the short arm of chromosome 6 (5). These molecules are coding for cell surface glycoproteins, needed for self-recognition by immune cells and presentation of antigens (5, 6). Most common antigen-presenting cells that present HLA molecules are B-cells, dendritic cells and macrophages (7).

Possessing HLA-DQ alleles is necessary, but not sufficient, for development of the disease (8). 90-95% of patients are carriers of HLA-DQ2 with an α-chain that is encoded by HLA-DQA1*05 and a β-chain that is encoded by HLA-DQB1*02, being either in cis or trans configuration (5). The other 5-10% of patients possess the HLA-DQ8 gene, with one chain being HLA-DQA1*0301 and the other HLA-DQB1*0302, in cis configuration (9).

Data indicate that 25-30% of healthy controls also possess the HLA-DQ2 genes and another 5-15% the HLA-DQ8 genes, which may suggest that there are additional, non-HLA genetic and especially environmental factors that determine the manifestation of

celiac disease. Some identified low penetrance genes are related to cell adhesion molecules, cytokines, chemokines and their receptors, B- and T-cell activators. Since their involvement in the disease is below 15%, they are not used for determination of genetic risk (5). Positive celiac disease associated HLA genetic test means that a person has a genetic predisposition, but only ~3% of gene carriers will develop the disease. In contrast, the absence of HLA-DQ2 or HLA-DQ8 genes excludes the possibility of developing the disease with 100% certainty (10). This is the reason for genetic testing to be used as a negative predictive factor for celiac disease, which is useful for patients on a gluten free diet, without a clear prior diagnosis of celiac disease (4).

1.1.2. **Pathogenesis**

After food ingestion, most proteins are broken down in the intestinal tract by enzymes into small fragments that are not immunogenic. Gliadin, glutenin, secalin and hordein, gluten peptides found in pathogenic (gluten containing) grains, have high content of proline and glutamine and are not completely digested by gastric and small intestinal enzymes. The resultant peptides can partly cross the intestinal epithelium by transcytosis or through epithelial tight junctions that have increased permeability, and then enter into the intestinal mucosa, where they become substrates for the enzyme transglutaminase 2 (TG2). This enzyme deamidates certain neutral glutamine residues, changing them via deamidation into glutamic acid with a negative charge. Gluten peptides with proline residues and a negative charge bind more tightly to HLA-DQ2 or HLA-DQ8 molecules that are presented on antigen-presenting cells (7, 11). Presentation of these peptides activates pathogenic cluster of differentiation 4 (CD4+) T helper cells. The T-helper cells type 1 (Th1) response leads to the release of inflammatory cytokines, such as interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α), which results in intestinal inflammation with villus atrophy and crypt hyperplasia (7). Activated CD4+ T-cells release interleukin-18 (IL-18) that polarizes and maintains Th1 response, and IL-21, that increases lamina propria T-cells production of IFN-γ through an autocrine amplification loop (12). IFN-γ enhances production and release of matrix metalloproteinases which degrade tissue matrix and cause mucosal remodelling (7).

Activated T-cells also enhance B-cells via the Th2 response to produce autoantibodies against gluten and TG2 (7).

On the other hand toxic gluten deamidated proteins activate innate immunity. They up-regulate secretion of interleukin 15 (IL-15) in enterocytes and lamina propria mononuclear cells. Released IL-15 leads to up-regulation of natural killer receptors and their ligands and activation of intraepithelial lymphocytes, which induces increased epithelial permeability and apoptosis. Another pathway of innate immunity is via the Toll-like receptor 4 (TLR-4) on dendritic cells or macrophages. TLR-4 recognizes gluten peptides leading to maturation of dendritic cells or macrophages and production of inflammatory cytokines (7).

1.1.3. **Diagnosis**

The standard diagnose is based on the presence of serum antibodies, specific for celiac disease, together with villus atrophy and crypt hyperplasia found with concomitant intestinal biopsy. Individuals with celiac disease display different symptoms, which are not necessarily intestinal, therefore it is often to be diagnosed very late or remain undiagnosed. Symptomatic children can be diagnosed without biopsy if they have signs typical for malabsorption, as well as test IgA transglutaminase 2 (IgA-TG2) antibody levels more than 10-times higher than the upper limit of normal, and if they have positive result for either anti-endomysial (EMA) antibodies or deamidated gliadin peptide (DGP) antibodies in second blood sample (13).

1.1.3.1. **Biopsy and histology**

Small intestinal endoscopy with biopsy is important tool to recognize lesions in celiac disease patient and get samples for histological analysis. For an accurate diagnosis, patients have to be on a normal diet with gluten for at least 2 weeks before examination, while consuming 3 or more grams of gluten per day (13).

Villous atrophy can usually be seen with endoscopy, but histology is needed to prove the diagnosis. Sampling of mucosa is done four to six times in second part of duodenum and duodenal bulb (13). Samples, fixed with formalin, embedded in paraffin and stained with haematoxylin and eosin, are checked under light microscope, where typical histology changes can be seen, such as an increased number of intraepithelial lymphocytes, villous atrophy and crypt hyperplasia with decreased villi/crypt ratio, lamina propria infiltration with mononuclear cells and changes of epithelium with abnormal epithelial cells structure. Mucosal damage is characterized by Marsh classification, using types from 0, which means normal mucosa, to 3c, that is used to describe complete villous atrophy (13, 14).

1.1.3.2. Serological tests for serum autoantibodies

a. Anti-endomysial antibodies (IgA-EMA)

Serological test with anti-endomysial antibodies is used as one of the tests for the diagnosis of celiac disease. It is also performed to follow up the disease and successfulness of a gluten-free diet. The test is based on the binding of endomysial antibodies to monkey esophagus sections (13). The substrate is incubated with patient serum in different dilutions. Anti-IgA antibodies which are conjugated with fluorescein are added after incubation and the fluorescence is measured with a fluorescent microscope using indirect immunofluorescent assay (13, 15). Results of the tests with this method have around 80% diagnostic sensitivity and almost 100% diagnostic specificity for untreated celiac disease (13).

b. Anti-transglutaminase 2 antibodies (IgA-TG2 and IgG-TG2)

Anti-TG2 antibodies have high diagnostic specificity and diagnostic sensitivity for celiac disease and are also used for diagnosis and follow-up. Due to high diagnostic sensitivity (93%) of the results, this test is used as the main screening method. It is not as expensive as anti IgA-EMA and easier to perform. Because of its availability, it is often used, but there are some variabilities between different commercial kits. The procedure is based on enzyme-linked immunosorbent assay (ELISA), using a standard curve of recombinant human TG2.

For patients that are IgA deficient, the IgG-TG2 test is used (13, 16).

c. Anti deamidated gliadin peptides antibodies (IgA-DGP and IgG-DGP)

Detection of anti deamidated gliadin peptides antibodies is based on enzyme-linked immunosorbent assay (ELISA), which detects synthetically developed deamidated gliadin peptides (DGPs) (13). Results obtained by this test are highly accurate for diagnosis in low-risk and high-risk populations. The test is used as a diagnostic tool in combination with an anti-transglutaminase 2 antibodies test, as it was discovered that some of the patients who tested negative for TG2 antibodies had a positive anti-DGP test, which was followed by biopsy that confirmed diagnosis of celiac disease (17).

1.1.3.3. Genetic test

For a correct diagnosis of celiac disease, a test of the genetic predisposition is necessary (4). The genetic test that is being used is HLA-DQ2/-DQ8 genotyping. Results from the

testing have an almost 100% negative predictive value, which means that a person who tests negative cannot develop the disease. Positive results do not confirm the disease, but only show the possibility for a patient to develop it (4).

1.1.4. **Clinical signs and symptoms**

Typical clinical signs are gastrointestinal problems, including chronic diarrhea, abdominal pain, vomiting, distension, steatorrhea, gaseousness and malabsorption. Extraintestinal indicators of the disease are usually the consequence of malabsorption, which eventually leads to weight and muscle loss, growth failure and failure to thrive, as well as iron-deficiency causing fatigue and malaise. Lack of vitamins and minerals due to poor absorption in damaged intestinal tissue can result in bone- or joint-related disorders, typically osteoporosis, osteopenia and inflammation of joints. The disease can further manifest itself in non-classical signs and symptoms, such as neurological disorders, dermatological manifestations and gynaecological problems (13, 18, 19).

1.1.5. **Therapy**

Since there is no other treatment than the gluten-free diet, pharmaceutical industry and researchers are trying to find different ways to prevent damaging effects of gluten on patient's intestine and develop a medication to treat the disease.

1.1.5.1. **Gluten-free diet**

Following a gluten-free diet means that patients should not consume any kind of wheat, barley, bran, bulgur, kamut, malt, rye, spelt, triticale and products made of them. Oats are allowed only if labelled gluten-free, since they are often contaminated with gluten (13).

a. FOOD LABELLING

The European commission defines labelling of food in the ″COMMISSION IMPLEMENTING REGULATION (EU) No 828/2014 of 30 July 2014 on the requirements for the provision of information to consumers on the absence or reduced presence of gluten in food″. The document determines labelling as followed:

″Very low gluten″: food that contains less than 100 mg of gluten per kilogram.
″Gluten-free″: food that contains less than 20 mg of gluten per kg (20).

1.1.5.2. Treatments under-development

Many different ways of treatment are being under development, but none of them are used in therapy yet. Some research groups have focused on conversion of toxic gluten cereals into harmless, which would allow patients to eat them. Ancient or genetically modified variants of wheat that have low immunogenicity have the potential to be used in a diet without causing immune response. In order to detoxify flour, researchers are trying to do the transamidation of gliadin or pre-treat flour with lactobacilli, which would result in peptidase hydrolisation of peptides rich with proline. Hydrolysed peptides would not be recognized by TG2, so there would not be a toxic immune response (7, 21).

Other possible treatments are targeting ingested gliadin peptides. Polymers that would bind intraluminal gliadin and prevent its digestion to immunogenic peptides are being tested, but there is a problem with specificity, as some other proteins could also bind to these polymers. Clinical studies are being performed with cow's milk autoantibodies that would neutralize gluten and with prolyl endopeptidases from different microorganisms to degrade it. To prevent transepithelial uptake of toxic gluten, zonula occludens toxin-receptor antagonist was synthetized, targeting epithelial tight junctions. If used in therapy it should be combined with other treatments, as its effect is expected to be limited (7).

Adaptive immune response plays a big role in pathogenesis of celiac disease, so attempts to inhibit human leukocyte antigen HLA-DQ2 by analogues of a peptide have been performed, but there were problems with specificity, affinity, toxicity and immunogenicity of the compounds, so there are still a lot of researches to be done (7).

Different systemic T-cell or cytokine blockers are now used in clinical studies as they could be relevant as forms of biological therapy. To modulate the immune system and stop pathological reactivity, Hookworm infection and gluten vaccination are being tested. Another part of autoimmune response is based on TG2 activation, which could be inhibited with inhibitory innate gluten peptides or transglutaminase 2 inhibitors (7).

1.1.5.3. Transglutaminase 2 inhibitors

TG2 upregulation is known to be connected to many disease states, also celiac disease, so there have been approaches to develop transglutaminase 2 inhibitor that could be used in treatment of TG2-associated diseases. Since all transglutaminase isoenzymes play a role in

the human body, it is important that the inhibitor is selective for transglutaminase 2 and does not affect functions of other members of transglutaminase family (22).

Depending on the mechanism of enzyme inactivation, inhibitors have been divided into three subclasses: competitive amine inhibitors, reversible and irreversible inhibitors (7).

a. Competitive amine inhibitors

Competitive amine inhibitors interfere in the reaction of transamidation. The mechanism of their action is to compete with natural amine substrates, such as protein bound lysine residues, in the formation of an isopeptide crosslink with natural glutamine substrate (23). Inhibitor potency of this subclass of inhibitors is characterized by the specificity constant that is defined as $\frac{k_{cat}}{K_M}$, where k_{cat} is catalytic constant which represents the turnover rate and K_M stands for the Michaelis constant (23). The inhibitors from this class have a typical chemical structure with an aliphatic unbranched chain of around 4 to 5 carbon atoms that are saturated and a primary amine that is bound to the carbon chain. Shorter amines like methylamine and hydroxylamine can also act like substrates for TG2 (23).

b. Reversible inhibitors

Reversible transglutaminase inhibitors do not covalently modify the enzyme, but block its active site, so the substrate does not have access to it. Their potency is characterized by the inhibitor dissociation constant-K_I (23).

Guanosine-5'-triphosphate (GTP) and guanosine 5′-diphosphate (GDP) block TG2 activity and some of GTP analogues have been found to inhibit the enzyme reversibly, such as GTPγS, GMP-PCP and thieno [2,3-d]pyrimidin-4-one acylhydrazide. As TG3, TG5 and TG6 also possess GTP binding sites, these molecules are not specific for TG2 (22, 23, 24).

Divalent Zn^{2+} ions compete with positive regulators of enzyme activity-Ca^{2+} ions for a metal binding site and can also inhibit the activity reversibly (23).
Another group of reversible inhibitors are cinnamoyl-based inhibitors that bind to the open conformation and are more specific for TG2 than the previous groups (22).

c. Irreversible inhibitors

Irreversible inhibitors bind covalently to TG2, which totally inactivates the enzyme. Structurally they have an electrophilic head that reacts with highly nucleophilic sulfur atom

from cysteine group and form relatively stable covalent bonds. This reaction modifies the enzyme and prevents substrate binding, resulting in irreversible inhibition (23).

The potency of irreversible inhibitors is characterized by $\frac{ki}{KI}$. The kinetic parameter k_i represents the reaction rate for inhibitor-enzyme covalent bond formation and K_I is inhibitor-enzyme complex dissociation constant (23).

ZED 1227: One representative of irreversible inhibitors that has been developed for the treatment of celiac disease is ZED1227, a peptidomimetic blocker synthesized by ZEDIRA company. It is a direct acting low-molecular transglutaminase 2 inhibitor. Prof. Schuppan and his working group at the Institute of Translational Immunology, University of Mainz, are working in collaboration with ZEDIRA company to construct a mouse model for ″in vivo″ evaluation of the drug (25).

Chemical information

Name: ZED1227

Molecular Formula: $C_{26}H_{36}O_6N_6$

Molecular Weight: 528.60 (26)

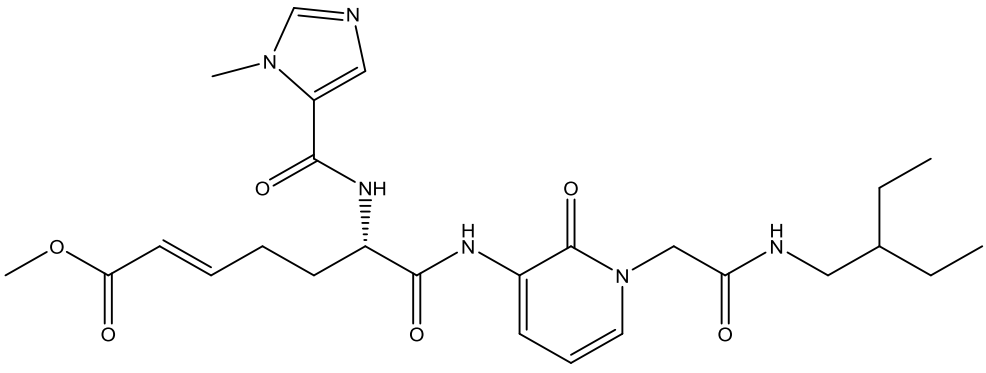


Figure 1: Irreversible inhibitor ZED1227 (61)

1.2. The role of transglutaminase 2 in CD

Transglutaminase 2 (protein-glutamine gamma-glutamyltransferase; enzyme code EC 2.3.2.13. (27)) belongs to the superfamily of papain-like enzymes-transglutaminases, which includes one catalytically inactive protein and 8 transglutaminase isoenzymes. All of them are cysteine proteases with 2 possible catalytic triads, Cys-His-Asp or Cys-His-Asn (28). Their main function is catalysing post translational modifications of different proteins in the body via cross-linking reactions that are calcium-dependent. Other mechanisms of modifying proteins include incorporation of amines, deamidation reactions and formation of esters (22). In surface epithelia expressed transglutaminase 1 and 3 are important for cell envelope formation in differentiation of keratinocytes. Prostate isoenzyme, transglutaminase 4, has a function in reproduction. Transglutaminase 5 is responsible for epidermal differentiation, while transglutaminase 6 plays a role in neurogenesis (22). The function of transglutaminase 7 is not yet characterized, but there are findings of its increased levels in relation to breast cancer (29). Plasma transglutaminase or Factor XIIIa is responsible for wound healing and blood clotting (22).

Transglutaminase 2 or tissue transglutaminase is an ubiquitous protein connected with autoimmune and inflammatory disorders. It is especially important in celiac disease, where it catalyses post translational modifications of gluten peptides, which then become immunogenic and trigger autoimmune reaction in the body (22).

1.2.1. Localisation and functions of TG2 in the body

Transglutaminase 2 is a monomeric protein made up of 687 amino acids that is found in several mammalian organs, for example heart, liver, intestine and erythrocytes. It is present inside and outside the cells. Intracellularly it is mostly localised in cytosol, but also mitochondria and nucleus, while extracellularly it binds to fibronectin in matrix (22, 30). The gene that is coding for transglutaminase 2 is found on chromosome 20q11-12 (22).

