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Tissue copper – AGE interactions in the aetiopathogenesis of tissue damage in the kidney and the heart in diabetes and their experimental treatment by selective Cu(II)-chelation

Sebastian Brings

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biological Sciences

The University of Auckland
2013
Abstract

Diabetes is a disease characterised by high blood glucose levels that affects over 300 million people worldwide. Moreover, more than 3 million people die from diabetic complications of the heart and kidney each year. Pathophysiologic changes in the kidneys and hearts of diabetic patients have been shown to correlate with the enhanced formation of a class of post-translational protein modifications termed advanced glycation end-products (AGEs), which are implicated as key factors in the pathogenesis of tissue damage in diabetes and related diseases. For example, enhanced AGE formation in collagen has been linked to corresponding decreases in pepsin digestibility and acid solubility, which may well contribute to in vivo fibrosis in the kidney and heart. Transition metals such as copper can catalyse in vitro AGE formation, in part through increased oxidative or ‘glycoxidative’ stress. Altered copper metabolism occurs in diabetes, wherein the Cu(II)-selective chelator triethylenetetramine (TETA) has been shown to ameliorate diabetic tissue damage in both kidney and heart.

The objective of this thesis has been to investigate changes in the structure and function of the extracellular matrix in the kidneys and hearts of healthy and diabetic rats, which had received TETA or corresponding placebo treatments. It was hypothesized that this approach would add to existing understanding of the molecular mechanisms of copper-catalysed AGE formation in diabetes, and of those by which TETA can prevent or reverse organ damage. Analysed here were alterations in collagen structure, the behaviour of enzymes of collagen metabolism and the integrity of defences against oxidative/glycoxidative stress in the heart and kidneys. In addition, collagen extracts from these organs were characterised by quantitative and qualitative methods that included measurement of post-translational modification.

Dysregulated collagen metabolism was detected in diabetic kidneys, wherein TETA treatment normalised protein levels without altering the transcription of corresponding genes: these protein-level effects may have been mediated at least in part through actions on collagen-degrading proteases. Collagen from diabetic kidneys displayed altered post-translational modifications whereas TETA treatment partially lowered collagen-AGE levels towards control values. Evidence for altered collagen metabolism was also detected in diabetic hearts at the transcriptional but not the protein level. By contrast, no changes in post translational modification were detected in collagen from diabetic hearts. TETA modified the
transcription of some proteases in the diabetic heart but had no measurable action at the protein level.

In summary, the occurrence of dysregulated collagen metabolism has here been shown to occur in the hearts and kidneys of diabetic rats, and TETA treatment partially normalized some of the observed changes. Furthermore, a previously unrecognised link between copper homeostasis, collagen-AGEs and collagen-degrading proteases in the diabetic kidney has been identified and partially characterized in this work.
Acknowledgements

First I would like to acknowledge my supervisor Garth Cooper for sharing his knowledge and giving me the opportunity to undertake the studies in his group. It was a great experience. In this regard I would also like to thank Shaoping Zhang for the supervision and constant encouragement.

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Last but not least I would like to thank my parents Marietta and Bernd as well as my siblings Anna and Olaf. While keeping in touch over a long distance is hard it is very comforting to know that there are people who support you and care for you no matter what.
# Table of Contents

Abstract ....................................................................................................................................... I
Acknowledgements ................................................................................................................... III
Table of Contents ...................................................................................................................... IV
List of Figures ........................................................................................................................... IX
List of Tables ............................................................................................................................. XII
Abbreviations ........................................................................................................................... XIII

Chapter 1 - Introduction ......................................................................................................... 1

1.1 Diabetes Mellitus ............................................................................................................... 1
  1.1.1 Overview, prevalence and classification ................................................................. 1
  1.1.2 Complications in diabetes mellitus and associated pathological features .......... 3
    1.1.2.1 Diabetic nephropathy - the major form of chronic kidney disease .......... 3
    1.1.2.2 Cardiovascular complications in diabetes - diabetic cardiomyopathy ....... 5
    1.1.2.3 Other chronic complications ....................................................................... 6
  1.1.3 Molecular biology and biochemistry of diabetic complications ......................... 7
    1.1.3.1 Introduction and relation to genetics ......................................................... 7
    1.1.3.2 A unifying hypothesis for the molecular biology and biochemistry of diabetic complications ................................................................. 8

1.2 The extracellular matrix (ECM) in diabetic complications of the kidney and heart .... 9
  1.2.1 Protein components of the ECM ............................................................................ 10
  1.2.2 Altered mRNA- and protein levels of collagen and the involvement of growth factors .......................................................................................................................... 10
    1.2.2.1 Changes in the diabetic kidney ................................................................... 10
    1.2.2.2 Changes in the diabetic heart ..................................................................... 12
  1.2.3 Degradation of the ECM and changes present in diabetes mellitus .................... 14
  1.2.4 Antioxidant defence in the extracellular matrix - Relation to diabetic complications of the kidney and the heart ................................................................. 16

1.3 Advanced glycation end-products and diabetic complications .............................. 18
  1.3.1 Advanced glycation end-product formation and structure ................................... 18
  1.3.2 AGE levels in diabetes mellitus ........................................................................... 24
  1.3.3 The role of AGEs in the development of diabetic complications ..................... 25
    1.3.3.1 Physicochemical and biological properties of glycated proteins .............. 25
    1.3.3.2 AGEs in the development of complications in the diabetic kidney and heart with a focus on the ECM ................................................................. 28
1.3.4 Cellular defences against AGE formation .............................................................. 30
1.3.4.1 The Glyoxalase system and its alterations in diabetes ................................... 30
1.3.4.2 Other potential enzymatic defence systems ................................................... 31

1.4 Further post-translational modifications .......................................................................... 32
1.4.1 Carbonyl formation and oxidative stress in diabetic complications ...................... 32
1.4.2 Enzymatic cross-linking of the extracellular matrix via lysyl oxidase ..................... 33
  1.4.2.1 Lysyl oxidase: Properties, functions and regulation ....................................... 33
  1.4.2.2 Alterations of LOX and the resulting enzymatic cross-links in diabetes .......... 33

1.5 The role of copper in the development of diabetic complications - potential treatments .34
  1.5.1 Copper metabolism in healthy mammals ............................................................... 34
  1.5.2 Altered copper metabolism in diabetes mellitus ..................................................... 35
    1.5.2.1 Changes of tissue copper levels in diabetes .................................................. 35
    1.5.2.2 Potential causes and consequences of perturbed copper metabolism .......... 36
  1.5.3 Copper chelation as a treatment for diabetic complications ................................... 38
    1.5.3.1 Treatment of diabetic complications with AGE-inhibitors and AGE-breakers -
         relation to copper chelation ......................................................................................... 38
    1.5.3.2 The Cu(II) chelator triethylenetetramine as a treatment for diabetic
         complications .............................................................................................................. 39

1.6 Hypothesis and experimental approach .......................................................................... 40
  1.6.1 Hypothesis .............................................................................................................. 40
  1.6.2 Experimental approach .......................................................................................... 40

Chapter 2 - Materials and methods ..................................................................................... 42
  2.1 STZ induced diabetes model and treatment regime ................................................... 42

  2.2 Collagen preparation from kidney and heart ................................................................. 44
    2.2.1 Hydroxyproline assay ............................................................................................. 44
    2.2.2 Collagen extraction ................................................................................................. 45
    2.2.3 Collagen solubilisation ............................................................................................ 46
      2.2.3.1 Solubilisation by pepsin digestion ................................................................... 46
      2.2.3.2 Solubilisation by Liberase DH digestion ......................................................... 47

  2.3 Measurement of mRNA levels by RT-qPCR ................................................................. 47
    2.3.1 RNA isolation and cDNA synthesis ........................................................................ 47
      2.3.1.1 Column based RNA isolation .......................................................................... 48
      2.3.1.2 Detection of RNA quality and quantity ......................................................... 49
      2.3.1.3 Synthesis and quantification of cDNA.......................................................... 49
    2.3.2 Measurement of relative mRNA levels by RT-qPCR .............................................. 50
      2.3.2.1 Primer design.................................................................................................. 50
2.3.2.2 Reference genes ................................................................. 50
2.3.2.3 RT-qPCR ........................................................................... 51

2.4 Protein analysis ........................................................................... 54
2.4.1 Determination of protein concentration by BCA assay ................. 54
2.4.2 Western Blotting ................................................................. 54
  2.4.2.1 Tissue lysates for SDS-PAGE followed by Immunoblotting 54
  2.4.2.2 SDS-PAGE ................................................................. 55
  2.4.2.3 Blotting ................................................................. 56
  2.4.2.4 Immunodetection ............................................................ 56
2.4.3 Staining of SDS-PAGE and mPAGE gels with colloidal Coomassie 57
2.4.4 LC-MS/MS based identification of proteins ................................. 58
2.4.5 Detection of protease activity by gelatine zymography .................. 60
2.4.6 Cathepsin L activity assay .................................................... 61

2.5 Analysis of collagen extracts for post-translational modifications .................... 63
  2.5.1 Synthesis of artificially glycated BSA as a standard .......................... 63
  2.5.2 Detection of CML levels via dot blot ........................................... 64
  2.5.3 Measurement of carbonyl levels ................................................... 65
  2.5.4 Measurement of the digestibility of collagen ................................... 66
  2.5.5 Mpba based gel electrophoresis for the analysis of glycated proteins ..... 66
    2.5.5.1 Synthesis of 3-methacrylamido phenylboronic acid .......... 66
    2.5.5.2 Analysis of glycated proteins by mPAGE .................................... 70

2.6 Statistical analysis ........................................................................ 72

2.7 List of standard reagents and equipment ........................................ 73

Chapter 3 – Collagen extraction from kidney and heart of healthy and diabetic rats treated with placebo or TETA and its preliminary characterisation .................... 74

3.1 Introduction .................................................................................. 74

3.2 Results ......................................................................................... 76
  3.2.1 Characteristics of the STZ-induced diabetes model ....................... 76
  3.2.2 Extraction of collagen from rat hearts and kidneys ......................... 77
    3.2.2.1 Determination of purity of collagen extracts by hydroxyproline measurement 77
    3.2.2.2 Solubilisation of collagen extracts employing a collagenase mixture ....... 78
    3.2.2.3 LC-MS/MS analysis of pepsin/acetic acid digested extracts from the LV and kidneys... 80

3.3 Discussion ..................................................................................... 85
Chapter 4 – Diabetes-induced changes in the ECM of the kidney - the effect of TETA

4.1 Introduction ................................................................................................................................. 87

4.2 Results ........................................................................................................................................ 90

4.2.1 Transcriptional analysis of genes relevant to ECM metabolism and oxidative/glycoxidative stress in the kidney .............................................................................................................. 90

4.2.1.1 Relative mRNA levels of collagen, genes involved in collagen processing and degradation and growth factors ........................................................................................................... 91

4.2.1.2 Relative mRNA levels of genes related to oxidative and glycoxidative stress 97

4.2.2 Amounts and enzyme-activity measurements of ECM- and ROS-related proteins in non-diabetic and diabetic rat kidney tissue, and the effects of TETA treatment ........... 101