This multifunctional, 78-kDa big protein acts as an enzyme, cell adhesion and cell signalling molecule or as G-protein. Its multiple roles include assembly of matrix, wound healing, proliferation, invasion, motility and apoptosis, as it is pro-apoptotic or anti-apoptotic factor (30, 31).

Despite all the functions of TG2, it has been shown in the study with the TG2-knockout mice that the absence of this enzyme is tolerated in mice (32), which means that the inhibition of the enzyme is possible and compatible with life.

1.2.1.1. Enzymatic activity

The enzymatic function of transglutaminase 2 is post-translational modification of proteins by deamidating or transamidating them, depending on the presence or absence of amine substrate. As shown in the figure 2, the cysteine from the active site of an enzyme forms thioester with glutamine from the acyl-donor substrate, which can be a protein or small peptide. If the concentration of amine substrate is high enough, it binds to thioester and forms an isopeptide bond. When the concentration is too low, the enzyme deamidates the thioester by hydrolysis that results in formation of glutamate (30, 31).

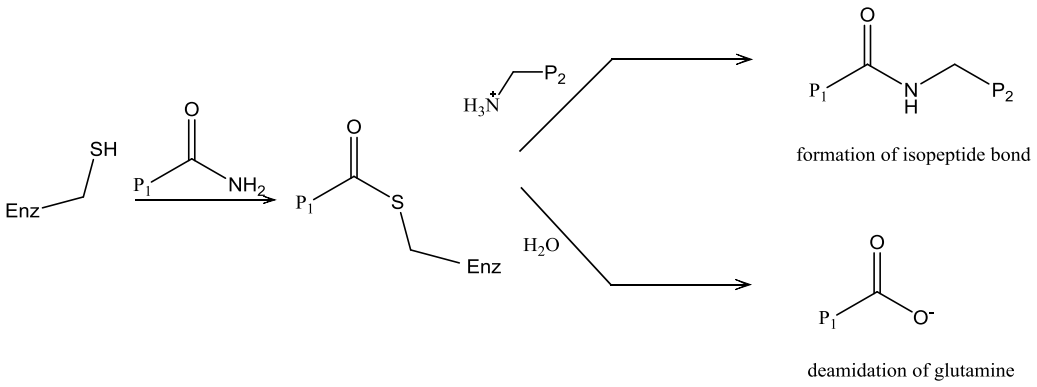


Figure 2: Enzymatic activity of transglutaminase 2 (30)

The enzyme is active when it is in open conformation and inactive when in a closed form (Figure 3). The conformation is regulated by three allosteric factors. Positive regulators are Ca^{2+} ions, six of which can bind to a transglutaminase 2 molecule with a complete dissociation constant of 90 μM. After their binding, the molecule changes into an open form. Second, negative regulators are guanine nucleotides, guanosine-5'-triphosphate (GTP) and guanosine 5′-diphosphate (GDP). They inactivate the enzyme when they bind to it with the dissociation constant of 1,6 μM. By binding they block the access to active site, which makes the enzyme adopt a close form. The third factor is a redox potential that influences binding of vicinal Cys370 and Cys371 to thiol groups of enzyme with a disulfide bond. When the potential is high, which is a usual condition in extracellular matrix, the molecule is in bonded, inactive conformation. This inactive form can be reversed by redox protein cofactor thioredoxin that keeps enzyme in open, active

conformation. Secretion of thioredoxin can be induced by interferon γ, abundantly secreted pro-inflammatory cytokine in damaged intestinal tissue of celiac patients. This mechanism promotes an auto-amplificatory gluten-induced inflammation loop (30, 31, 33).

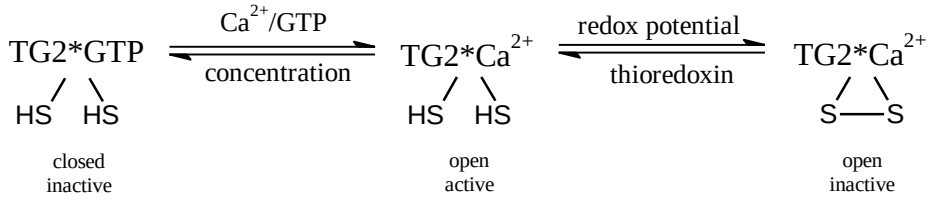


Figure 3: Modulation of transglutaminase 2 activity (30)

At physiological conditions, there is low concentration of Ca^{2+}, high concentration of GTP/GDP and low redox potential intracellularly, so TG2 is inactive. Due to trauma, apoptosis or cell stress the Ca^{2+} concentration is disturbed, which leads to activation of the enzyme inside the cells (34). Extracellularly there is a high concentration of Ca^{2+} and lower concentration of GTP/GDP, but it is mostly not enzymatically active because of high redox potential that keeps the molecule in inactive conformation. Extracellular TG2 can be activated by inflammation or injury (33).

1.2.2. **Measurement of TG2 activity**

1.2.2.1. **A homogeneous fluorescence anisotropy assay**

This assay is based on measurement of change in fluorescence anisotropy after cross-linking of fluorescence labelled TG2 substrate peptide to a larger protein. The acceptor protein is BSA, because it has a lot of surface lysines, is significantly larger than TG2 substrate and in experiments showed high labelling with the substrate mediated by TG2.

The reaction is initiated with combining a substrate, protein and TG2, and subsequently the level of anisotropy is measured over time. With increasing concentrations of enzyme, the reaction rate increases linearly (35).

1.2.2.2. **Colorimetric assay**

Colorimetric assay is based on the measurement of colour developed after the protein is incubated with substrate, enzyme and chromogenic substrate.

The reaction mixture contains biotin-HQSYVDPWMLDH peptide, dithiothreitol (DTT), $CaCl_2$, NaCl, Tris–HCl and standard tranglutaminases or samples. The mixture is added to plates that are covalently coated with acyl-acceptor. The plates are incubated at 37°C and

washed. After washing streptavidin-labeled horseradish peroxidase (HRP) is used for detection of the cross-linked biotinylated peptide. After a next washing step the chromogenic substrate is added which starts the colour reaction. Colour development is stopped with H_2SO_4 and the level of absorbance of the plate is measured by a spectrophotometer (36).

1.2.2.3. **Electrochemical assay based on nanochannels**

This voltammetric method is performed on a golden surface with amino-functionalized nanochannels of thin mesoporous silica film in the presence of glutamine-donor substrate.
Nanochannels are prepared by electrodepositions of silica film, which later functionalize with amino-donor groups. The assay is based on enzyme-controlled diffusion of electro-active probe ($Fe(CN)_6^{3-/4-}$) through the nanochannels and measurement of voltammetric signal. When glutamine-donor substrate is added in presence of a transglutaminase sample, the enzyme catalyzes binding of the substrate which selectively gates the nanochannels and results in decrease of the signal (37).

1.2.3. **Induction of TG2 activity**

As explained before, TG2 is an enzyme that is released from the injured and inflamed tissue. It has been reported that intraperitoneal administration of polyinosinic-polycytidylic acid (poly I:C), the Toll-like receptor 3 (TLR-3) ligand, induced injury in the mouse small intestine, including villous atrophy, an increase in IL-15 concentrations in serum and induction of TG2 activity, which was dose-dependent. Intestinal injury and activation of the enzyme recovered in 1-2 days, showing that it was just a temporary condition (38).
Researchers at the Institute of Translational Immunology (Universitätsmedizin Mainz) have evaluated the ″in vivo″ inhibition of TG2 activity by reproducing the work of Khosla et al (38). However, the outcomes of these experiments have not been consistent and lead to shed more light about the enzymatic activity of TG2 on an ″in vivo″ model. Therefore the institute is involved in optimisation of ″in vivo″ model of TG2 activity and collaborates in the development of an animal model for celiac disease. The induction of intestinal inflammation ″in vivo″ and the enhancement of TG2 activity have been explored essentially with two compounds: polyinosinic-polycytidylic acid and indomethacin.

1.2.3.1. **Indomethacin**

IUPAC: 2-[1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-yl]acetic acid

Molecular formula: $C_{19}H_{16}ClNO_4$

Molecular weight: 357.79 g/mol

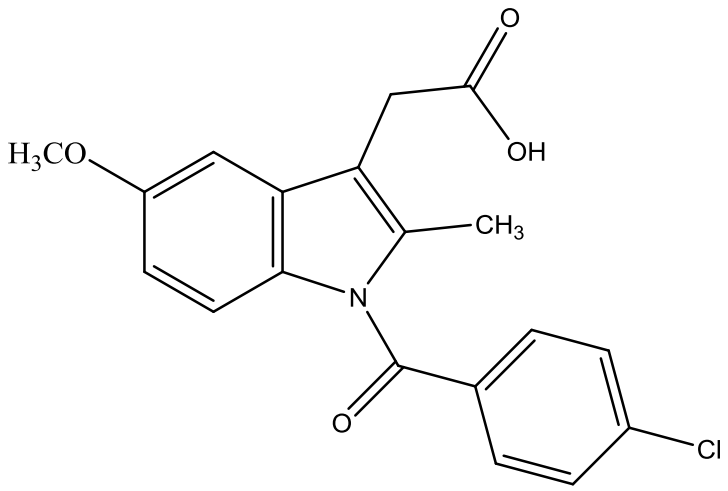


Figure 4: Structure of indomethacin (64)

Non-steroidal anti-inflammatory drugs (NSAID) are widely used as treatment for inflammatory and rheumatic diseases, but also as antipyretics and analgesics. Their disadvantage is the high possibility of side effects, which can cause other medical conditions and are responsible for low adherence in patients. One of the main side effects is the damage of the intestine, more specifically inflammation, ulcerative changes, perforations and consecutive bleeding. If these symptoms are not treated, they can cause anemia, malabsorption of vitamin B12 and bile acids, diarrhoea, abdominal pain and hypoalbuminemia (43).

Indomethacin is one of the most commonly used NSAIDs, having a big spectrum of possible usage. Mechanism of its action is inhibition of the enzyme cyclooxygenase that is responsible for production of prostaglandins (44). Like other drugs from this group, indomethacin also causes damage of the small intestinal mucosa. Some researches have been done over the pathophysiology of the mechanism of injury. It has been proposed that after NSAIDs are absorbed into the enterocytes, they uncouple oxidative phosphorylation of the mitochondria, which causes disruption of intracellular tight junctions. Consequently, the permeability of intestine increases and the luminal contents can reach the enterocytes.

Bile acids, enzymes, secretions of pancreas and intestinal bacteria cause chemotaxis of the neutrophil and their activation, leading to nonspecific intestinal inflammation with ulcerations, perforations and bleeding (45).

A study has shown that simultaneous administration of gliadin and albumin/globulin fractions from wheat in HLA-DQ8 transgenic mice that were preliminarily treated with indomethacin caused decrease of villi/crypt ratio, number of lamina propria-activated macrophages was higher and the basal interferon-γ secretion in mesenteric lymph nodes has increased, suggesting that indomethacin could have a role in TG2 activation. All the effects were reversible (46).

1.2.3.2. **Polyinosinic-polycytidylic acid (POLY I:C)**

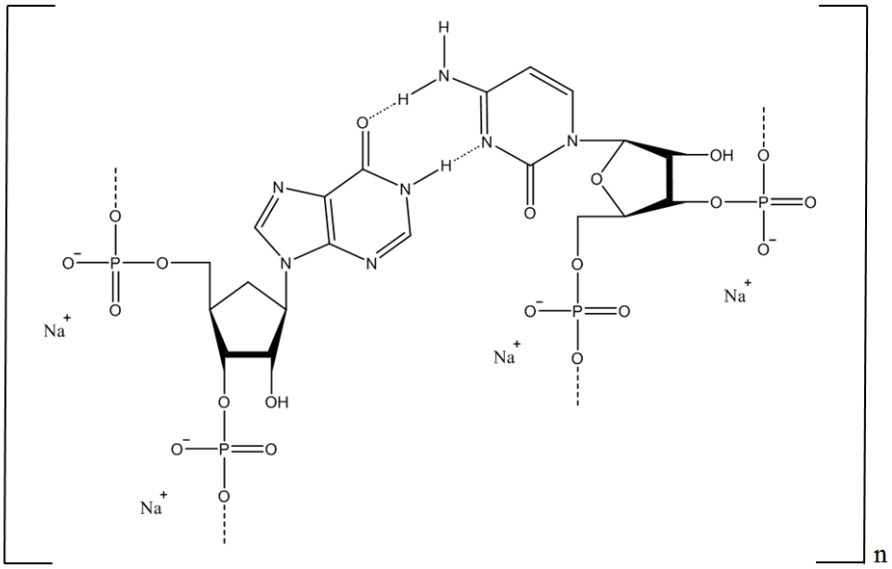


Figure 5: Structure of poly I:C (65)

Double stranded RNA (dsRNA), usually viral, is recognized in the body by TLR-3. To mimic dsRNS its synthetic analogue poly I:C can be used. When TLR-3 receptor recognizes poly I:C, the toll/interleukin-1 receptor (TIR) domain recruits TIR-domain-containing adapter-inducing interferon-β (TRIF). Interaction with TLR-3 leads to TRIF oligomerization and its recruitment of TRAF-family-member-associated NF-κB activator binding kinase 1 (TBK1), that together with IkappaB kinases-epsilon (IKK-ε), phosphorylates interferon regulatory factor 3 (IRF-3) (39). IRF-3 after dimerization translocates to the nucleus, where it recognises interferon-stimulated regulatory element

(ISRE) and induces expression of interferon stimulating genes (40). TRIF also interacts with receptor-interacting serine/threonine-protein kinase 1 (RIP1), which mediates activation of nuclear factor kappa B (NF-κB) (41).

Consequently, this cascade of reactions causes inflammation in the affected tissue. The effect of poly I:C in the intestine was seen to be reversible and result in release of inflammatory mediators (IFN-β after 2 hours and TNF-α after 4 hours), activation of TG2 within few hours and small intestinal villi atrophy after 12 hours, which were all similar effects to gut damage in celiac disease (42).

2. AIM OF THE STUDY

The aim of the study is to evaluate, optimise and measure transglutaminase 2 activity ″in vivo″. In addition, chemical triggers to enhance transglutaminase 2 activation in the intestine will be studied in mouse models. This study is a part of the project for the evaluation of a novel transglutaminase 2 inhibitor for the treatment of celiac disease.

The method to be optimised is an ELISA-like test, where different parameters of the experiment that could have an influence on the results will be evaluated to find the most optimal conditions for a reliable activity testing. The final aim in which my studies are embedded will be to reliably measure transglutaminase 2 activity in three different mouse strains and in response to two different proinflammatory triggers (poly I:C and indomethacin). Transglutaminase 2 gene expression will be evaluated by polymerase chain reaction.

3. MATERIALS AND METHODS

3.1. Chemicals and solvents

- Triton X-100; Sigma Aldrich, Darmstadt, Germany
- tris(hydroxymethyl)aminomethane for buffer solutions; AppliChem Panreac, Darmstadt, Germany
- sodium chloride (NaCl); Carl Roth, Karlsruhe, Germany
- ethylenediaminetetraacetic acid (EDTA); Carl Roth, Karlsruhe, Germany
- Phosphatase Inhibitor Cocktail Tablets PhosSTOP®; Roche Applied Sciences, Penzberg, Germany
- forene 100% V/V; Abbvie, Wiesbaden, Germany
- Dulbecco's Phosphate Buffered Saline, modified; Thermo Fisher Scientific GmbH, Dreieich, Germany
- Roti®-Histofix 4%; Carl Roth, Karlsruhe, Germany
- potassium chloride (KCl); Carl Roth, Karlsruhe, Germany
- calcium chloride ($CaCl_2$); Sigma Aldrich, Darmstadt, Germany
- Tween® 20 (Polysorbate) for synthesis; Merck KGaA, Darmstadt, Germany
- disodium hydrogen phosphate (Na_2HPO4); Carl Roth, Karlsruhe, Germany
- potassium dihydrogen phosphate (KH_2PO_4) ; Carl Roth, Karlsruhe, Germany
- dithiothreitol (DTT) 0,25M; Promega, Mannheim, Germany
- 5-(biotinamido)pentylamine (5-BP) 10mg/ml; Thermo Fisher Scientific GmbH, Dreieich, Germany
- Streptavidin-HRP 1:200; R&D Systems, Wiesbaden, Germany
- 3,3',5,5'-Tetramethylbenzidine (TMB); R&D Systems, Wiesbaden, Germany
- sulfuric acid, ACS reagent, 95.0-98.0%; Sigma Aldrich, Darmstadt, Germany
- Pierce™ BCA Protein Assay Kit; Thermo Fisher Scientific GmbH, Dreieich, Germany
- ethanol absolut 99,7%; VWR, Radnor, Pennsylvania, USA
- qScript cDNA synthesis kit; Quantabio, Beverly, MA 01915, USA
- SYBR® Green PCR Master Mix and SYBR® Green RT-PCR Reagents Kit; Applied Biosystems, Thermo Fisher Scientific GmbH, Dreieich, Germany
- PCR primer TG2 (sense, antisense), Metabion GmbH, Steinkirchen, Germany

- sodium bicarbonate (NaHCO3), Carl Roth, Karlsruhe, Germany
- glucose; Sigma Aldrich, Darmstadt, Germany
- Mid-binding 96-well plate, Greiner Bio-One GmbH, Frickenhausen, Germany
- 96-Well Plates, Thermo Fisher Scientific GmbH, Dreieich, Germany
- GeneMATRIX Universal RNA Purification Kit containing buffers (RL, wash DN1, wash RBW) and spin-columns (homogenization, RNA binding), RNAse free water; EURx, Gdansk, Poland
- indomethacin, Sigma Aldrich, Darmstadt, Germany
- dimethyl sulfoxide (DMSO), Sigma Aldrich, Darmstadt, Germany
- polyinosinic:polycytidylic acid(poly I:C), Sigma Aldrich, Darmstadt, Germany

3.2. **Solutions**

Lysis buffer (pH 7,5)

COMPOUND	AMOUNT
TRIS	1,21 g
NaCl	14,61 g
EDTA	146,12 mg
Triton	5 mL
distilled water	to 500 ml

Phosphate buffer solution–PBS (pH 7,4)

COMPOUND	AMOUNT
NaCl	40,0 g
KCl	1,0 g
Na_2HPO_4	14,4 g
KH_2PO_4	2,4 g
distilled water	to 500 ml

Coating buffer (pH 7,5)

COMPOUND	AMOUNT
TRIS	3,0 g
NaCl	4,4 g
EDTA	730,6 g
distilled water	to 500 ml

Diluent buffer (pH 7,4)

COMPOUND	AMOUNT
TRIS	3,0 g
NaCl	4,4 g
$CaCl_2$	298,3g
distilled water	to 500 ml

Washing buffer (pH 7,4)

COMPOUND	AMOUNT
Tween	0,5 mL
PBS	499,5 mL

KREBS solution: We weighted 0,63g of $NaHCO_3$ and 1,351g of glucose into a glass beaker, added 30 ml of KHB and a stir bar. We placed the beaker on a stir plate until

complete dissolution, then added 220mL of distilled water and adjusted pH to 7,42 with NaOH. Then we added distilled water to a total content of 300mL.