4.2.2.1 Collagen protein levels in rat kidney cortices ........................................................................ 102

4.2.2.2 Protein level and activity measurements of collagen-degrading proteases ......................................................... 103

4.2.2.3 Protein level of the collagen cross-linking enzyme LOX .................................................................................. 109

4.2.2.4 Protein level of glutathione reducing anti-oxidant enzyme GSR ........................................... 110

4.2.3 Biochemical analysis of collagen isolated from kidneys of healthy and diabetic rats and the effect of TETA ......................................................................................................... 111

4.2.3.1 Changes in pepsin digestibility of collagen extracts ........................................................................ 111

4.2.3.2 Collagen CML levels in the four groups of rats ........................................................................ 112

4.2.3.3 Measurement of collagen carbonyl levels in the four groups of rats ........................................ 114

4.2.3.4 Analysis of collagen via mPAGE – Characterisation of one additional collagen band ......................... 115

4.2.4 Correlation analysis of the data ............................................................................................. 119

4.3 Discussion .................................................................................................................................. 129

Chapter 5 – Diabetes-induced changes in the ECM of the LV of the heart - the effect of TETA

5.1 Introduction .................................................................................................................................. 137

5.2 Results ........................................................................................................................................ 139

5.2.1 Transcriptional analysis of genes relevant to ECM metabolism and oxidative/glycoxidative stress in the heart .............................................................................................................. 139

5.2.1.1 Relative mRNA levels of collagen and genes involved in collagen processing and degradation, and growth factor responses ................................................................................. 140

5.2.1.2 Relative mRNA levels in cardiac LV of genes relevant to oxidative and glycoxidative stress .......................................................................................................................... 146

5.2.2 Protein levels of genes relevant to the ECM and anti-ROS defence in the healthy and diabetic rat heart, and the effect of TETA ........................................................................ 150

5.2.2.1 Collagen protein level in the cardiac LV of four groups of rats ................................................... 151

5.2.2.2 GAPDH as a normaliser for Western blot measurements in the cardiac LV of
List of Figures

Figure 1-1: Schematic overview of the pathways of formation of CML and glucosepane......19
Figure 1-2: Structure of AGEs that form protein cross-links..............................................22
Figure 1-3: Structure of AGEs that do not form protein cross-links.....................................23
Figure 1-4: The glyoxalase system ....................................................................................30
Figure 1-5: TETA in the form of a dihydrochloride salt called trientine.................................39
Figure 2-1: Setup and timeline of the animal trial ................................................................42
Figure 2-2: Representative amplification curve of an RT-qPCR measurement .................53
Figure 2-3: Normalisation procedure for RT-qPCR ..............................................................53
Figure 2-4: 3-mpba synthesis reaction 1 ............................................................................67
Figure 2-5: 3-mpba synthesis reaction 2 ............................................................................67
Figure 2-6: 3-mpba synthesis reaction 3 ............................................................................68
Figure 2-7: 3-mpba synthesis reaction 4 ............................................................................69
Figure 2-8: 3-mpba synthesis reaction 5 ............................................................................70
Figure 3-1: Blood glucose level and body weight ..................................................................76
Figure 3-2: Collagen content of collagen extracts from rat kidneys and hearts ..................78
Figure 3-3: Time and concentration dependent collagen digestibility ...............................79
Figure 3-4: Liberase DH solubility of collagen extracts from rat kidney and LV ...............80
Figure 3-5: SDS-PAGE and LC-MS/MS analysis of collagen extracts from the LV ..........81
Figure 3-6: SDS-PAGE and LC-MS/MS analysis of collagen extracts from rat kidneys .......83
Figure 3-7: SDS-PAGE of collagen extracts from kidney and the left ventricle (LV) in parallel ........................................................................................................................................84
Figure 4-1: Relative mRNA levels of basement membrane collagens in cortices of rat kidneys ........................................................................................................................................91
Figure 4-2: Relative mRNA levels of interstitial collagens from cortices of rat kidneys .......93
Figure 4-3: Relative mRNA levels of growth factors in the cortex of rat kidneys ...............94
Figure 4-4: Relative mRNA levels of pro-collagen proteinases and the collage cross-linking enzyme Lox in the cortex of rat kidneys ........................................................................95
Figure 4-5: Relative mRNA levels for extracellular and lysosomal proteases in rat kidney cortices .........................................................................................................................................96
Figure 4-6: Relative mRNA levels for enzymes involved in the detoxification of ROS in the cortex of rat kidneys ........................................................................................................98
Figure 4-7: Relative mRNA levels of the two genes of the glyoxalase system in the cortex of rat kidneys ..................................................................................................................................99
Figure 4-8: Relative mRNA levels for additional methylglyoxal and 3-deoxyglucosone detoxification enzymes in rat kidney cortices ............................................................... 100
Figure 4-9: Relative mRNA levels for \textit{Aoc3} and \textit{Rage} in rat kidney cortices ...................... 101
Figure 4-10: Collagen protein levels in rat kidney cortices of the four groups.......................... 102
Figure 4-11: Relative CTSL protein level in rat kidney cortices ............................................. 103
Figure 4-12: Relative CTSB protein level in rat kidney cortices ........................................... 104
Figure 4-13: CTSL activity level in rat kidney cortices .......................................................... 105
Figure 4-14: Effect of CuCl$_2$ on relative CTSL activity in the four groups of rat kidney cortices ................................................................................................................ 106
Figure 4-15: Effect of CuCl$_2$ and TETA on CTSL activity in lysates of rat kidney cortices \textit{in vitro}.......................................................................................................................... 107
Figure 4-16: Relative gelatinase activity measured in lysates from rat kidney cortices ... 108
Figure 4-17: Relative LOX protein levels in rat kidney cortices ............................................ 110
Figure 4-18: Relative GSR protein levels in rat kidney cortices ........................................... 111
Figure 4-19: Pepsin digestibility of collagen extracts from rat kidneys .................. 112
Figure 4-20: Relative CML levels of collagen extracts from rat kidneys ................................ 113
Figure 4-21: Carbonyl levels of collagen extracts from rat kidneys ....................................... 114
Figure 4-22: Analysis of collagen and glycated BSA by mPAGE ........................................ 116
Figure 4-23: Comparison of SDS-PAGE analysis and mPAGE analysis of collagen extracts – Identification of a previously unrecognised collagen band via LC-MS/MS .............................................. 118
Figure 4-24: Linear regression analysis for CTSL protein level vs. CML level ................. 120
Figure 4-25: Linear regression analysis for CTSL protein level vs. pepsin digestibility of collagen ......................................................................................................................... 122
Figure 4-26: Linear regression analysis of collagen content vs. CTSL protein level .......... 123
Figure 4-27: Linear regression analysis for collagen content vs. gelatinase activity at 60 kDa (MMP2) and 72 kDa (pro-MMP2) ................................................................. 124
Figure 4-28: Linear regression analysis of relative gelatinase activity ...................... 125
Figure 4-29: Linear regression analysis of collagen carbonyl level vs. CML level .......... 126
Figure 4-30: Linear regression analysis of pepsin digestibility vs. Lox protein level ............ 127
Figure 4-31: Regression analysis of pepsin digestibility of collagen vs. gelatinase activity .128
Figure 5-1: Relative mRNA levels of collagen type I, III and VI in the LV of the four groups of rats ........................................................................................................................ 141
Figure 5-2: Relative mRNA levels of collagen type IV in the cardiac LV of the four treatment groups .................................................................................................................. 142
Figure 5-3: Relative mRNA levels of growth factors in the cardiac LV ............................ 143
Figure 5-4: Relative mRNA levels of the pro-collagen proteinases and the collagen cross-linking enzyme \textit{Lox} in the LV of four groups of rats ................................................................. 144