For preparation of KREBS solution we used KHB 10x, that has already been prepared before by the protocol: 7,34g of $CaCl_2*2H_2O$ was dissolved in 1L of ultrapure water to form solution 1. 7,46g of KCl, 138g of NaCl, 5,42g of $MgSO_4*7H_2O$ and 3,26g of KH_2PO_4 were dissolved in ultrapure water to a volume of 1L to form solution 2. Then solution 1 and solution 2 were mixed and filtered through a 0,45µm filter.

HEPES solution 0,1M: We weighted 4,76g of HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) to a glass beaker, added 160 ml of distilled water, a stir bar and placed it on a stir plate until dissolved. Then we adjusted pH to 7,4 with solid NaOH, added distilled water up to 200ml and sterile-filtered the solution.

3.3. Laboratory equipment

- vortex, Vortex-Genie 2, Scientific Industries
- battery powered pipette filler, pipetus, 9907200, Hirschmann Laborgeräte
- freezers -20°C, -80°C; fridge 4°C, Liebherr
- TissueLyser II, Qiagen
- centrifuge, Thermo Scientific, Heraeus Fresco 21
- incubator, Thermo Scientific, MaxQ 6000
- plate washe, TECAN, HydroSpeed™
- repetitive pipet, HandyStep® S, Brand
- set of pipetts 10 µL//200 µL/300 µL multichannel/1000 µL, ErgoOne® Single & Multi-Channel Pipettes, STARLAB GmbH; 20 µL, PIPETMAN Classic™, Gilson; 20 µL multichannel, PIPETMAN L MULTI, Gilson; repetitive pipette
- spectrophotometer, TECAN, Infinite M200 PRO
- short-spin, Fischer Scientific
- thermal cycler, BIORAD, T100™ Thermal Cycler
- StepOnePlus™ Real-Time PCR System, Applied biosystems
- balance, Sartorius - M-Pact
- temperature block, Eppendorf, Thermomixer comfort
- magnetic stirrer, IKA RCT Basic
- centrifuge for plates, Eppendorf Centrifuge 5804 R

3.4. **Animal experiments**

Certified and authorised personnel performed the manipulation of the living animals and sacrifice. The permission of the Institute for working with the laboratory animals is 23 177-07/G 14-1-092 and it is valid from 18.03.2015 to 31.03.2020.

The Translational Animal Research Center (TARC) of Johannes Gutenberg University Mainz provided the animals used for this research. They were housed 4-5 per cage and fed with gluten-free food (Black 6) or normal food (TLR4 -/- and Balb/C), depending on the group of animals. They were supplied with water in the cages. The age of sacrifice was between 6 and 8 weeks.

All together 41 mice were used for the experiments.

3.4.1. **Mouse strains**

We evaluated 3 different strains of mice in order to compare differences in the enhancement of TG2 activity. One of them was a wild type and the other two were genetically modified.

3.4.1.1. **C57BL/6**

C57BL/6, also named as Black 6, C57 Black or B6, is a wild type mouse that is most often used inbred line, known for its black colour. Its advantage is that mice are easy to breed, can highly express many mutations, but are also resistant to numerous tumours. They are common for experiments in diabetes, obesity, neurology, cardiovascular studies, and researches in immune and sensory system. Black 6 mice are very useful as background for genetic modifications (47, 48).

3.4.1.2. **B6 TLR4 -/-**

B6 TLR4 -/- (later in text as TLR 4-/-) is a genetically modified knockout mouse with C57BL/6 background that was deleted of the locus for Toll-like receptor 4 (TLR4), the protein that recognizes pathogens and is responsible for innate immune response. This mouse strain is used for researches in immunology, inflammation and autoimmunity (49).

3.4.1.3. **BALB/c**

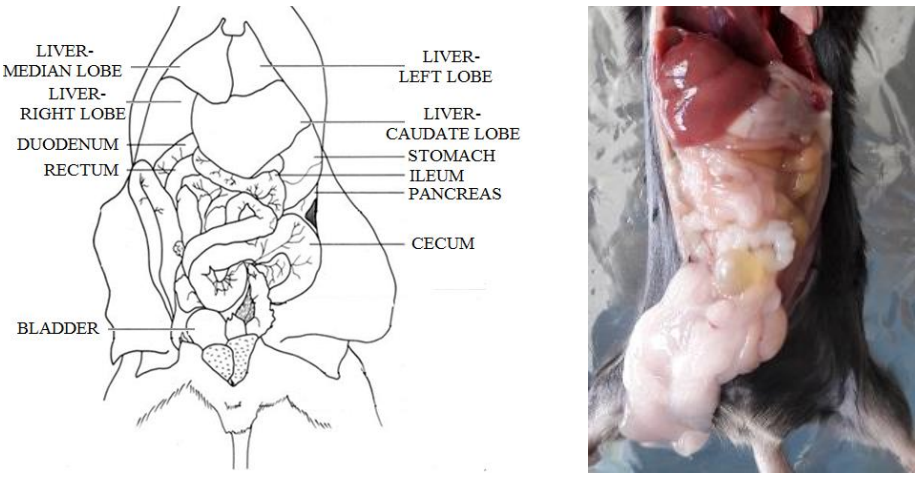
BALB/c is an inbred line that is known for perceptivity to development of the demyelinating disease after it is infected with Theiler's murine encephalomyelitis virus.

These mice are applicable for researches about cancer, cardiovascular disease, neurobiology, sensorineural researches and immunology, inflammation and autoimmunity (50).

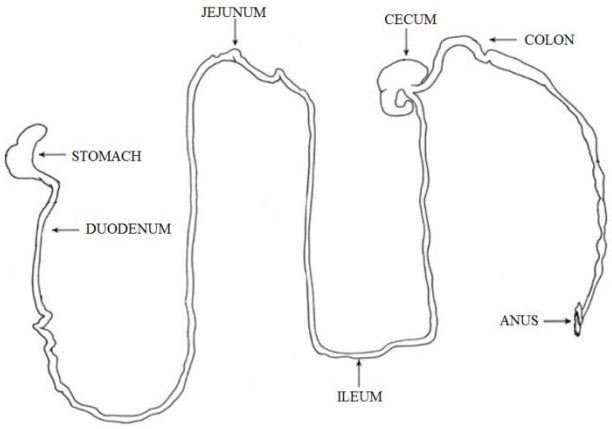
3.4.2. **Sacrifice procedure and harvesting of the tissue**

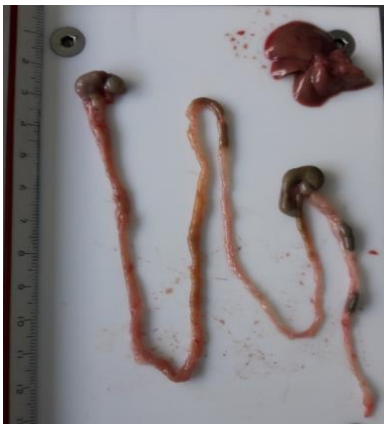
The mice were anaesthetised by inhalation of isoflurane in a hermetic glass box. When no signs of consciousness were seen anymore, depth of anaesthesia was monitored by reflex and response test with the pressure on nerve endings in pads or ears and by palpebral reflex testing with touching the inner corner of the eye. If the animal did not react and the anaesthesia was deep enough then they were sacrificed by cervical dislocation.

We cut the body transversally on the abdominal side (figures 6, 7) and harvested hepatic and intestinal tissue, from stomach to the end of the colon (figures 8, 9). All the harvesting procedure was performed on melting ice. We took a part of the liver and immediately put it in a tube and snap froze it in liquid nitrogen. One part we put into tube with 1,5ml of histofix 4% and stored into 4°C fridge for histology analysis. During harvesting procedure, we kept the whole intestine in cold Dulbecco's modified phosphate buffer solution (PBS) to preserve the tissue and the enzymatic activity of TG2. Then we divided it into duodenum, jejunum, terminal ileum and colon. First, we washed each part with ice-cold PBS buffer to clean all the content that was in lumen. Very precisely we cut any fat off the tissue and then did the sectioning. We put each section in different 2ml tube and immediately snap froze them in liquid nitrogen. Then we stored the samples in the freezer at -80°C.



Figures 6 and 7: Abdominal viscera-scheme (63) **and photo**





Figures 8 and 9: Mouse intestine from stomach to anus-scheme (62) **and photo (including liver)**

3.5. Transglutaminase extraction

Transglutaminase extraction was done according to an established protocol for the extraction of transglutaminases from animal tissue; it is used to isolate enzymes for further evaluations, in our case for transglutaminase activity assays. It is not a specific method for transglutaminase 2 extraction, but for all transglutaminases.

3.5.0.1. Preparation

Working lysis buffer: We added 1 Phosphatase inhibitor tablet per 10mL of lysis buffer and vortexed until dissolved.

Samples: We took tissue samples from the -80°C freezer and weighted them. After that, we added 400µL of working lysis buffer and a metal grinding ball to each tube.

3.5.0.2. Assay

At the beginning, we set the centrifuge to cool down to 4°C. We performed the whole assay on melting ice.

We placed the tubes in cold boxes of the Tissue lyser and ran the equipment three times at the frequency of 30/s for 1 minute each. Then we centrifuged the samples at 21,0 x G for 2 minutes. We divided supernatant into 3 aliquots, snap frozen them in liquid nitrogen and stored them in the -80°C freezer until further use.

3.6. In-house transglutaminase activity assay

The aim of this assay is to test the activity of transglutaminases in the intestinal and hepatic tissue. This method is not specific for transglutaminase 2, but it has been observed, that

levels of transglutaminase 2 are in higher proportion than the other transglutaminases (51), so we used it as a preliminary evaluation of TG2 activity.

Procedure of the in-house assay is similar to ELISA test, with the difference that the substrate here is dimethylcasein, while in ELISA the substrates are antibodies.

3.6.0.1. **Assay**

- One day before the assay we coated mid-binding ELISA 96-well plates with 100µL/well of coating buffer containing 0,1% of dimethyl casein and then incubated the plate over-night at 40°C.
- After incubation we washed the plates in a plate washer three times with 200µL/well of washing buffer. Then we added in each well 50µL of Master mix, consisting of 47µL of diluent buffer, 1µL of dithiothreitol (DTT) with concentration 0,25M and 2µL of 5-(biotinamido)pentylamine (5-BP) with concentration 10mg/ml. For negative control we added 10µL of EDTA in selected wells. We pipetted the samples, diluted 1:50 in diluent buffer, on the plate in duplicates or triplicates, depending on the number of samples to evaluate. For negative control, we used repeated samples. We incubated the plate for 4 hours in dark at room temperature (18-26°C), without any movements.
- We washed the plates as described before and added 100µL of 0,1% HRP- labelled streptavidin, diluted in washing buffer, to amplify the signal and make it more visible in later steps. We incubated the plates on room temperature (18-26°C) for 30 minutes.
- After incubation we washed the plates again and added 100µL/well of chromogenic substrate-TMB. This step we had to do in dark circumstances as TMB is photosensitive. Again we incubated the plates for maximum 30 minutes.
- The samples with active transglutaminase should have turned blue, depending on the activity of TG2. We added 50µL /well of stop solution-1 M H_2SO_4, to stop reaction and change the blue colour into yellow-orange.
- We measured the strength of colorimetric reaction spectrophotometrically at 450 nm.
- We calculated the transglutaminase activity relatively per 1mg of the tissue.

3.7. **Protein assay-BCA protocol**

The BCA (bicinchoninic acid) protocol is an assay that is used to quantify overall proteins in tissue sample. This was important if the results from transglutaminase 2 assay were too

low, so we checked if the extraction was successfull and there was some tissue in the sample. The protocol is based on biuret reaction. Proteins reduce Cu^{2+} to Cu^{1+}, which results in formation of bicinchoninic acid, turning the green colour of reagent into purple.

3.7.0.1. **Preparation**

First, we mixed 50 parts of BCA Reagent A with 1 part of BCA Reagent B (depending on the volume needed) and vortexed until the formation of a clear green solution. We prepared standards in concentrations 0, 25, 125, 250, 500, 750, 1000, 1500 and 2000 (μg/mL) with serial dilutions using the stock solution and distilled water (Table I).

Table I: Scheme of preparation of the standards for BCA protein assay

TUBE	VOLUME OF DISTILLED WATER (μL)	VOLUME AND ORIGIN OF STANDARD (μL)	BSA CONCENTRATION IN TUBE (μg/mL)
I	0	400 from Stock solution	2000
II	125	375 from Stock solution	1500
III	325	325 from Stock solution	1000
IV	175	175 from tube II	750
V	325	325 from tube III	500
VI	325	325 from tube V	250
VII	325	325 from tube VI	125
VIII	400	100 from tube VII	25
IX	400	0	0

3.7.0.2. **Assay**

- We diluted samples 1:25, using 4μL of each sample with 100μL of distilled water.
- 25μL of each diluted sample or standard we pipetted in duplicates onto a plate. To each well we added 200μL of working reagent. Then we mixed the plate on a plate shaker, covered it with aluminium foil and incubated for 30 minutes at 37°C. After

incubation, we let the plate cool down to room temperature (18-26°C) and measured the absorbance spectrophotometrically at 562nm.

- We calculated protein concentration in relation to the standard curve.

3.8. **RNA purification**

The RNA purification protocol is used to isolate RNA from animal tissue. We performed the procedure with GeneMATRIX Universal RNA Purification kit.

3.8.0.1. **Preparation**

First, we prepared working RL buffer in laminar flow hood by adding β-mercaptoethanol (β-ME) to RL buffer in proportion 1:100 and vortexing.

3.8.0.2. **Assay**

At the beginning, we set the centrifuge to cool down to 4°C. We performed the whole assay on melting ice.

- We placed 1,5ml of working RL buffer, tissue (approx. 0,5cm) and a metal grinding ball into 2mL tubes.
- Then we placed the tubes in cold boxes of Tissue lyser and ran the equipment three times at a frequency of 30/s for 1 minute.
- We centrifuged the samples at 21,1 x G for 3 minutes, transferred the supernatant to the homogenization spin-columns placed in the 2mL tubes and centrifuged the tubes at 21,1 x G for 2 minutes.
- We discarded the homogenization spin-columns, added 350µL of 70% ethanol to the flow-through, mixed by pipetting and transferred it to the RNA binding spin-columns in 2mL tubes. The tubes we centrifuged at 11 x G for 1 minute.
- We discarded the flow-through, added 400µL of wash DN1 buffer on top of the columns and centrifuged the tubes at 11 x G for 1 minute.
- We again discarded the flow through, added 650µL of wash RBW buffer on top of the columns and centrifuged the tubes at 11 x G for 1 minute.
- As before, we discarded the flow-through, then added 350µL of wash RBW buffer on top of the columns and centrifuged the tubes at 11 x G for 2 minutes.
- At the end we placed the columns in new 1,5mL receiver tubes, added 40µL of RNA-se free water on the membranes and centrifuged the tubes at 11 x G for 1 minute.

- We determined the concentration of RNA by measuring the absorbance at 260nm in a spectrophotometer. The ratio of the readings at 260nm and 280nm provides an estimate of the purity of RNA with respect to contaminants.
- RNA we stored at -80 °C for further use.

3.9. cDNA synthesis

3.9.0.1. Preparation

We calculated volumes of samples, equal to 500ng of RNA, from measured concentrations of RNA and pipetted them into PCR-tubes. Then we added RNA-se free water to reach 8μL and 2μL of cDNA synthesis mix containing reverse transcriptase and centrifuged the tubes for 20 seconds in short-spin.

3.9.0.2. RNA to cDNA conversion

- We placed the samples into BIO-RAD Thermal cycler on following program:
  - 22°C 5 minutes
  - 42°C 30 minutes
  - 85°C 5 minutes
  - 4°C up to 2 hours.

Then we stored cDNA at -20°C for long term or at 4°C if we used samples in the same day.

3.10. Polymerase chain reaction in real time

Polymerase chain reaction in real time (RT-PCR) is an in-vitro technique for DNA amplification and evaluation of gene expression in particular tissue. Compounds needed for reaction are original cDNA, sense and anti-sense chain of gene primer, oligonucleotides and enzyme DNA polymerase. For quantitation of the product, special dye is also needed in the mixture. The reaction consists of 3 stages: denaturation of cDNA into single strands, annealing of primers to single-stranded DNA and elongation by DNA polymerase. The process is repeated 45 times. Amplified DNA is measured by fluorescence of a reagent, which was in our case SYBR Green.

We used the reaction to determine gene expression of transglutaminase 2. For quantitative analysis we always used comparative C_T ($\Delta\Delta C_T$) method that is based on comparison of the selected gene with the housekeeping gene, GAPDH.

Formula for ratio calculation: **ratio=$2^{-\Delta\Delta CT}$**

In order to select the most suitable primers we compared c_T values at 6 different temperature zones for 2 pairs of primers that we have found in the literature (52). The one that performed better and we used for later experiments was:

TG2 sense: 5′-ACT TCG ACG TGT TTG CCC ACA T-3′
TG2 antisense: 5′-TTG ATG TCC TCA GTG CCA CAC T-3′

3.10.0.1. **Preparation**

We diluted cDNA samples 5 times with 0,1mM EDTA and centrifuged the tubes for 30 seconds in short-spin.
The gene-mix we prepared for each gene, using (per sample) 5μL of Master mix, 0,25μL of sense gene primer, 0,25μL of anti-sense gene primer and 2,5μL of RNA-se free water.

3.10.0.2. **Assay**

- We pipetted 2μL of samples into 96-well PCR-plate in duplicates, added 8μL of gene mix per well, covered the plate and centrifuged at 700 x G for 2 minutes.
- After centrifuge we placed the plate into the StepOnePlus Real-Time PCR System on the Quantitation-comparative c_T ($\Delta\Delta c_T$) program:
  - 95°C 10 minutes
  - 45 cycles : 95°C 15 seconds
    60°C 1 minute
  - 95°C 15 seconds
    60°C 1 minute
    95°C 15 seconds.

3.11. Storage conditions of the samples before TG2 extraction

After the harvesting of duodenum, jejunum, colon and hepatic tissue (as described before), we divided each tissue into 4 parts. We placed part F (to be extracted fresh) into a tube

with metal grinder and lysis buffer and then put in the 4°C fridge until extraction. Parts A and B (to be extracted after freezing) we placed into tubes, immediately deep froze in liquid nitrogen and subsequently transferred to the freezer at -80°C, where they were kept for one day until we did the extraction. We placed part C (to be extracted after freezing in liquid nitrogen) into a tube, deep frozen in liquid nitrogen and extracted after we took the tube out of it. Scheme of the assay is in the figure 10.

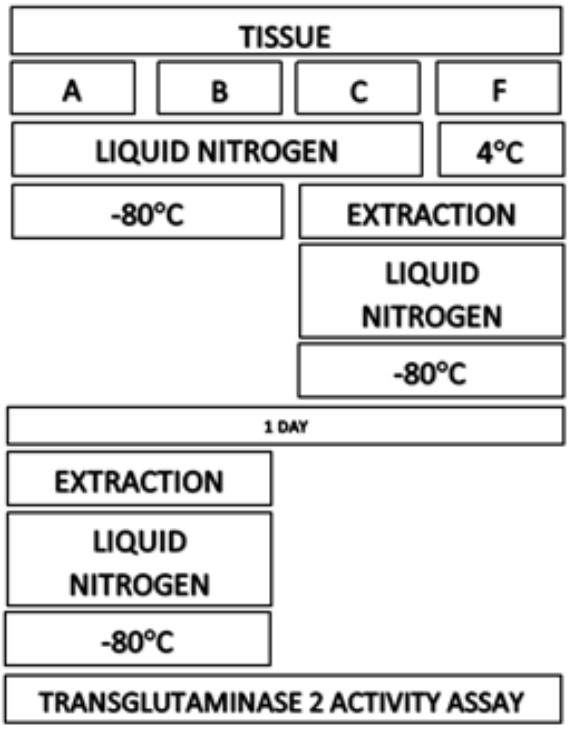


Figure 10: Scheme of the assay-determination of storage conditions

We did the extractions as described before, divided the samples into aliquots and immediately frozen in liquid nitrogen and transferred to the -80°C freezer. We measured transglutaminase 2 activity in duplicates, using transglutaminase activity assay.

3.12. **Determination of buffers**

With the purpose to find the optimal buffer that would keep transglutaminase activity after washing the tissue with it, we explored three different buffers: Dulbecco's phosphate buffer solution, KREBS solution and HEPES buffer.

Triton in lysis buffer is responsible for cell lysis. Two different concentrations, 0,5% and 1%, were tested.

Transglutaminase 2 activity is dependent on calcium concentration in the diluent buffer. To determine the optimal, 3 concentrations of $CaCl_2$ were tested.

3.12.1. Buffer used for washing the tissue

We harvested the tissue as described before, using PBS, KREBS or HEPES buffer for washing the tissue, depending on the group. Between the experiments with each group, we cleaned the instruments with 70% ethanol. We did the sectioning and stored the tissue as described before.

We performed transglutaminase 2 extraction and activity assay using the described protocols.

3.12.2. Triton concentration in lysis buffer

We separately prepared two lysis buffers, one with 0,5% Triton and one with 1% Triton and performed transglutaminase extraction and transglutaminase activity assay as described before, using each buffer for selected group of mice. For transglutaminase activity assay we diluted extracted samples in 1:50. To compare if there was any difference in response after change of Triton concentration, we tested also the samples in dilution 1:100.

3.12.3. Calcium concentration in diluent buffer

We prepared three diluent buffers with three different $CaCl_2$ concentrations: 5mM, 7mM and 9mM. We used them for the dilution of the samples and preparation of the Master mix. Then we performed transglutaminase activity assay as described before.

3.13. Induction of intestinal inflammation

(TG2 activity evaluation)

3.13.0.1. Preparation of compounds

- We dissolved indomethacin powder in DMSO in concentration 2mg/ml.
- We dissolved poly I:C in PBS in concentration 4mg/ml and heated to 85°C for 3 minutes. Then we cooled it down to 25°C with the speed of 1°C per minute, using a temperature block. This procedure results in annealing of poly I:C molecules, which after application to animals produce reproducible small intestinal damage.

3.13.0.2. **Experiment**

- For each compound we used two groups of mice, control and treated. Animals from control group were treated with a vehicle only, which was DMSO (control for indomethacin) or PBS (control for poly I:C).
- 12 hours before sacrifice, the animals were intraperitoneally treated with the selected compound or with a vehicle. Indomethacin was administered in concentration 20 mg per kg of the mouse and poly I:C in concentration 40 mg per kg of the mouse, amounts were calculated depending on the weight of the animal. After treatment, the food was discarded and they were fastened until sacrifice, to provide equal conditions for all of them.
- Mice were sacrificed as described before. We did the harvesting, TG2 extraction and TG2 activity assay as described before.

4. RESULTS AND DISCUSSION

Data from spectrophotometrical measurements that have been used for the graphs are presented in the Appendix.

4.1. Optimisation of TG2 activity ″in vivo″

4.1.1. Sectioning and flushing

The way of sectioning was very important, since the enzyme has a different level of activity in various organs or its distribution varies in different tissues. These were also the reasons that we tested the activity in liver and intestine, as high TG2 expression has been observed in them (30). We tried to optimise sectioning in order to get the most consistent and comparable results, avoiding variations due to the section of the tissue that was examined. As preliminary results of transglutaminase 2 activity in the tissue where sectioning was not optimised were not consistent, we evaluated the exact length of the tissue taken, determined the way of washing the tissue and conditions in which sectioning has been done.

4.1.1.1. Liver

The liver is constructed of 4 lobes, each with its own function in the body. To standardise the procedure we decided to always section in the same way, taking a 0,5cm to 1cm big part of the left lateral lobe (figure 11). We recognised it by the round shape and the size.

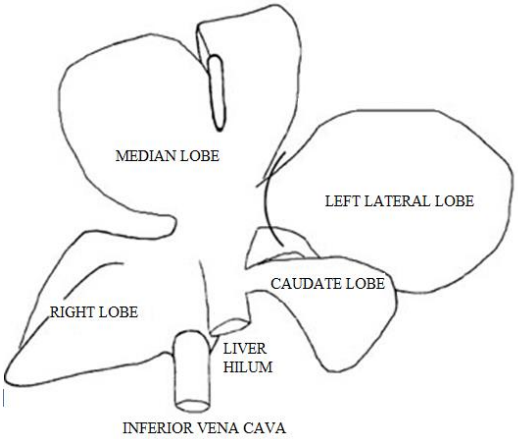


Figure 11: Structure of the murine liver (66)

4.1.1.2. **Intestine (figure 12)**

Mice intestine is approximately 35cm long. The first 15cm after the stomach is in the literature considered as duodenum and has a muscular composition. Jejunum starts after the duodenum and after jejunum is the ileum, the last part of small intestine. As there is no visible difference between jejunum and ileum, we collected the proximal part of the jejunum in relation to the stomach and terminal part of ileum, to ensure that the part we were taking was from the right part of the intestine. The first part after cecum is colon, which is approx. 6-8cm long, depending on the size of the animal. The last part of large intestine is rectum (53).

The aim of the intestine is to digest food, absorb nutrients and form feces. Its formation can be seen in harvested tissue. Intestinal content in duodenum is chime that was formed in stomach. It is a compact greenish liquid with some undigested parts of the food. Down the small intestine it becomes denser and starts forming sections of approx. 0,5cm. After cecum, the major difference is seen when solid feces are formed.

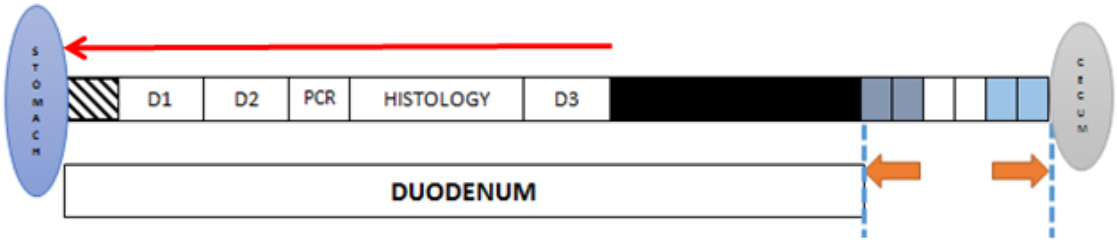
4.1.1.3. **Optimisation of the methodology**

First, we tried to avoid the influence of pH, which might interact with TG2 activity especially in the proximal part of the duodenum where gastric acid still remains. Importance of the pH has been proposed in the study where they found out that the TG2 activity and also GTPase activity was the highest at pH 7 and decreased at pH 5 and pH 9, so too acidic or too basic conditions were not optimal for TG2 (54).
We flushed duodenal tissue with ice cold Dulbecco's modified PBS from the proximal parts in the direction of the stomach, to ensure that remaining acidic content did not affect the stability and the activity of TG2 from the duodenal section. In the jejunum, we flushed the tissue in the direction to ileum, as we used first part after the duodenum for the assay. We flushed terminal ileum from distal to proximal part and the colon from proximal to distal part, referring to the stomach. With this standard procedure we tried to ensure that the intestinal content might not influence on the activity of the TG2. The way of sectioning and sizes of the tissue taken are shown in figure 12, red arrows represent direction of flushing of the tissue.

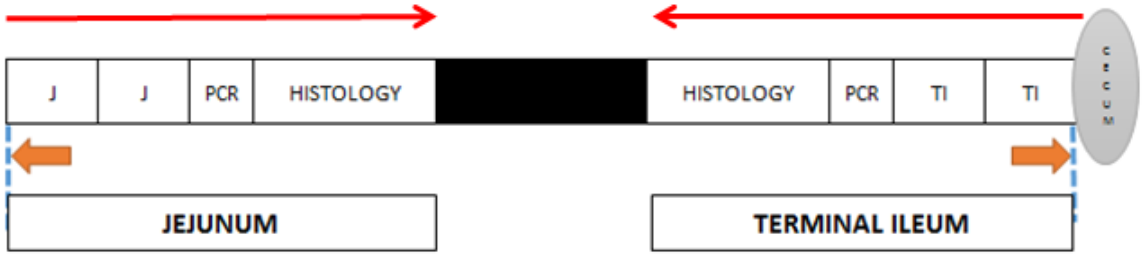
In order to preserve the viability in the intestinal cells and to slow down metabolic demand, we always kept the tissue and the sections in the ice-cold buffer on melting ice, therefore

we might prevent enzymatic activity loss. In previous evaluations it has already been observed (data not shown) that enzyme activity will be lower if the tissue is exposed to higher temperatures. For the same reason, immediately after sectioning, we snap froze the sections in liquid nitrogen, and then stored samples at -80°C for further use.

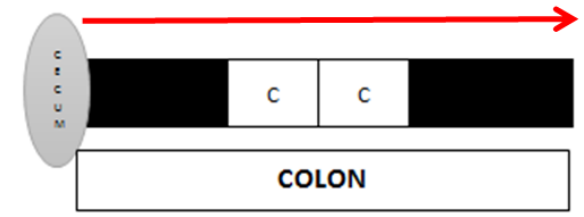
We have also collected samples for histology analysis (figure 12). As we cut the sections, we immediately put them into tubes containing 1,5ml of 4% histofix each and stored them into 4°C fridge. Due to the time limitation, we did not do the histology analysis within this project, but the samples have been stored and then embedded if needed for further evaluation. It would be important to see whether there have been some histological changes in the tissue and compare them with the TG2 activity results.



- Discard (1cm)
- Discard until jejunum
- D1, D2, D3-Duodenum section 1, section 2, section 3 (each 1,5cm)
- PCR-Duodenum PCR (1cm)
- Duodenum histology (3cm)



- Discard
- J-Jejunum / TI-Terminal ileum (2 x 1,5cm)
- Jejunum / Terminal ileum PCR (1cm)
- Jejunum / Terminal ileum histology (3cm)



Discard

C-Colon (2 x 1,5cm)

Figure 12: Sectioning of the intestine

This part of our research contributed to our understanding of regional and cellular differences in the distribution of the TG2 activity along the intestine. We have observed that the activity of TG2 was higher in most of the groups in the jejunum, terminal ileum and the colon than in the duodenum. This could be explained with the hypothesis of the influence from gastric acid on the enzymatic activity, so the flushing did not nullify the influence and further analysis is required. Another possible explanation of low transglutaminase 2 activity in duodenum is that the activation of TG2 may occur at different time frames along the intestine, so 12 hours from treatment may already be the time when the injury is recovering in the duodenum, but not yet in other tissues where the effect of poly I:C could have started later.

In further experiments, we always used the second part of duodenum, duodenum II, to ensure uniformity of the results and avoid the differences in the activity within whole duodenum. It will be termed as ″duodenum″.

4.1.2. **Buffer used for washing the tissue**

The pH in the body and particular organs is an important determinant in many physiological processes. Some of them are either activated or inhibited by the change of pH. As we discovered during the initial experiments that transglutaminase 2 activity in the first part of duodenum was very variable, we tried to hypothesise a reason for these inconsistent results. This part of duodenum is right after the stomach, so one of the possibilities might be the level of pH, which changes under the influence of the release of gastric juices in the duodenum and its subsequent neutralization by pancreatic HCO^{3-}. To standardise the conditions, we tested three buffers that keep pH in physiological range:

Dulbecco's phosphate buffered saline (PBS) modified, Krebs-Henseleit buffer (KHB) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). As this was a preliminary evaluation and we were limited with the time, we were able to use only 1 mouse for PBS, 2 for KHB and 2 for HEPES.

Transglutaminase activity assay was performed three times to avoid systematical errors and to ensure a higher reliability of the results. Each time we used the same extracted samples, the first and the second time it was the same aliquot that was immediately snap frozen in liquid nitrogen after first use and then stored in -80°C, and the third time the second aliquot.

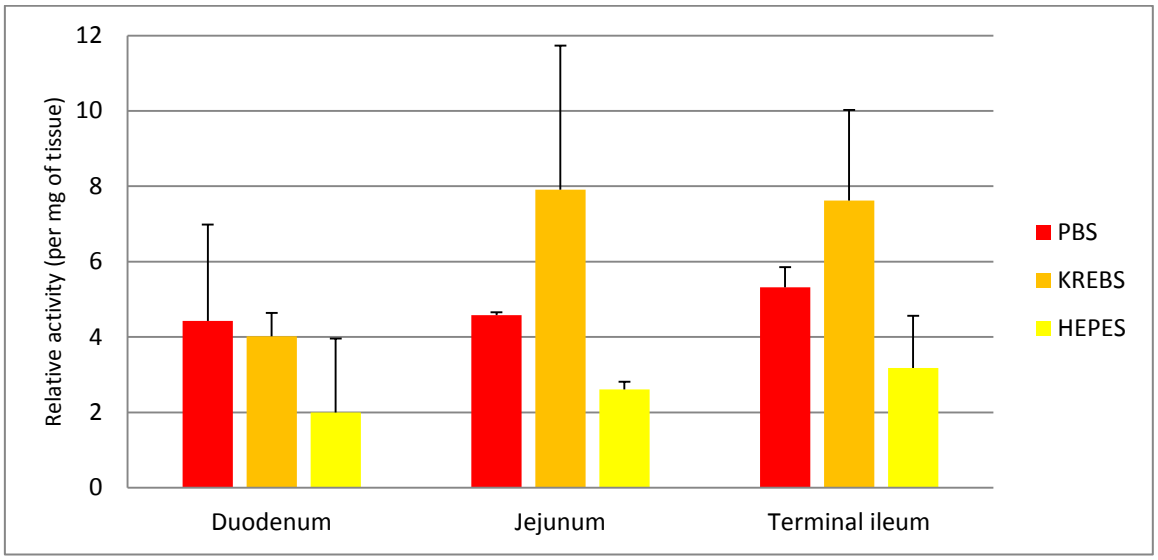


Figure 13: Relative transglutaminase activity in duodenum, jejunum and terminal ileum, depending on the buffer used for washing the tissue-PBS, KREBS or HEPES.