X
Figure 5-5: Relative mRNA levels of extracellular and lysosomal proteases in the cardiac LV of rats ................................................................................................................................... 145
Figure 5-6: Relative mRNA levels of genes implicated in the detoxification of ROS in the cardiac LV of rats ................................................................................................................................... 147
Figure 5-7: Relative mRNA levels of the two genes of the glyoxalase system in the cardiac LV of rats ................................................................................................................................... 148
Figure 5-8: Relative mRNA levels of additional enzymes involved in the detoxification of methylglyoxal and 3-deoxyglucosone ................................................................................................................................... 149
Figure 5-9: Relative mRNA levels of Aoc3 and Rage in the LV of rats ................................ 150
Figure 5-10: Collagen content in the cardiac LV of rats from the four treatment groups ...... 151
Figure 5-11: Beta-actin vs. GAPDH as a normaliser for Western Blot measurements in the LV of rats ................................................................................................................................... 152
Figure 5-12: Relative protein levels of CTSL in the cardiac LV of rats ................................ 153
Figure 5-13: Relative protein levels of CTSB in the cardiac LV of rats ................................ 154
Figure 5-14: Relative protein levels of CAT in the cardiac LV of rats ................................ 155
Figure 5-15: Relative protein level of GSR in the cardiac LV of rats ................................ 156
Figure 5-16: Pepsin digestibility of collagen extracts from the LV of rat hearts ............... 157
Figure 5-17: Carbonyl levels of collagen extracts from the LV of rats ............................... 158
Figure 6-1: Summary of essential results in the kidney ......................................................... 167

Appendix figure 1: Certificate of analysis for the lot of STZ used ........................................ 173
Appendix figure 2: NMR of 3-methacrylamido phenylboronic ester .................................... 177
Appendix figure 3: NMR of 3-methacrylamido phenyl trifluoroborate ............................... 178
Appendix figure 4: NMR of 3-methacrylamido phenylboronic acid .................................... 179
List of Tables

Table 2-1: Details of the qPCR programme ..........................................................52
Table 2-2: List of antibodies used for Western Blotting .........................................57
Table 2-3: Resolving gel recipe sufficient for 1 gel ..............................................71
Table 2-4: Stacking gel recipe sufficient for 1 gel ...............................................72
Table 2-5: List of standard reagents ....................................................................73
Table 2-6: List of standard equipment .................................................................73

Appendix table 1: Target primer sequences (continued on next page) ..................174
Appendix table 2: Reference primer sequence ......................................................176
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAMTS2</td>
<td>A disintegrin and a metalloproteinase with thrombospondin motifs</td>
</tr>
<tr>
<td>AER</td>
<td>Albumin excretion ratio</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced glycation end-products</td>
</tr>
<tr>
<td>AKR1A1</td>
<td>Aldehyde reductase</td>
</tr>
<tr>
<td>AKR1B1</td>
<td>Aldose reductase</td>
</tr>
<tr>
<td>AKR1C1</td>
<td>Dihydropyridone dehydrogenase</td>
</tr>
<tr>
<td>ALDH1A1</td>
<td>2-oxoaldehyde dehydrogenase</td>
</tr>
<tr>
<td>ALE</td>
<td>Advanced lipoxidation end-products</td>
</tr>
<tr>
<td>AOC3</td>
<td>Amine oxidase copper containing 3</td>
</tr>
<tr>
<td>BAPN</td>
<td>Beta-aminopropionitrile</td>
</tr>
<tr>
<td>BBW</td>
<td>Biobreeding Wistar</td>
</tr>
<tr>
<td>BCA</td>
<td>Bichinchoninic acid</td>
</tr>
<tr>
<td>BMP1</td>
<td>Bone morphogenetic protein 1</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>CML</td>
<td>Nε-carboxymethyllysine</td>
</tr>
<tr>
<td>COL</td>
<td>Collagen</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>CTS</td>
<td>Cathepsin</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>Cu</td>
<td>Copper</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton; atomic mass unit</td>
</tr>
<tr>
<td>DCM</td>
<td>Diabetic cardiomyopathy</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DHLNL</td>
<td>Dihydroxy-lysiononorleucine</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DN</td>
<td>Diabetic nephropathy</td>
</tr>
<tr>
<td>DNP</td>
<td>Dinitro phenyl hydrazone</td>
</tr>
<tr>
<td>DNP</td>
<td>2,4-dinitro phenyl hydrazine</td>
</tr>
<tr>
<td>DPN</td>
<td>Diabetic peripheral neuropathy</td>
</tr>
<tr>
<td>DR</td>
<td>Diabetic retinopathy</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation end-products</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Reverse transcription quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RRT</td>
<td>Renal replacement therapy</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecylsulphate</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>T1D</td>
<td>Type 1 diabetes</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 diabetes</td>
</tr>
<tr>
<td>TETA</td>
<td>Triethylenetetramine</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transcription growth factor beta</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>VEGFA</td>
<td>Vascular endothelial growth factor alpha</td>
</tr>
</tbody>
</table>
Chapter 1 - Introduction

1.1 Diabetes Mellitus

1.1.1 Overview, prevalence and classification

More than 346 million people worldwide currently have diabetes, a number originally predicted to be reached by the year 2030 (Wild et al. 2004; WHO 2011). The estimated death toll from diabetes was 3.4 million people in 2004, and is expected to double from 2005 to 2030. Diabetes mellitus is characterised by high blood glucose levels. The diagnostic criteria advanced by the World Health Organisation (WHO) is a venous plasma glucose level >11.1 mmol/L measured 2 h after a 75 g glucose challenge (Wild et al. 2004). A long-term indicator of the blood glucose level is the fraction of glycated haemoglobin (HbA1c) present in the blood which lies at around 6% for healthy people and is elevated in diabetic patients (Walinder et al. 1982).

Four main types of diabetes have been defined (Harris 1995). The pathogenesis of type 1 diabetes mellitus (T1D), formerly known as insulin-dependent diabetes mellitus is considered to involve the autoimmune destruction of insulin producing pancreatic islet β-cells. The resulting near-total lack of insulin leads to highly elevated blood glucose levels and accompanying metabolic defects that (in the absence of insulin therapy) always culminate in diabetic ketoacidosis, a uniformly fatal metabolic state. Type 1 diabetes (T1D) can only be treated by the lifelong administration of insulin. The risk of iatrogenic hypoglycaemia (too-low blood glucose levels due to excess insulin), is one of the main obstacles to achieving a normal blood glucose level in T1D patients and is responsible for up to 10% of deaths in these patients (Realsen et al. 2011).

Type 2 diabetes (T2D) mellitus is the most prevalent type worldwide. It accounts for ~90 - 95% of all cases in the US and 85% - 90% of the cases in Europe (OECD 2010). In contrast to T1D, the body still produces substantial amounts of insulin in the early stages of T2D, where the peripheral tissues (particularly skeletal muscle, liver and adipose) have become insensitive towards insulin-stimulated glucose uptake that leads to a high postprandial blood glucose values. Furthermore, increased hepatic glucose output leads to high blood glucose levels in the fasting state (Inzucchi et al. 2012). Fortunately a healthy diet, regular exercise and the abstinence of smoking in combination with a normal body weight can delay or possibly even prevent the occurrence or progression of T2D (Tuomilehto et al. 2001; Ritz et
al. 2005). While these changes are desirable, studies show that an altered lifestyle is often not achieved in the daily routine (Friedman et al. 2007).

Metformin is the drug treatment of choice for T2D, which acts mainly by reducing hepatic glucose output. Metformin is frequently combined with a range of other medications including insulin at later stages of the disease while the path of treatment should be centred on the “preference, needs and values of the patient” (Inzucchi et al. 2012). The goal of treatment is the lowering of the blood glucose to a target level previously agreed upon by the doctor and the patient. The standard recommendation for T2D patients by the American Diabetes Association is to target a mean blood glucose level of around 8.3-8.9 mmol/L, with a fasting plasma glucose level ideally below 7.2 mmol/L and an HbA1c of <7.0%.

Gestational diabetes mellitus (GDM) is defined as any degree of glucose intolerance that starts or is first diagnosed during pregnancy, wherein there is no evidence of antecedent (pre-pregnancy) diabetes (ADA 2003). It may be difficult in the absence of evidence to distinguish GDM from previously undiagnosed T2D and it is currently considered that many persons with GDM are at high risk of developing T2D and related cardiovascular disease later in life.

These first three common classes of diabetes comprise those for which the causative disease processes remain uncertain, and they are therefore categorized as primary forms of the disease.