In the figure 13 we can see that the transglutaminase activities in all three tissues were the lowest when we used HEPES for washing the tissue. Measurements of the absorbance on the spectrophotometer were in the same range as of the other buffers, but the weights of the tissues were higher than of the tissues washed with PBS and KREBS, so the relative activity per mg of the tissue was calculated as lower. To check if higher weight correlates with higher protein content, which would consequently mean higher TG2 concentration, we performed BCA protein assay (figure 14). We observed that there was higher protein concentration in HEPES samples, which would mean that the TG2 was less active.

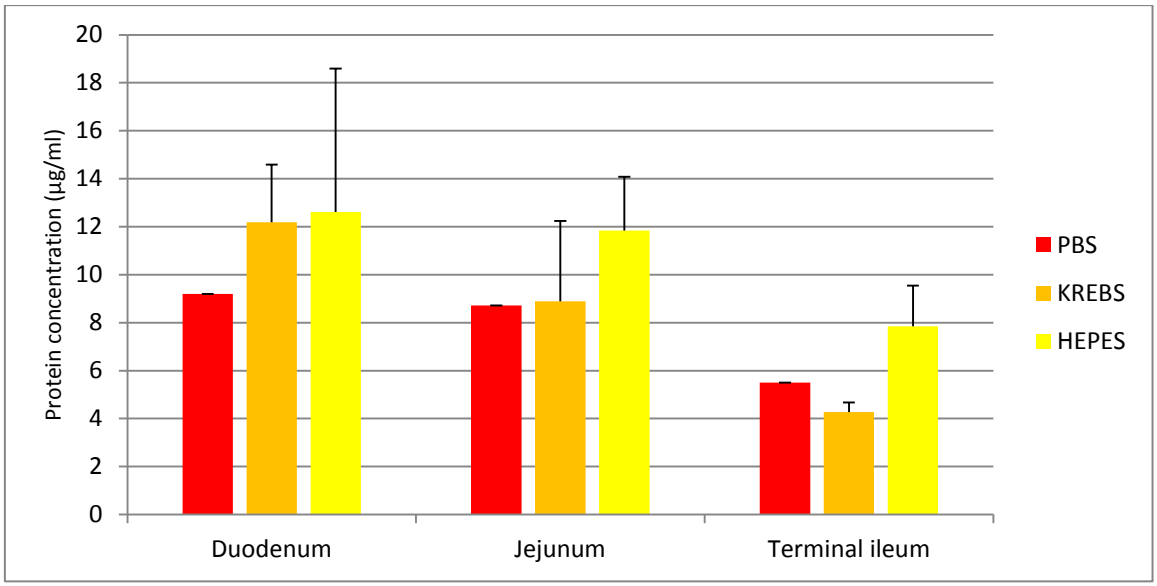


Figure 14: BCA protein assay-protein concentration in µg per ml of duodenum, jejunum and terminal ileum, depending on the buffer used for washing the tissue-PBS, KREBS or HEPES.

In jejunum and terminal ileum there was higher average TG2 activity in tissue washed with KREBS buffer, but there were high deviations, which could be due, for instance, to non-homogenous distribution of the protein in the tissue homogenate, among others. As we only took 50µL of the whole sample for the assay, this non-homogenous distribution could have a role. High variability of the results is also possible to be because of activation of inactive TG2 in the tissue homogenate, due to Ca^{2+} concentration in buffer, which has been reported to influence the TG2 activity if present in the right concentration (55).

We decided to go on with the experiments using PBS buffer for washing because the results were more consistent and between the range of detection.

4.1.3. **Storage conditions of the samples before TG2 extraction**

Ideally, the extraction of the enzyme and TG2 activity assay would be done immediately after the tissue has been taken, so that the enzyme would remain active. This is hard to do when there is a large number of animals in one experiment and since the harvesting itself takes approximately 15 minutes per animal. To find out if it is possible to store the tissue and do extractions later we tested three different conditions of storing the tissue and evaluated the loss of activity. Our aim was to determine the best way to preserve the active enzyme and at the same time perform the experiment in conditions that allow us to work with a larger number of animals. We used two mice for this experiment.

As seen in the figure 15, change in the storage conditions did not have a significant influence on transglutaminase 2 activity in the tissue. In all samples except from the duodenum the average TG2 activity was slightly higher in freshly extracted tissue than in other two. Although lower values were obtained for the activity in frozen tissue, the signals were high enough for a proper evaluation in the range of detection considered in our experiments.

High variability of the results in freshly extracted tissue might have occurred due to greater activation of inactive intracellular TG2 during extraction as a result of tissue disruption. We have observed low TG2 activity in duodenum in relation to other parts of the intestine which makes the hypothesis of the pH influence on TG2 activity more plausible. The hepatic tissue was much thicker than intestinal, so the same procedure for the extraction of the enzyme might have not been suitable for these samples, therefore lower TG2 activity in the liver.

Considering the fact that studies with the inhibitors will have to be done with big groups of animals and that the results from different conditions of the storage were comparable, we have chosen for further experiments to use liquid nitrogen immediately after harvesting and then store the tissue in freezer at -80°C until extraction and measurement.

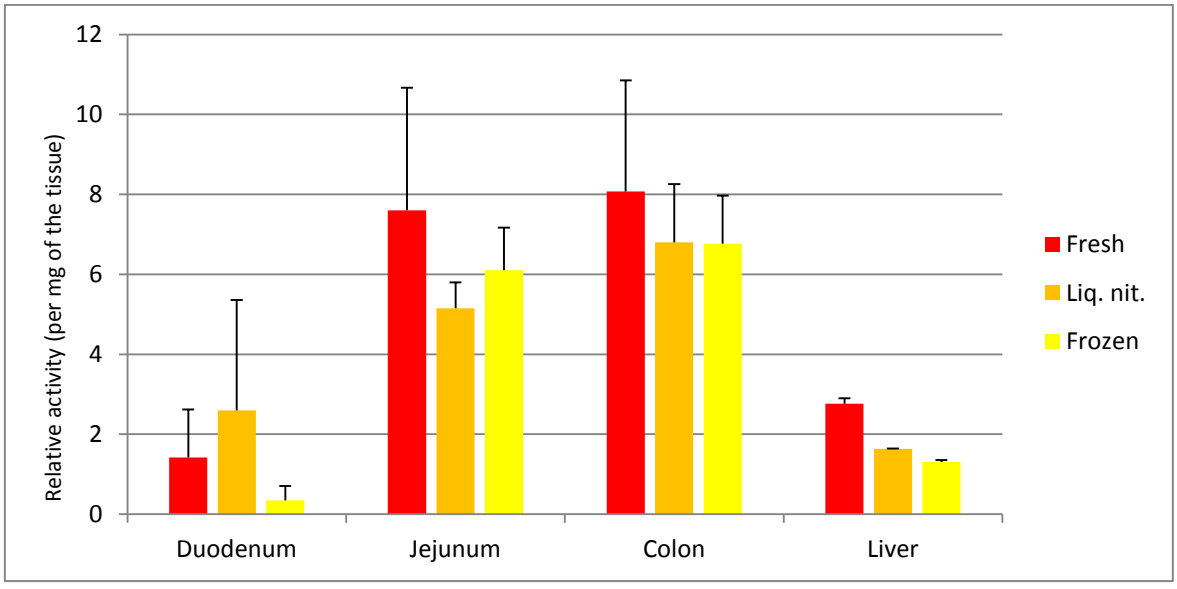


Figure 15: Relative transglutaminase activity in duodenum, jejunum, colon and liver, depending on the storage conditions before TG2 extraction-fresh extracted, extracted after being frozen in liquid nitrogen, extracted after being frozen to 80°C.

4.1.4. **Triton concentration in lysis buffer and dilution of samples**

Triton (polyethyleneglycol) is a non-ionic detergent that lysis cells and is used for protein extractions. It is useful for assays where structure and activity of proteins should remain the same during extraction, because it has low affection to proteins denaturation and the breaking up of protein complexes.

Two concentrations of Triton in lysis buffer were used to determine if lysis affects the later extraction of protein and its activity. As we could not predict the range of the values, we tested the samples in dilution that was in the original protocol and in a higher dilution. Due to the availability, the assay was performed with hepatic tissue, where TG2 is known to be highly expressed (30).

As seen in the figure 16, transglutaminase activity in samples extracted with lysis buffer containing 1% of Triton was approximately 6-fold higher than in samples extracted with 0,5% Triton when we diluted them 1:100, and approximately 2,5-fold higher when the dilution was 1:50. Low activity in 0,5% Triton could be the consequence of incomplete lysis in lower concentration of detergent, which was not enough to release the proteins from the cells. In the literature, 1% Triton is also mentioned for the optimal lysis (56, 57).

Based on our results we decided to use 1% Triton in the original dilution 1:50, since the activity in these conditions was in the most optimal range for further evaluation.

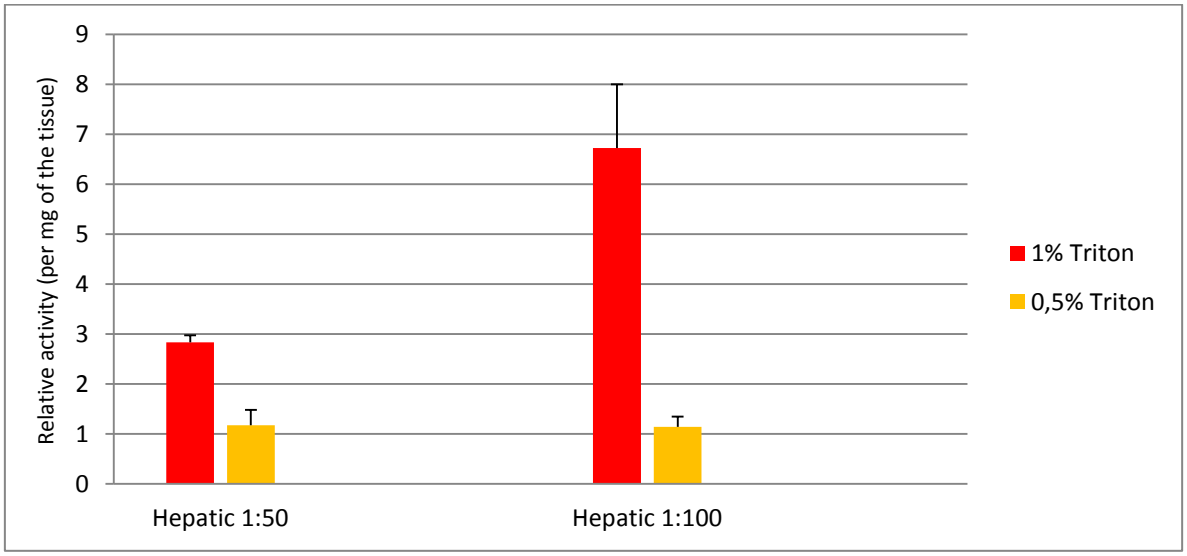


Figure 16: Relative transglutaminase activity in hepatic tissue, depending on the concentration of Triton and dilution of the samples.

4.1.5. **Calcium concentration in diluent buffer**

Calcium is an important co-factor for transglutaminase 2 activity, changing TG2 to an active or inactive stage, depending on the concentration. As it has been proven ″ex vivo″ on cells that low concentrations of calcium activate TG2, but high concentrations inactivate it (55), we evaluated three different concentrations of Ca^{2+} in diluent buffer to provide and maintain the most adequate activation of the enzyme.

The determination of calcium concentration was performed using hepatic, duodenal, jejunal and colon tissue of two animals. The samples were tested in duplicates. The activity in each tissue using three different concentrations did not show any significant difference (figure 17). There were higher results with 5mM buffer in jejunum and colon, but due to the variability we could not conclude it was significant. This means that TG2 involved in inflammation is not influenced by the buffers enriched with calcium.

However, it is possible that we did not measure the active TG2 that is involved in the intestinal inflammation on ″in vivo″ model but also the intracellular enzyme that has been activated by calcium after tissue homogenization.

As we could not determine which one could be considered to be the best, we continued the experiments using 5mM concentration of calcium in diluent buffer, as it has been suggested in the original protocol.

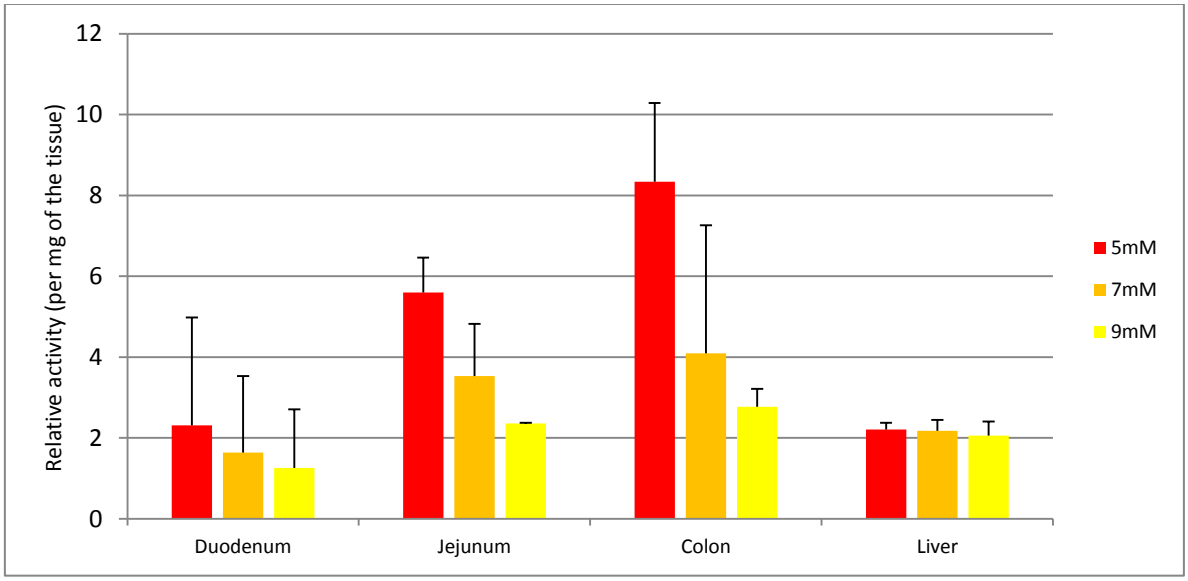


Figure 17: Relative transglutaminase activity in duodenum, jejunum, colon and liver, depending on the calcium concentration in diluent buffer.

4.1.6. **Handling with the aliquots after the extraction and use**

An important part of the optimisation of the method was the way we stored and used homogenate of the tissue. Various reasons can cause a loss of activity of TG2, in our experience oxidative processes, degradation by contaminating proteases and elevated temperature promote deterioration of the activity. Therefore, the harvested tissue should be kept cool, preferentially on ice. Based on previous outcomes (data not shown) we evaluated if this loss of activity also happens when the enzyme has already been extracted. We used the same aliquots of extracted samples for TG2 activity assay on three different days. The samples were defrosted, kept on ice and immediately after use snap frozen in liquid nitrogen.

We have shown with our experiments (figure 18) that the same aliquot can only be used twice, if it is immediately deep frozen in liquid nitrogen and stored at -80°C after first use. The values from second time used aliquots were in the same range as from the first measurement, some were even slightly higher, which was due to deviations between measurements. In the samples 18DIII and 19DIII the values of second measurements were around 30% lower than in the first measurement. This could be due to not precise sampling, not homogenising the aliquot before sampling or bigger loss of activity than in other samples because of inappropriate handing during first measurement. When the same aliquots were used for a third time, values were much lower. This can be explained with the change of the temperature while defrosting and freezing the samples, especially room temperature during sampling, which accelerates the loss of activity. On the other hand, freezing and thawing of the proteins in the PBS buffer have been studied and the results showed that it results in changes of the pH that can affect the enzyme stability and lead to its denaturation (58).

In the sample 20 D III that was incubated with EDTA as a negative control, we can see that all three measurements showed no activity.

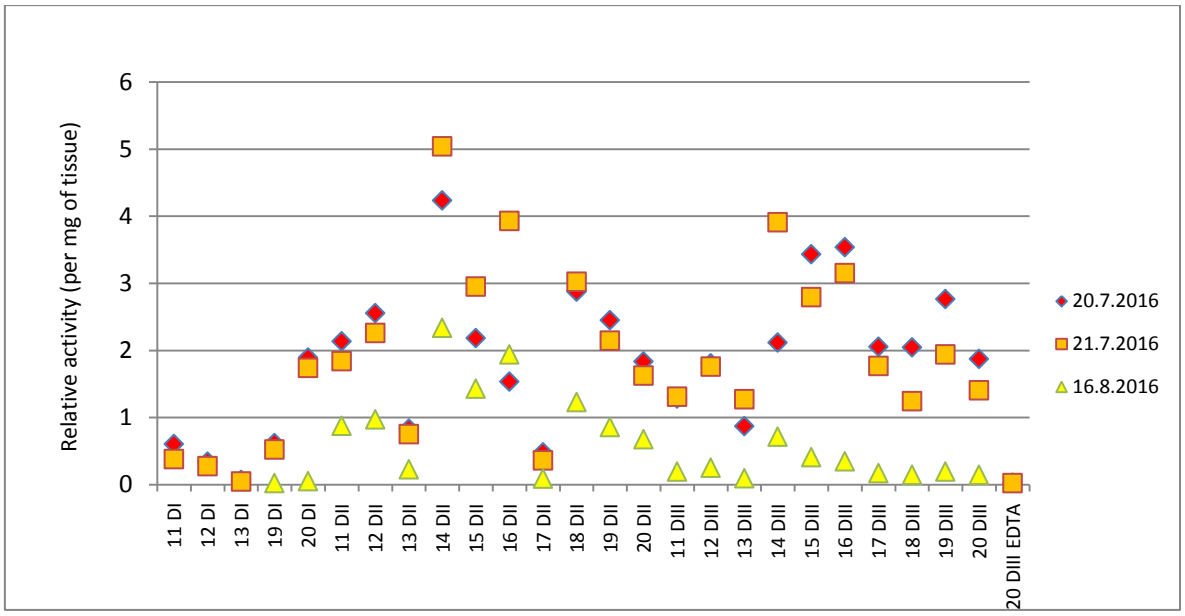


Figure 18: Relative transglutaminase activity in the same aliquots measured 3 times-loss of activity after second defrosting and freezing.

Therefore, we did all the procedures on ice and stored samples into 2 or 3 smaller aliquots, to avoid multiple defrosting of the same aliquot in use in the assays.

4.2. ″In vivo″ evaluation of TG2 activity

We focused on providing a proper ″in vivo″ model of transglutaminase 2 activity. This model should show enhancement of TG2 activity, which is the first step for testing the inhibitor. First we compared the poly I:C model of B6 mice with two other mouse strains, TLR 4-/- and Balb/C, to check whether there were some differences in the baseline of transglutaminase 2 activity, and also response to the treatment with poly I:C.

As it has been mentioned before that TG2 is released from damaged tissue, we compared poly I:C and indomethacin, compounds that injure intestinal tissue and cause inflammation, to induce TG2 activity (31, 42, 46).

4.2.1. **Mouse strain**

Comparison of TG2 activity and its enhancement as a response to the treatment with poly I:C gave interesting results in three mouse strains (figure 19). Number of animals per group was 3 for B6, 5 for TLR 4-/-and 2 for Balb/C.

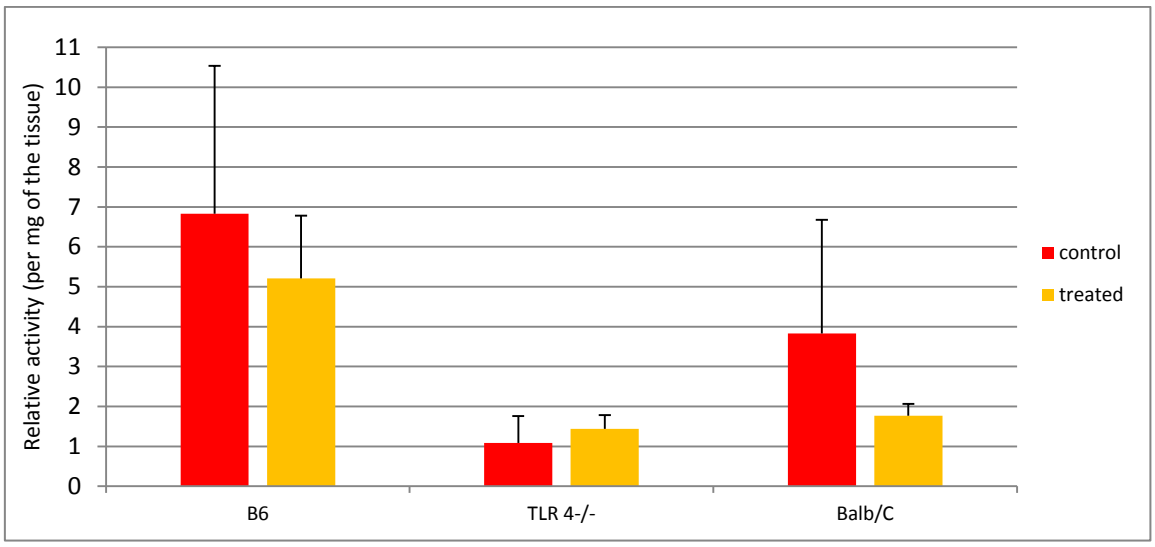


Figure 19: Relative transglutaminase activity per mg of the tissue of duodenum in B6, TLR 4-/- and Balb/C mice, control and treated.

In duodenum of B6 and Balb/C mice the treatment caused reduction of the activity, which was opposite of what we have expected. For TLR 4-/- mice the treated group had a slightly higher average activity of TG2, but some results of control group were even higher than the treated, so the difference was not significant. We have already observed that the activity in duodenum was influenced by gastric acid and tried to reduce this influence by flushing the tissue, but the activity was still not induced in TLR 4-/- mice after treatment with poly I:C. In humans, duodenal tissue should be the one to evaluate the TG2 activity as it is the main tissue affected in celiac disease, but our results and some published data show that in murine the activity of TG2 in intestine is different. It has been reported, that tissue injury activated TG2 in the mice colon (59), but there is not so much evidence of duodenal activation of TG2.

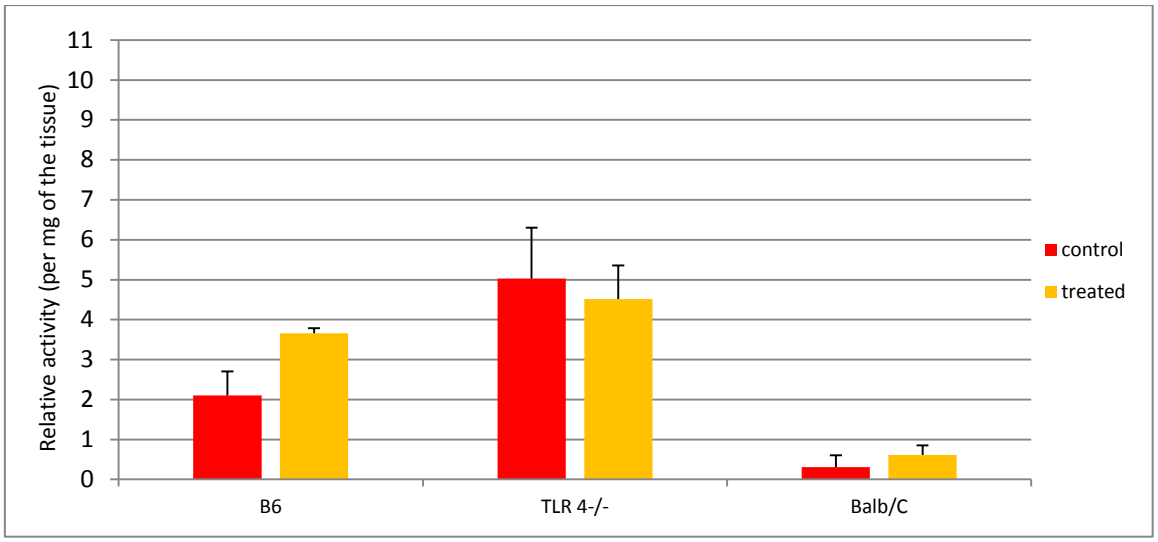


Figure 20: Relative transglutaminase activity per mg of the tissue of jejunum in B6, TLR 4-/- and Balb/C mice, control and treated.

Results of the TG2 activity in jejunum (figure 20) were opposite to the duodenum for B6 and Balb/C mice, showing the tissue difference of the enzyme activity. In the B6 group the signals were in the range that we expected and also the TG2 activity of treated mice was higher than in control, so the activation of the TG2 occurred. In Balb/C strain the treated group had higher TG2 activity than the untreated; however the signals were very low. In TLR4 -/- strain the activities in control and treated group were comparable. The level of activity in both groups was high, indicating possible activation of the intracellular TG2 during extraction.

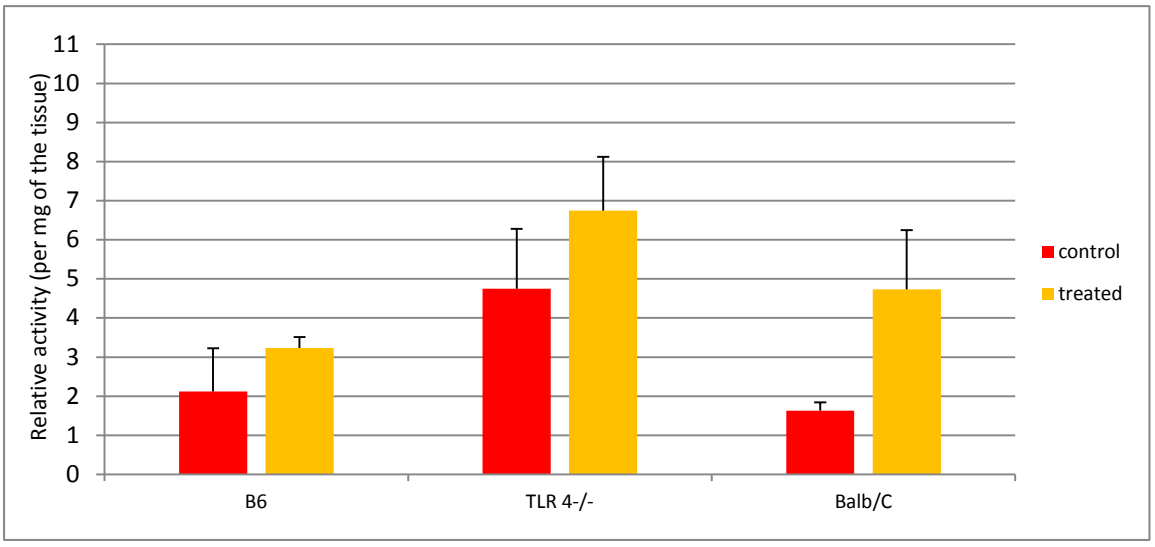


Figure 21: Relative transglutaminase activity per mg of the tissue of terminal ileum in B6, TLR 4-/-and Balb/C mice, control and treated.

In the figure 21 the results of terminal ileum are presented. The activity of the enzyme was increased in the treated groups of all three strains, the biggest response compared to the control was in Balb/C line.

Comparing all three graphs we have observed that the activity of TG2 in the B6 mice decreased down the intestine in both, control and treated groups. This is accordant with the data of Khosla et al. (38) who did the histological analyses of the duodenum, jejunum and ileum that have been treated with poly I:C. They have found out that the TG2 activity was mostly expressed in the duodenum and decreased in jejunum and terminal ileum. In the TLR4 -/- mice the baseline TG2 activity was very low in duodenum, but in jejunum and terminal ileum it was approximately 4-5 times higher. The activities after the treatment were comparable to the controls, only in terminal ileum there was a higher activity in the treated mice. Strong TLR 4 expression was found in distal small intestine and colon of the normal mouse, so there could be compensatory upregulation of other TLR receptors due to TLR 4 knockout in TLR 4-/- mice, that would still cause inflammation and consequently TG2 response (60). In Balb/C strain the activity was high in duodenum of control group and terminal ileum of treated, for jejunum the results were very low. As we only had 2 mice per group for Balb/C, it would be reasonable to repeat the experiments with a bigger number of animals, to confirm that the results in terminal ileum were not coincidental, which would make this part interesting for the study.

Overall, we could not conclude that one of the mouse strains is better for our experiment, as in none of them the results were appropriate for the study of inhibitors in all the tissue.

4.2.2. ″In vivo″ activation of TG2

INDOMETHACIN

Five animals per group were used for the experiment. Due to problems with RNA extraction, four animals per group were used for TG2 expression in terminal ileum.

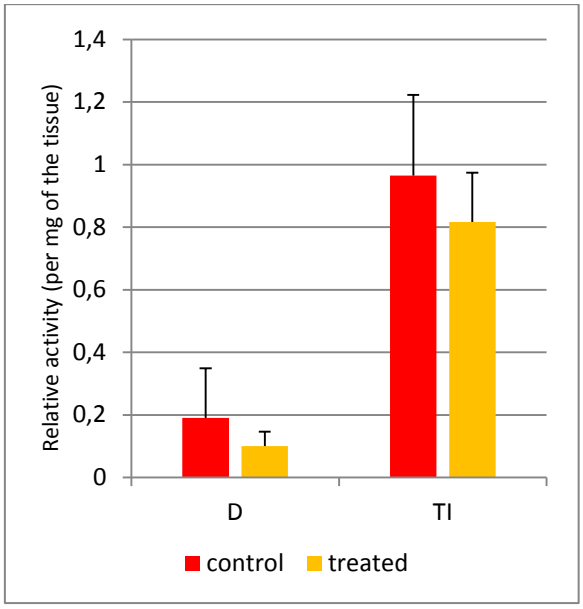


Figure 22: Relative transglutaminase activity per mg of the tissue in duodenum (left) and terminal ileum (right) of B6 mice treated with indomethacin and control.

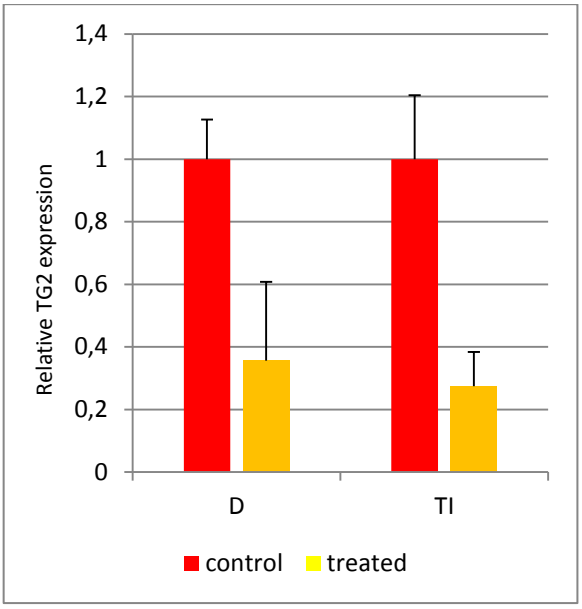


Figure 23: Relative TG2 expression in duodenum (left) and terminal ileum (right) of B6 mice treated with indomethacin and control.

The activity of transglutaminase 2 in animals that were treated with indomethacin (figure 22) shows no significant difference compared to the control group, as it did not induce the activity of the enzyme, but even decreased it. Indomethacin is a non-steroidal anti-inflammatory drug that is used to treat inflammation, so it is possible that the anti-inflammatory effect takes place at this time point more than producing intestinal damage. Another possible explanation for these results can also be the single dose of indomethacin administered. It has been reported in the studies that the treatment lasted for 10 days before the mice were sacrificed and the tissue evaluated, seeing histological changes and enhancement of TG2 activity (46). Results from PCR for TG2 gene in B6 mice treated with indomethacin (figure 23) show that the expression of the gene in duodenum and terminal ileum was lower than in control, which correlates with the results for activity.

POLY I:C

3 animals per group were used for the experiment.

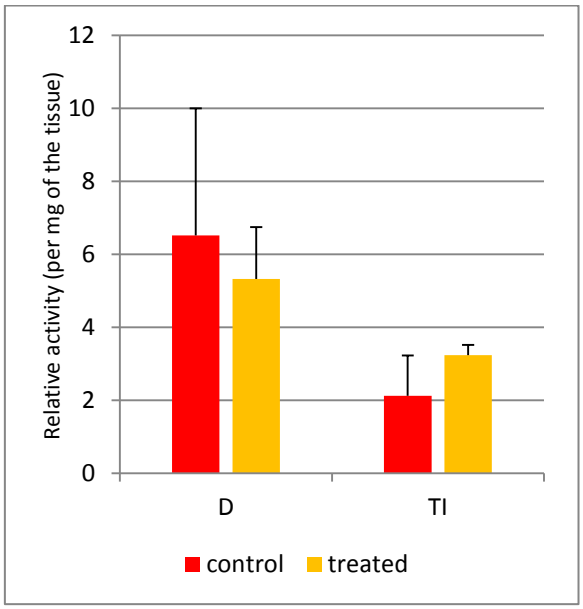


Figure 24: Relative transglutaminase activity per mg of the tissue in duodenum and terminal ileum of B6 mice treated with poly I:C and control.