The fourth and final category is that of secondary diabetes, which comprises all other types of diabetes mellitus that occur secondary to other defined genetic, metabolic, hormonal or other systemic diseases, or due to the diabetogenic effect of drugs or toxins (Harris et al. 1995). The fundamentally important implication of secondary diabetes is that, unlike the previous three categories, in many cases it is curable or at least treatable by diagnosis and treatment of the underlying primary cause. There are many examples of this principle, two of which are the treatment of diabetes accompanying Cushing’s syndrome by the removal of a causative adrenal cortical adenoma, and of bronze diabetes by the treatment of the underlying haemochromatosis (Nieman et al. 2005; Bacon et al. 2011).
1.1.2 Complications in diabetes mellitus and associated pathological features

Chronic complications develop in many diabetic patients. It is estimated that 50% of people with diabetes die of cardiovascular disease while 10-20% die of kidney failure (WHO 2011). Other complications that affect diabetic patients include retinopathy, neuropathy and arteriopathy while the overall risk of death in diabetic patients is more than twice as high as in the corresponding non-diabetic population.

1.1.2.1 Diabetic nephropathy - the major form of chronic kidney disease

Diabetes is the major cause of chronic kidney disease (CKD), called diabetic nephropathy (DN), worldwide (Pyram et al. 2011). African-Americans with diabetes of a duration >5 years are at a 4.6-fold increased risk for CKD compared to those with diabetes of <1 year. CKD development is divided into five stages according to the estimated glomerular filtration rate (eGFR) with stage 5 being overt kidney failure, also called end-stage renal disease. Patients are not only at risk of dying from CKD but also have a concomitantly higher mortality rate from cardiovascular disease (DuBose 2007; Aso 2008). Prevalence rates of CKD are rising globally, in both the developed and the developing world (Atkins 2005). However, the comparison of CKD prevalence between different ethnic groups is difficult due to problems with normalising the applied eGFR and albuminuria measurements (Zhang et al. 2008). It is worth noting that, while the prevalence rates are still increasing in Western countries, the incidence rate of stage 5 CKD in the USA and renal replacement therapy (RRT) in Australia and New Zealand has decreased slightly in recent years whereas final-stage CKD and RRT incidence rates in Europe were stable over the last few years (Friedman et al. 2007; Zoccali et al. 2009). RRT incidence rates for Canada have also been stable while whereas a slight increase was still noted in Japan (Jager et al. 2007).

DN and CKD in general are currently diagnosed via the measurement of eGFR and albuminuria while fibrosis is considered be a hallmark of DN but is not frequently used as a diagnostic marker as it involves biopsies. The eGFR is calculated from the measurement of the urinary and serum creatinine level. The most recently adapted "modification of diet in renal disease" (MDRD) formula used for these calculations is dependent on the age of the person and contains a correction term for gender and ethnicity, in particular Afro-American as opposed to Caucasian but not for other ethnicities (Zhang et al. 2008). The eGFR is reported in [mL/min/1.73 m²] and varies from >90 for CKD stage 1, where the eGFR can be even higher than in healthy persons, to <15 for CKD stage 5, considered to be the failure of kidneys. CKD stage 1, in which the eGFR is within the normal range, is usually diagnosed by
Chapter 1: Introduction

the presence of diagnostic levels of albuminuria or by the finding of structural abnormalities detected via ultrasound (Pyram et al. 2011).

The classical view is that albuminuria, measured as the albumin excretion rate (AER), is present at an early stage of the disease (CKD stage 1). This initial stage with slightly elevated AER is characterised by moderately increased amounts of urinary albumin, termed microalbuminuria. Accordingly, the eGFR rate is thought only to decrease when the AER is already very high and macroalbuminuria, as detectable for example by a dipstick method, is present (Macisaac et al. 2011). This view has been challenged recently as it was found that approximately 20% of people with T2D develop stage 3 CKD with a normal AER. Thus it was concluded by Macisaac et al. that “changes in AER and eGFR are [being] increasingly recognized as complementary to rather than obligatory manifestations of diabetic CKD” (Macisaac et al. 2011).

The mesangial matrix and the basement membrane of the glomerulus as well as the tubulointerstitium frequently develop fibrosis in the course of DN (Simonson 2007; Jefferson et al. 2008). Possibly due to the fact that the glomerulus represents the functional filtration unit of the kidney, mesangial matrix expansion and glomerular basement membrane thickening have been the focus of intensive research. The interstitium is another prominent site for the development of fibrosis in DN. Interestingly, it is fibrosis in the tubulointerstitium of the kidney which is associated with albuminuria and the magnitude of renal dysfunction in T1D and T2D (Nath 1998; Gilbert et al. 1999). Another hint for the importance of fibrosis in the interstitium comes from a study by Bader et al. (Bader et al. 1980). For several patients with severe glomerular lesions, normal creatinine clearance was found in patients where the interstitium showed no fibrotic change. At the same time, patients with small glomerular lesions displayed elevated serum creatinine concentrations if accompanied by fibrosis of the tubulointerstitium, highlighting the importance of the interstitium in the disease process. Fioretto et al. reported that only 30% of T2D patients with microalbuminuria developed typical features of glomerulopathy such as glomerular basement membrane thickening and mesangial expansion (Fioretto et al. 1996). Other microalbuminuric patients displayed fibrosis of the interstitium or arterial lesions with near-normal glomerular structure. Thus glomerular basement-membrane thickening, mesangial matrix expansion and in particular tubulointerstitial fibrosis appear to be involved in the pathogenesis of DN.

There is currently no effective treatment available that is particularly designed and therefore effective for the condition of DN. All current therapies target known risk factors rather than the disease itself. Thus the goal is to normalise the blood glucose level as well as blood
Chapter 1: Introduction

pressure in order to slow the progression of the disease (Thomas et al. 2011). In addition to a treatment for DN, better biomarkers are needed in order to improve diagnosis and to follow the progression of the disease with a better comparability between different ethnicities as well as genders (Valk et al. 2011).

1.1.2.2 Cardiovascular complications in diabetes - diabetic cardiomyopathy

According to the Framingham Heart Study, diabetes mellitus is an independent risk factor for heart failure. Men and women with diabetes have a 2.4-fold and 5-fold higher risk of heart failure, respectively (Kannel et al. 1974). This finding has been confirmed by several other epidemiological studies including a combined analysis of 102 studies in which a 2-fold higher risk for coronary heart disease and a 2.3-fold higher risk for ischaemic stroke was reported (The Emerging Risk Factors Collaboration 2010; Ernande et al. 2012).

A distinct pathology of cardiomyopathy in diabetes was first described by Rubler et al. and later on re-confirmed (Rubler et al. 1972; Regan et al. 1977). The term “diabetic cardiomyopathy (DCM)” has since been defined as “the existence of left ventricular dysfunction in diabetic patients without coronary artery disease, hypertension or other potential aetiological conditions” (Ernande et al. 2012). The definition of DCM by inclusive criteria is more difficult as abnormalities seen in DCM can also be found in other forms of cardiomyopathy. In recent reviews structural and morphological as well as functional abnormalities were used to define DCM (Fang et al. 2004; Aneja et al. 2008; Ernande et al. 2012; Miki et al. 2013):

Myocardial fibrosis
Fibrosis, the accumulation of extracellular matrix (ECM) proteins such as collagen, is one hallmark of DCM. In a clinical setting, fibrosis can be detected by an altered echocardiographic signal, namely backscatter reduction, and related phenomena can be detected by magnetic resonance imaging. Additional data stems from post mortem studies where alterations in the ECM can be detected by histology including immunohistochemistry (Asbun et al. 2006). As mentioned above (see section 1.1.2.1), fibrosis also occurs in DN making this a hallmark of the two major diabetic complications.

Impaired diastolic dysfunction
Left ventricular diastolic dysfunction detected via Tissue Doppler Imaging is another characteristic of DCM (Miki et al. 2013). It is already present at early stages of the disease in asymptomatic subjects. The ejection fraction in subjects with left ventricular diastolic
dysfunction is not impaired at early stages but subsequently decreases towards the middle or late stages of the disease (Fang et al. 2004).

Concentric left ventricular hypertrophy
In concentric left ventricular hypertrophy, cardiac mass is increased alongside the relative wall thickness (Opie et al. 2006; Hill et al. 2008). This condition is present in the more advanced stages of DCM (Aneja et al. 2008). Cardiomyocyte hypertrophy is associated with left ventricular hypertrophy in diabetes. However, the diameter of cardiomyocytes was shown to be unaltered in two studies while other studies found a mixture of atrophic and hypertrophic cardiomyocytes, which suggests that cardiomyocyte hypertrophy is not a prerequisite for left ventricular hypertrophy (Miki et al. 2013).

Altered cardiac metabolism
Energy metabolism in the diabetic heart is altered and a switch to the oxidation of fatty acids rather than glucose frequently takes place as a probable consequence of hyperglycaemia (Fang et al. 2004). One mechanism that causes the reduced utilisation of glucose is the inhibitory effect of free fatty acid oxidation on the pyruvate dehydrogenase complex. Studies from genetically diabetic mice showed a decrease of glucose oxidation to 16% while the oxidation of palmitate was increased two-fold (Fang et al. 2004).

As for DN, there is currently no therapy available for DCM and thus treatment is limited to the lowering of high blood glucose levels at early stages, and the general treatments available for heart failure at later stages of the disease. One major shortcoming of all available treatments for heart failure is that no existing therapies are effective in cases of diastolic heart failure (or heart failure with preserved ejection fraction), which comprises about half of all cases, including in diabetes.

1.1.2.3 Other chronic complications

Diabetic peripheral neuropathy (DPN) and diabetic retinopathy (DR) (Klein et al. 1984; Antonetti et al. 2012) are complications frequently encountered in diabetic patients. While neither is as lethal as DCM and DN, they nevertheless represent further major burdens for the patient and for society as a whole.

DPN, the most common neuropathic syndrome associated with diabetes, is estimated to affect up to 50% of people with diabetes mellitus (Tesfaye et al. 2012). The first symptom is sensory loss in the lower limbs due to diabetes-related nerve damage (neuropathy), while the upper limbs are affected subsequently. DPN can eventually lead to the formation of foot
Chapter 1: Introduction

ulcers due to insensitivity to noxious stimuli and also puts the patient at elevated risk for burns due to a lack of heat sensitivity both of which may result in the need for limb amputation. Painful neuropathic symptoms are present in around 20% of diabetic patients. They are particularly prominent at night and may lead to the disturbance of sleep and thus additionally affect the quality of life.

DR is caused by alterations of the retinal blood vessels, and is a leading cause of blindness in diabetic patients. The incidence and progression has been decreasing steadily within the last 30 years in the US in T1D as well as T2D patients (Antonetti et al. 2012). These improvements have been ascribed to better management of blood glucose, blood pressure and lipid levels. Impairments from DR include impaired contrast sensitivity, visual acuity and visual fields and may culminate in marked impairment of vision or clinical blindness.