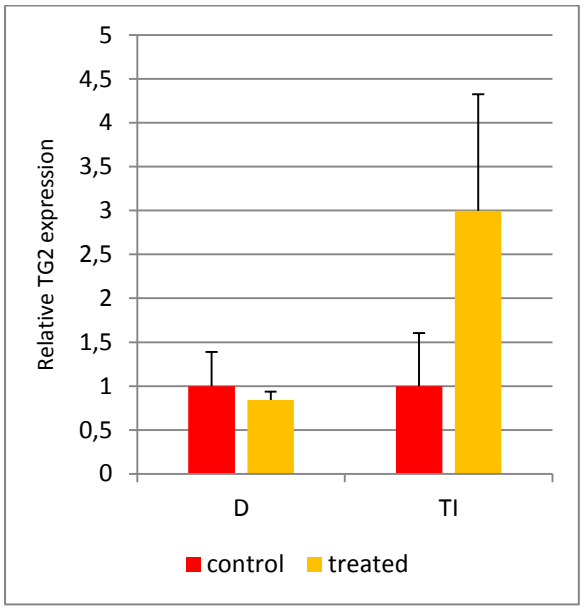


Figure 25: Relative TG2 expression in duodenum and terminal ileum of B6 mice treated with poly I:C and control.

In comparison, experiments with the B6 line of mice treated with poly I:C (figure 24) gave us interesting results, as the activity and expression were comparable in duodenum between treated and control group, but in terminal ileum there was higher expression (figure 25) in treated than in control group. In connection with the previous results of TG2 activity in terminal ileum of different mouse strains, we can see again that terminal ileum is the part of the intestine where TG2 reacts to the treatment with poly I:C. High TG2 gene expression shows that there were already processes on the gene level. It is possible that the activity would be higher if we had sacrificed the animals later, and the gene expression could manifest in higher production of transglutaminase 2.

B6 mice that were used in experiments for the TG2 induction were on a gluten-free diet, so that could have an influence on the TG2 activity that did not rise as it was expected. If the mice were fed with normal food containing gluten, it is possible that there would be more tissue destruction, which would result in higher concentrations of TG2. Namely, it has been reported in the study where the mice received indomethacin in drinking water, that there was higher TG2 expression in the group that was intraperitoneally administered

gliadin than in the group that has been on a gluten-free diet during the whole experiment (46).

5. CONCLUSION

In the first part of my thesis, the focus was on optimising the method of measurement of TG2 activity ″in vivo″ in two mouse models of intestinal inflammation. Our conclusions were that harvesting and sectioning had to be done on ice to avoid loss of activity due to a short half-life of TG2 activity of ~15 min at room temperature. Using always the same scheme of sectioning, differences in baseline TG2 activity were found between parts of the intestine. For washing the tissue, the best buffer was determined to be PBS. Moreover, storage of the harvested sections had to be performed immediately in liquid nitrogen followed by transfer to a -80°C freezer. For extraction, the use of 1% Triton X-100 in lysis buffer and 5 mM calcium concentration in diluent buffer preserved optimal enzyme activity. Calcium activates TG2, but also causes its rapid inactivation (55). Therefore, the next step towards an optimised method was to test the influence of calcium on TG2 activation ″ex vivo″, not only during the procedure of extraction but also during measurement of the activity in the tissue homogenate with ELISA-like assay.

The half-life of the TG2 protein once secreted is around 11 hours (28), but the activity diminishes much faster, so the procedures must be done as quickly as possible and under conditions that we established as optimal to maintain the activity and the enzyme. Therefore, each aliquot could only be used twice, as with a third use the activity was much decreased. As there were tissue differences, especially between intestine and liver, which is much more compact, some other buffers and concentrations of the constituents need to be adjusted for an organ-specific correct extraction.

The second part of the project was focused on the ″in vivo″ model of enzyme activation. No mouse strain dependent differences in enzyme activity were detected. Moreover, with the methods used, there was no increased activity after treatment in all examined parts of the intestine, neither duodenum, jejunum not terminal ileum. The treatment with indomethacin caused even a decrease of TG2 activity and gene expression, while the treatment with poly I:C increased TG2 activity and gene expression, but only in the more distal parts of the intestine.

Another important subject to be evaluated in the future is the possibility of the differentiation between the intra- and extracellular TG2. It would be important to distinguish these forms in the extracted samples and also how long the extracellular

fractions that may get activated during intestinal inflammation remain active after the extraction. Since we destroyed the tissue and the cells, it is possible that we measured both of them, so the results would not be relevant for testing the inhibitor for TG2 for the treatment of celiac disease, where the extracellular TG2 is responsible for the pathogenesis. To determine the extracellular TG2 only, the treatment of tissue slices and histology appears to be the most promising approach, as ″ex vivo″ evaluation could provide an assessement of the ″in vivo″ activated extracellular TG2.

REFERENCES

1. Dhalwani NN, West J, Sultan AA, Ban L, Tata LJ: Women with celiac disease present with fertility problems no more often than women in the general population. Gastroenterology 2014; 147: 1267-1274.
2. Cappello M, Morreale GC, Licata A: Elderly onset celiac disease: A narrative Review. Clinical Medicine Insights: Gastroenterology 2016; 9: 41-49.
3. Vilppula A, Kaukinen K, Luostarinen L, Krekelä I, Patrikainen H, Valve R, Mäki M, Collin P: Increasing prevalence and high incidence of celiac disease in elderly people: A population-based study. BMC Gastroenterology 2009; 9:49.
4. Lie BA, Sollid LM: Celiac disease genetics: Current concepts and practical applications. Clinical Gastroenterology and Hepatology 2005; 3: 843–851.
5. Megiorni F, Pizzuti A: HLA-DQA1 and HLA-DQB1 in celiac disease predisposition: practical implications of the HLA molecular typing. Journal of Biomedical Science 2012; 19:88.
6. Sollid LM: Molecular basis of celiac disease. Annual Review of Immunology 2000; 18: 53-81.
7. Schuppan D, Junker Y, Barisani D: Celiac disease: From pathogenesis to novel therapies. Gastroenterology 2009; 137(6): 1912–1933.
8. Karell K, Louka AS, Moodie SJ, Ascher H, Clot F, Greco L, Ciclitira PJ, Sollid LM, Partanen J: Hla types in celiac disease patients not carrying the DQA1*05-DQB1*02 (DQ2) heterodimer: results from the european genetics cluster on celiac disease. Human Immunology 2013; 64(4): 469–477.
9. Meresse B, Ripoche J, Heyman M, Cerf-Bensussan N: Celiac disease: from oral tolerance to intestinal inflammation, autoimmunity and lymphomagenesis. Mucosal Immunology 2009; 2(1):8-23.
10. Wolters VM, Wijmenga C: Genetic background of celiac disease and its clinical implications. The American Journal of Gastroenterology 2008; 103(1):190-195.
11. Qiao SW1, Sollid LM, Blumberg RS: Antigen presentation in celiac disease. Current Opinion in Immunology 2009; 21(1): 111–117.
12. Meresse B, Verdier J, Cerf-Bensussan N. The cytokine interleukin 21: a new player in coeliac disease. Gut 2008; 57(7): 879-881.

13. Bai JC, Ciacci C, Corazza GR, Fried M, Olano C, Rostami-Nejad M, González A, Green P,Gutierrez-Achury J, Schultz M, Verdú E, Barada K, Gibson P, Koletzko S, Coton T, Mulder C, Makharia G, LeMair A: Celiac disease. World Gastroenterology Organisation Global Guidelines 2016.
14. Datta Gupta S: Pathology of celiac disease: a brief review. Tropical Gastroenterology 2013; 34(4):207-226.
15. Laboratory Procedure Manual, Mayo Clinic: Department of Dermatology https://www.cdc.gov/nchs/data/nhanes/nhanes_11_12/tgema_g_met_ema.pdf (Access: 11.7.2017).
16. Dahlbom I, Olsson M, Forooz NK, Sjöholm AG, Truedsson L, Hansson T: Immunoglobulin G (IgG) anti-tissue transglutaminase antibodies used as markers for iga-deficient celiac disease patients. Clinical and Diagnostic Laboratory Immunology 2015; 12(2): 254-258.
17. Benson BC1, Mulder CJ, Laczek JT: Anti-Gliadin antibodies identify celiac patients overlooked by tissue transglutaminase antibodies. Hawai'i Journal of Medicine & Public Health 2013; 72(9 Suppl 4): 14–17.
18. Gujral N, Freeman HJ, Thomson AB: Celiac disease: Prevalence, diagnosis, pathogenesis and treatment. World Journal of Gastroenterology 2012; 18(42): 6036–6059.
19. Taylor AK, Lebwohl B, Snyder CL, et al. Celiac Disease. GeneReviews, 2008, (updated 2015). Available from: https://www.ncbi.nlm.nih.gov/books/NBK1727/ (Access: 11.7.2017).
20. Commission Implementing Regulation (EU) No 828/2014 of 30 July 2014 on the requirements for the provision of information to consumers on the absence or reduced presence of gluten in food. Official Journal of the European Union L 2014; 228: 5–8.
21. Di Cagno R, De Angelis M, Auricchio S, Greco L, Clarke C, De Vincenzi M, Giovannini C, D'Archivio M, Landolfo F, Parrilli G, Minervini F, Arendt E, Gobbetti M: Sourdough bread made from wheat and nontoxic flours and started with selected lactobacilli is tolerated in celiac sprue patients. Applied and Environmental Microbiology 2004; 70(2): 1088-1096.
22. Sulic AM, Kurppa K, Rauhavirta T, Kaukinen K, Lindfors K: Transglutaminase as a therapeutic target for celiac disease. Expert Opinion on Therapeutic Targets 2014; 19(3): 335-348.

23. Siegel M, Khosla C: Transglutaminase 2 inhibitors and their therapeutic role in disease states. Pharmacology & Therapeutics 2007; 115(2): 232–245.
24. Badarau E, Collighan RJ, Griffin M: Recent advances in the development of tissue transglutaminase (TG2) inhibitors. Amino Acids 2013; 44(1): 119–127.
25. http://zedira.com/News/Press-Release-Dr-Falk-Pharma-GmbH-and-Zedira-enter-a-phase-1b-clinical-trial-for-a-celiac-disease-drug_90 (Access: 11.7.2017).
26. http://www.ligandchiral.com/index.php?_m=mod_product&_a=view&p_id=274 (Access: 11.7.2017).
27. http://enzyme.expasy.org/EC/2.3.2.13 (Access: 11.7.2017).
28. Griffin M, Casadio R, Bergamini CM: Transglutaminases : Nature's biological glues. Biochemistry 2002; 368(Pt 2): 377-396.
29. Jiang WG, Ablin R, Douglas-Jones A, Mansel RE: Expression of transglutaminases in human breast cancer and their possible clinical significance. Oncology Reports 2003; 10(6): 2039-2044.
30. Klöck C, DiRaimondo TR, Khosla C: Role of transglutaminase 2 in celiac disease pathogenesis. Seminars in Immunopathology 2012; 34(4): 513-522.
31. Siegel M, Strnad P, Watts RE, Choi K, Jabri B, Omary MB, Khosla C: Extracellular TG2 is catalytically inactive, but is transiently activated upon tissue injury. Plos One 2008; 3(3):e1861 (Access: 11.7.2017).
32. De Laurenzi V, Melino G: Gene disruption of tissue transglutaminase. Molecular and Cellular Biology 2001; 21: 148-155.
33. Jin X, Stamnaes J, Klöck C, DiRaimondo TR, Sollid LM, Khosla C: Activation of extracellular transglutaminase 2 by thioredoxin. The Journal of Biological Chemistry 2011; 286(43), 37866-37873.
34. Mehta K, Kumar A, Kim HI. Transglutaminase 2: A multi-tasking protein in the complex circuitry of inflammation and cancer. Biochemical Pharmacology 2010; 80(12): 1921-1929.
35. Kenniston JA, Conley GP, Sexton DJ, Nixon AE: A homogeneous fluorescence anisotropy assay for measuring transglutaminase 2 activity. Analytical Biochemistry 2013; 436(1): 13-15.
36. Perez Alea M, Kitamura M, Martin G, Thomas V, Hitomi K, El Alaoui S: Development of an isoenzyme-specific colorimetric assay for tissue transglutaminase 2 cross-linking activity. Analytical Biochemistry 2009; 389(2): 150-156.

37. Fernández I, Sánchez A, Díez P, Martínez-Ruiz P, Di Pierro P, Porta R, Villalonga R, Pingarrón JM: Nanochannel-based electrochemical assay for transglutaminase activity. Chemical Communications 2014; 50(87): 13356-13358.
38. Dafik L, Albertelli M, Stamnaes J, Sollid LM, Khosla C: Activation and inhibition of transglutaminase 2 in mice. PLoS One 2012; 7: e30642 (Access: 11.7.2017).
39. Chattopadhyay S, Sen GC: dsRNA-activation of TLR3 and RLR signaling: Gene induction-dependent and independent effects. Journal of Interferon & Cytokine Research 2014; 34(6) 427–436.
40. Hiscott J, Pitha P, Genin P, Nguyen H, Heylbroeck C, Mamane Y, Algarte M, Lin R: Triggering the interferon response: the role of IRF-3 transcription factor. Journal of Interferon & Cytokine Research 2004; 19(1): 1-13.
41. Meylan E, Burns K, Hofmann K, Blancheteau V, Martinon F, Kelliher M, Tschopp J: RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. Nature Immunology 2004; 5(5): 503-507.
42. Araya RE, Jury J, Bondar C, Verdu EF, Chirdo FG: Intraluminal administration of poly i:c causes an enteropathy that is exacerbated by administration of oral dietary antigen. PLoS ONE 2014; 9(6): e99236 (Access: 11.7.2017).
43. Shi S, Wang H, Gao H, Li Z, Chen FX, Zuo XL, Li YQ: Increased gap density predicts weakness of the epithelial barrier in vivo by confocal laser endomicroscopy in indomethacin-induced enteropathy. Digestive Diseases and Sciences 2014; 59(7): 1398–1405.
44. https://pubchem.ncbi.nlm.nih.gov/compound/indomethacin#section=Top (Access: 11.7.2017)
45. Matsui H, Shimokawa O, Kaneko T, Nagano Y, Rai K, Hyodo I: The pathophysiology of non-steroidal anti-inflammatory drug (NSAID)-induced mucosal injuries in stomach and small intestine. Journal of Clinical Biochemistry and Nutrition 2011; 48(2): 107-111.
46. Mazzarella G, Bergamo P, Maurano F, Luongo D, Rotondi Aufiero V, Bozzella G, Palmieri G, Troncone R, Auricchio S, David C, Rossi M: Gliadin intake alters the small intestinal mucosa in indomethacin-treated HLA-DQ8 transgenic mice. The American Journal of Physiology - Gastrointestinal and Liver Physiology 2014; 307(3) G302-G312.
47. https://www.jax.org/strain/000664 (Access: 11.7.2017)

48. http://www.informatics.jax.org/external/festing/mouse/docs/C57BL.shtml (Access: 11.7.2017)
49. https://www.jax.org/strain/007227 (Access: 11.7.2017)
50. https://www.jax.org/strain/000651 (Access: 11.7.2017)
51. Odii BO, Coussons P: Biological functionalities of transglutaminase 2 and the possibility of its compensation by other members of the transglutaminase family. The Scientific World Journal 2014; ID 714561.
52. Ierardi E, Amoruso A, Giorgio F, Principi M, Losurdo G, Piscitelli D, Buffelli F, Fiore MG, Mongelli A, Castellaneta NM, Giangaspero A, De Francesco V, Montenegro L, Di Leo A: Mucosal molecular pattern of tissue transglutaminase and interferon gamma in suspected seronegative celiac disease at marsh 1 and 0 stages. The Saudi Journal of Gastroenterology 2015, 21(6): 379-385.
53. Pham BT, van Haaften WT, Oosterhuis D, Nieken J, de Graaf IA, Olinga P: Precision cut rat, mouse, and human intestinal slices as novel models for the early onset of intestinal fibrosis. Physiological Reports 2015; 3(4): e12323 (Access: 11.7.2017).
54. Chae KY, Kim JH, Park WJ, Kim YG, Yun HY, Kwon NS, Im MJ, Baek KJ: Distinct pH modulation for dual function of Gαh (Transglutaminase II). The Journal of Biochemistry 2005; 137 (3): 407-413.
55. Jeong EM, Kim CW, Cho SY, Jang GY, Shin DM, Jeon JH, Kim IG: Degradation of transglutaminase 2 by calcium-mediated ubiquitination responding to high oxidative stress. FEBS letters 2009; 583(4): 648-654.
56. Hwang B, Tsai K, Mitragotri S: Optimized lysis buffer reagents for solubilization and preservation of proteins from cells and tissues. Drug Delivery and Translational Research 2013; 3 (5): 428-436.
57. Peach M, Marsh N, MacPhee DJ: Protein solubilization: Attend to the choice of lysis buffer; Protocol-Protein electrophoresis. Methods in Molecular Biology 2012; 869: 37-47.
58. Pikal-Cleland KA, Rodríguez-Hornedo N, Amidon GL, Carpenter JF: Protein denaturation during freezing and thawing in phosphate buffer systems: monomeric and tetrameric beta-galactosidase. Archives of Biochemistry and Biophysics 2000; 384(2): 398-406.

59. Jeong EM, Son YH, Choi Y, Kim JH, Lee JH, Cho SY, Kim IG: Transglutaminase 2 is dispensable but required for the survival of mice in dextran sulfate sodium-induced colitis. Experimental & Molecular Medicine 2016; 48(11):e267 (Access: 11.7.2017).
60. Ortega-Cava CF, Ishihara S, Rumi MA, Kawashima K, Ishimura N, Kazumori H, Udagawa J, Kadowaki Y, Kinoshita Y: Strategic compartmentalization of Toll-like receptor 4 in the mouse gut. The Journal of Immunology 2003; 170 (8) 3977-3985.
61. http://www.chemexpress.cn/1542132-88-6.html (Access: 11.7.2017)
62. http://www.informatics.jax.org/cookbook/figures/figure57.shtml (Access: 11.7.2017)
63. http://www.informatics.jax.org/cookbook/figures/figure54.shtml (Access: 11.7.2017)
64. https://pubchem.ncbi.nlm.nih.gov/compound/indomethacin#section=Top (Access: 11.7.2017)
65. http://www.adipogen.com/iax-200-021/polyinosinic-polycytidylic-acid-poly-i-c-endotoxin-free-sterile.html (Access: 11.7.2017)
66. Mitchell C, Willenbring H: A reproducible and well-tolerated method for 2/3 partial hepatectomy in mice. Nature Protocols 2008; 3(7): 1167 - 1170.

APPENDIX

Raw data of the spectrophotometrical measurements-absorbance

Table II: Buffer used for washing the tissue

SAMPLE	Measurement 1	Measurement 2	Measurement 3
P1 D2	6,7178949	5,34169575	1,23766085
K1 D2	4,08001419	3,48389839	2,89382067
K2 D2	5,80199998	4,62410266	3,25266669
H1 D2	0,3684583	0,3183529	0,19780187
H2 D2	3,89891145	3,65893386	3,53697454
P1 J	4,55997971	4,67841953	4,51048628
P1 TI	4,85216345	5,99439097	5,12628203
K1 J	8,04125679	13,1531419	12,4696722
K1 TI	2,80695874	7,14166651	6,67242274
K2 J	5,41600008	3,29224232	5,09254545
K2 TI	10,2977567	8,22499997	10,5956728
H1 J	0,50698005	2,80332382	3,97782529
H1 TI	0,60319866	5,51540402	7,01523593
H2 J	0,24764632	2,4135497	5,70706119
H2 TI	0,47408088	0,2088848	5,25398277

Table III: BCA standards

STANDARD	CONC. (µg/ml)	Measurement 1	Measurement 2
STD A	2000	2,23769999	2,16269994
STD B	1500	1,99940002	2,09669995
STD C	1000	1,3549	1,37679994
STD D	750	1,09969997	1,09860003
STD E	500	0,7967	0,81290001
STD F	250	0,4605	0,44839999
STD G	125	0,29980001	0,2642
STD H	25	0,1252	0,1241
STD I	0	0,0877	0,0918

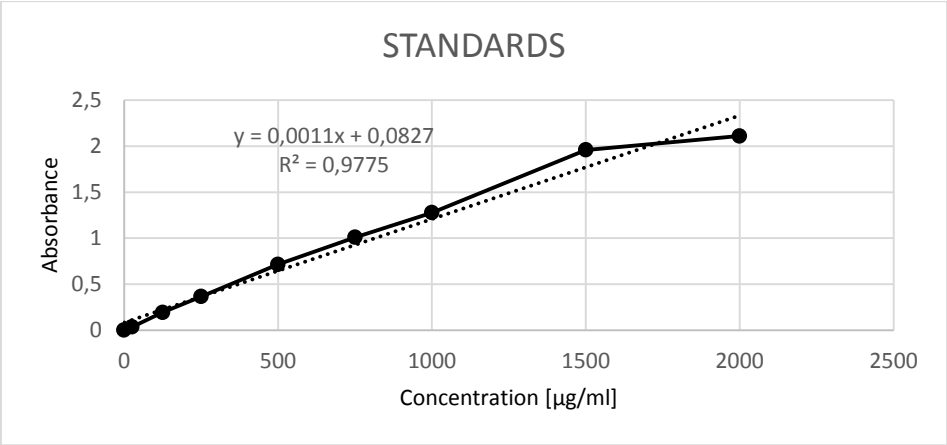


Figure 26: Calibration curve for BCA standards

Table IV: BCA assay

SAMPLE	BCA 1	BCA 2	SAMPLE	BCA 1	BCA 2
P1 D1	0,28580001	0,29069999	**H1 D4**	0,24609999	0,64480001
P1 D2	0,29620001	0,28799999	**H2 D1**	0,54769999	0,5327
P1 D3	0,2474	0,2383	**H2 D2**	0,2568	0,25029999
P1 D4	0,2282	0,2111	**H2 D3**	0,74940002	0,72719997
K1 D1	0,47150001	0,46810001	**H2 D4**	0,60049999	0,58249998
K1 D2	0,42820001	0,37889999	**P1 J**	0,28909999	0,27410001
K1 D3	0,48140001	0,43169999	**P1 TI**	0,20819999	0,2132
K1 D4	0,4447	0,4005	**K1 J**	0,25529999	0,1881
K2 D1	0,23190001	/	**K1 TI**	0,2448	0,13779999
K2 D2	0,31400001	0,31040001	**K2 J**	0,3504	0,34779999
K2 D3	0,40360001	0,3524	**K2 TI**	0,1777	0,1744
K2 D4	0,4962	0,46489999	**H1 J**	0,40549999	0,3804
H1 D1	0,66659999	0,6124	**H1 TI**	0,23029999	0,23029999
H1 D2	0,4831	0,47909999	**H2 J**	0,3118	0,30379999
H1 D3	0,69090003	0,68889999	**H2 TI**	0,29949999	0,28979999
Blank	0,0877	0,0918			

Table V: Storage conditions of the samples before TG2 extraction

SAMPLE	Measurement 1	Measurement 2
H1F1	2,48329997	2,51830006
D1F1	1,09389997	1,02489996
J1F1	2,56550002	2,73090005
C1F1	1,99450004	1,94739997
H2F1	1,86210001	2,47449994
D2F1	0,26210001	0,2938
J2F1	2,67470002	2,7572999
C2F1	1,78999996	2,13720012
H1C1	2,36310005	2,66869998
D1C1	0,2219	0,0479
J1C1	2,2039001	1,99730003
C1C1	2,42440009	1,86549997
H2C1	2,52469993	2,46950006
D2C1	2,8599999	2,83909988
J2C1	2,30669999	2,72460008
C2C1	2,36529994	2,18980002
H1B1	2,50230002	2,47670007
D1B1	0,29949999	0,32449999
J1B1	2,7118001	2,40310001
C1B1	2,07389998	2
H2B1	2,09179997	1,75849998
D2B1	0,1547	0,2155
J2B1	2,58430004	2,19230008
C2B1	2,13010001	1,51479995
D1F2	0,9307	1,47660005
D2F2	1,36300004	1,25940001
D1C2	0,087	0,0741
D2C2	2,19560003	1,83249998
D2A1	0,1644	0,28040001
D1A1	0,22319999	0,1829
EDTA	0,0989	0,0979
Blank	0,0768	0,0921

Table VI: Triton concentration in lysis buffer and dilution of samples

SAMPLE	Measurement 1	Measurement 2	Measurement 3
Liver 1- 1%	0,9813	1,15999997	1,08749998
Liver 2- 1%	1,24880004	1,04630005	1,84440005
Liver 1- 0,5%	0,56279999	1,12419999	2,42810011
Liver 2- 0,5%	1,25380003	1,17789996	2,28290009

Tables VII-IX: Calcium concentration in diluent buffer

Table VII: 5mM

SAMPLE	Measurement 1	Measurement 2
H1C2	2,90389991	2,92670012
D1C2	0,0507	0,0491
J1C2	2,33010006	2,46740007
C1C2	2,19619989	2,42799997
H2C2	3,0553	3,11360002
D2C2	2,64639997	2,57159996
J2C2	2,66969991	2,56550002
C2C2	3,03539991	2,51929998

Table VIII: 7mM

SAMPLE	Measurement 1	Measurement 2
H1C2	2,94260001	3,02600002
D1C2	0,05	0,0479
J1C2	1,21599996	2,32640004
C1C2	2,5933001	2,15739989
H2C2	3,18709993	2,64499998
D2C2	3,05830002	0,67680001
J2C2	2,23060012	0,42269999
C2C2	0,52039999	0,31290001

Table IX: 9mM

SAMPLE	Measurement 1	Measurement 2
H1C2	2,96799994	2,87809992
D1C2	0,0613	0,0565
J1C2	0,53710002	1,29620004
C1C2	1,25890005	0,98589998
H2C2	2,39310002	2,85010004
D2C2	1,90900004	0,97320002
J2C2	0,92760003	1,68570006
C2C2	0,8222	0,57520002

Table X: Handling with the aliquots after the extraction and use

SAMPLE	Measurement 1	Measurement 2	Measurement 3
11 DI	0,600559	0,379473	-0,000559
12 DI	0,339638	0,272336	-0,000183
13 DI	0,067150	0,046634	-0,000212
19 DI	0,619218	0,521032	0,022534
20 DI	1,892900	1,737395	0,050406
11 DII	2,134304	1,838113	0,873799
12 DII	2,552833	2,260198	0,970538
13 DII	0,830255	0,752402	0,226633
14 DII	4,235193	5,041102	2,333167
15 DII	2,183222	2,950597	1,428188
16 DII	1,532064	3,926490	1,939487
17 DII	0,480985	0,360615	0,086409
18 DII	2,871741	3,020531	1,227108
19 DII	2,447584	2,144263	0,855480
20 DII	1,832668	1,621853	0,674235
11 DIII	1,279249	1,309310	0,192580
12 DIII	1,805918	1,755403	0,250386
13 DIII	0,872272	1,269816	0,094643
14 DIII	2,115897	3,908615	0,714308
15 DIII	3,430512	2,792476	0,409199
16 DIII	3,538491	3,150725	0,347379
17 DIII	2,052404	1,766995	0,170574
18 DIII	2,042554	1,242325	0,146821
19 DIII	2,765841	1,940625	0,191649
20 DIII	1,872749	1,406695	0,144165
20 DIII EDTA	0,026025	0,020438	-0,000141

Mouse strain

Table XI: Duodenum TLR 4-/-

SAMPLE	Measurement 1	Measurement 2	Measurement 3
1 D	2,64310002	2,67589998	2,67459989
2 D	2,56559992	2,25609994	2,46479988
3 D	2,67510009	2,6322	2,6098001
4 D	2,41499996	2,51920009	2,36380005
5 D	2,29690003	1,82910001	2,4526999
6 D	2,55749989	2,59209991	2,58890009
7 D	2,69840002	2,43969989	2,6256001
8 D	2,54559994	2,48920012	2,58170009
9 D	3,0250001	2,95970011	2,9986999
10 D	0,47499999	0,551	0,5431

Table XII: Duodenum B6

SAMPLE	Measurement 1	Measurement 2	Measurement 3
1 D	3,43860006	3,64669991	3,52049994
2 D	3,31010008	3,63179994	3,48390007
3 D	3,70210004	1,45510006	3,72670007
4 D	3,74519992	3,56010008	3,71720004
5 D	3,704	3,75589991	3,77139997
6 D	3,5704999	3,62890005	3,62159991

Table XIII: Duodenum Balb/C

SAMPLE	Measurement 1	Measurement 2	Measurement 3
1 D	1,99189997	1,81509995	0,65880001
2 D	2,06570005	1,24440002	0,62779999
3 D	2,44330001	1,81840003	1,17260003
4 D	2,2729001	1,03509998	0,5309

Table XIV: Jejunum TLR 4-/-

SAMPLE	Measurement 1	Measurement 2	Measurement 3
1 J	3,39109993	3,26609993	3,34770012
2 J	3,28500009	3,21569991	3,16350007
3 J	3,00460005	2,66750002	2,63310003
4 J	2,9605999	2,83229995	2,80209994
5 J	2,17529988	2,77220011	2,82500005
6 J	1,53419995	2,78060007	2,70679998
7 J	2,61879992	2,38969994	2,72849989
8 J	3,27040005	3,12940001	3,05890012
9 J	3,52889991	3,45939994	3,45289993
10 J	3,07529998	2,7973001	3,02679992

Table XV: Jejunum B6

SAMPLE	Measurement 1	Measurement 2	Measurement 3
1 J	3,497800112	3,406300068	3,65260005
2 J	3,65199995	3,711600065	3,631599903
3 J	3,807899952	3,719599962	3,875400066
4 J	1,650099993	3,327199936	2,902400017
5 J	3,293600082	3,20600009	0,52700001
6 J	3,022399902	0,825900018	0,168899998

Table XVI: Jejunum Balb/C

SAMPLE	Measurement 1	Measurement 2	Measurement 3
1 J	0,702099979	0,231800005	0,430200011
2 J	0,934000015	0,431199998	0,310699999
3 J	0,608200014	0,263999999	0,194900006
4 J	0,124600001	0,071400002	0,0579

Table XVII: Terminal ileum TLR 4-/-

SAMPLE	Measurement 1	Measurement 2	Measurement 3
1 TI	3,02160001	2,94959998	2,89479995
2 TI	2,99979997	2,9460001	2,94580007
3 TI	2,75	2,61570001	2,50359988
4 TI	3,03279996	2,83990002	1,78980005
5 TI	2,88310003	2,7650001	2,66650009
6 TI	3,26749992	0,37639999	3,11619997
7 TI	3,47819996	0,498	3,33829999
8 TI	2,94959998	0,30410001	2,83240008
9 TI	2,0783999	0,66369998	2,29410005
10 TI	2,30850005	0,74010003	1,255

Table XVIII: Terminal ileum B6

SAMPLE	Measurement 1	Measurement 2	Measurement 3
1 TI	3,556099892	3,498800039	3,423000097
2 TI	3,344700098	3,308300018	3,297100067
3 TI	3,019500017	2,730600119	2,920200109
4 TI	3,496799946	3,457400084	3,27060008
5 TI	3,145999908	0,520900011	2,333899975
6 TI	0,54519999	0,169699997	2,121999979

Table XIX: Terminal ileum Balb/C

SAMPLE	Measurement 1	Measurement 2	Measurement 3
1 TI	1,991700053	1,639099956	2,584100008
2 TI	3,460999966	3,183900118	2,713099957
3 TI	0,999499977	0,696099997	0,833199978
4 TI	0,596800029	0,53579998	0,675999999

″In vivo″ activation of TG2

Table XX: INDOMETHACIN-duodenum TG2 activity

SAMPLE	Measurement 1	Measurement 2
1 D	0,179299995	0,216800004
2 D	0,1087	0,111400001
3 D	0,207599998	0,0594
4 D	0,194299996	0,241899997
5 D	0,197500005	0,214100003
6 D	0,114200003	0,126399994
7 D	0,2086	0,231000006
8 D	0,055199999	0,057599999
9 D	0,343800008	0,382400006
10 D	0,380299985	0,617200017

Table XXI: INDOMETHACIN-duodenum TG2 expression

SAMPLE	TG2	GAPDH
1 D	23,0489178	18,8332062
2 D	22,9562759	18,7649193
3 D	24,491663	21,5810928
4 D	23,6790009	18,2731819
5 D	28,9675179	23,8369217
6 D	23,8334007	21,2914047
7 D	23,7738304	21,2417011
8 D	23,1661644	20,1726074
9 D	25,4696331	22,9979057
10 D	24,5572891	22,0233192

Table XXII: INDOMETHACIN-terminal ileum TG2 activity

SAMPLE	Measurement 1	Measurement 2
1 TI	0,676299989	0,63319999
2 TI	0,60680002	0,546899974
3 TI	0,488000005	0,479600012
4 TI	0,412400007	0,426400006
5 TI	0,433099985	0,38440001
6 TI	0,753700018	0,644299984
7 TI	0,555700004	0,433099985
8 TI	0,65200001	0,504299998
9 TI	0,649299979	0,502799988
10 TI	0,493200004	0,407299995

Table XXIII: INDOMETHACIN-terminal ileum TG2 expression

SAMPLE	TG2	GAPDH
1 TI	20,87919235	18,03418732
3 TI	23,41018295	20,65691757
4TI	22,61302948	19,68848419
5 TI	32,61978149	28,04613495
6 TI	22,1907692	20,3657589
7 TI	20,63406372	19,4862709
9 TI	21,87124634	20,82618713
10 TI	29,66677856	28,54964828

Table XXIV: POLY I:C-duodenum TG2 activity

SAMPLE	Measurement 1	Measurement 2	Measurement 3
1 D	3,438600063	3,646699905	3,520499945
2 D	3,310100079	3,631799936	3,48390007
3 D	3,702100039	1,45510006	3,726700068
4 D	3,745199919	3,560100079	3,717200041
5 D	3,703999996	3,755899906	3,771399975
6 D	3,570499897	3,628900051	3,621599913

Table XXV: POLY I:C-duodenum TG2 expression

SAMPLE	TG2	GAPDH
1 D	28,49099159	21,36072731
2 D	29,08146095	22,31990623
3 D	27,38579178	20,40052605
4 D	28,34447861	21,69167709
5 D	26,10281944	19,90498161
6 D	29,88721657	22,32716942

Table XXVI: POLY I:C-terminal ileum TG2 activity

SAMPLE	Measurement 1	Measurement 2
1 TI	3,556099892	3,498800039
2 TI	3,344700098	3,308300018
3 TI	3,019500017	2,730600119
4 TI	3,496799946	3,457400084
5 TI	3,145999908	0,520900011
6 TI	0,54519999	0,169699997

Table XXVII: POLY I:C-terminal ileum TG2 expression

SAMPLE	TG2	GAPDH
1 TI	32,29432678	27,7391777
2 TI	29,37169266	23,35308266
3 TI	28,1282196	22,82005692
4 TI	27,91980362	21,98995018
5 TI	28,91265488	21,64887619
6 TI	30,5410881	22,8470993