1.1.3 Molecular biology and biochemistry of diabetic complications

1.1.3.1 Introduction and relation to genetics

Shedding light on the molecular background of the development of diabetic complications of the kidney and the heart is a major goal in an attempt to develop suitable treatments. This is a great challenge as these are multifactorial diseases affected by lifestyle choices, genetic background, glycaemia, blood pressure, lipid levels and other factors. Genetic background is a strong determinant in the development of complications. Even in the presence of severe, chronic hyperglycaemia, a large number of patients do not develop DN (Schena et al. 2005). It is known that the susceptibility to complications is related to ethnicity: this effect may be caused by the clustering of different loci. Siblings of T1D patients are also at a four-fold increased risk of developing DN. However, the identification of genes involved has proven difficult as a simple Mendelian inheritance pattern does not occur (Schena et al. 2005).

The strong and complex genetic component of the disease makes the findings from in vitro and in vivo experiments aimed at the elucidation of the molecular biology of the disease, difficult to interpret. Any single method employed will have significant limitations in certain respects. Tissue culture analyses look at the reaction of one specific type of cell in an artificial setting with a limited set of stimuli. The complex paracrine and endocrine signalling, which is essential in the interaction of different tissues and cell types is absent and makes the interpretation of data achieved in such ways difficult. Additional limitations are the
skewed time frame, with stimuli lasting for hours or days, rather than months and years and the absence of an extracellular matrix in this setting. More complex models like the well-established streptozotocin (STZ)-induced-diabetic rat used in this thesis have acknowledged limitations in their own right, which are discussed in more detail on page 40. Genetically-modified mice are also employed for the detailed analysis of individual genes and have their own limitations (Petermann et al. 2006; Aitken et al. 2010).

As such limitations will always be present, it is essential to interpret one’s findings within the known limitations of the particular model, and to compare current results with previous findings obtained in the applied model, and to interpret them in the context of other models, in order to further our understanding of the molecular biology of diabetic complications in humans.

1.1.3.2 A unifying hypothesis for the molecular biology and biochemistry of diabetic complications

Brownlee proposed a hypothesis in 2001 in an effort to link the molecular changes that have been observed in diabetes (Brownlee 2001). According to this theory, four major pathways: the polyol pathway, the hexosamine pathway, the protein kinase C pathway and the advanced glycation end-product (AGE) pathway are involved in the development of diabetic complications, all of which are triggered by hyperglycaemia and subsequently increased mitochondrial superoxide production. Unfortunately the experiments in support of the hypothesis were solely performed in vitro using tissue culture of bovine endothelial cells (Du et al. 2000; Nishikawa et al. 2000; Du et al. 2003). While such a system can yield interesting findings it may be inadequate to simulate the complex diabetic disease state. The AGE pathway which is discussed in more detail on page 18 ff., is detrimental to extracellular matrix proteins such as collagen. Fibrosis, which results mainly from the accumulation of collagen in the extracellular matrix, is a prominent pathogenic feature of diabetic complications of the kidney and the heart, but a model of the ECM is absent in most in vitro tissue culture systems (Asbun et al. 2006; Simonson 2007; Jefferson et al. 2008).

In support of the involvement of reactive oxygen species (ROS) in the pathogenesis of DCM, genetically-modified mice overexpressing superoxide dismutase have shown partial protection of cardiac mitochondria in diabetes (Shen et al. 2006). Administration of benfotiamine and thiamine has been shown to be beneficial for the treatment of DR and DN. Both drugs are thought to act by diverting substrates, in particular fructose-6-phosphate and glyceraldehyde-3-phosphate which supposedly trigger the pathogenic protein kinase C pathway, the AGE pathway and the hexosamine pathway in diabetes and have thus been
Chapter 1: Introduction

cited in support of Brownlee’s initial hypothesis (Giacco et al. 2010). However, the mechanism of action of these compounds in vivo is uncertain and both may also act via transition metal chelation as has recently been proposed for the so-called AGE-inhibitors and AGE-breakers recently (Nagai et al. 2012). A potential involvement of transition metals in the formation of ROS and the complications of diabetes was proposed by Wolff and Dean in 1987 and the beneficial effects of the Cu(II) chelator TETA were first shown in 1995 by Cameron and Cotter (Wolff et al. 1987; Baynes 1991; Cameron et al. 1995).

In the following sections, the connection between the extracellular matrix, advanced glycation end-products, other post-translational modifications and diabetic complications of the kidney and the heart will be reviewed. Furthermore, the relation to known defects of copper homeostasis in diabetes will be highlighted. Finally the current evidence for the treatment of diabetic complications employing the Cu(II) chelator TETA is discussed.

1.2 The extracellular matrix (ECM) in diabetic complications of the kidney and heart

Fibrosis, the pathogenic accumulation of the ECM, was described as a common pathologic hallmark of DCM and –nephropathy in sections 1.1.2.1 and 1.1.2.2. The ECM is present basolaterally, directly adjacent to endothelial and epithelial cells, and is a major component of the basement membrane, as well as of the interstitium (Alberts et al. 2002). To date, collagen has received the most attention in research as it is the main component of the ECM. It is also relatively easy to extract from tissues rich in ECM like skin and tendon in sufficient amounts to enable ex vivo analysis. Alterations of collagen in diabetes have been analysed by histochemistry using immunologically-based or classical chemical stains, as well as by the quantitative determination of collagen by hydroxyproline (hyp) measurement, western blotting and measurement of transcription levels. Functional analyses of collagen such as tendon-break time and tendon-break strength measurements as well as digestibility measurements have also been performed in order to elucidate post-translational alterations of the ECM in diabetes. Functional parameters have often been measured in parallel with post-translational modifications like advanced glycation end-products (AGEs). Thus these functional analyses are discussed in more detail in the context of AGE formation in section 1.3.3 while alterations at the transcriptional and protein levels are discussed in the sections below.
1.2.1 Protein components of the ECM

The main isoform of collagen found in the basement membrane of the glomeruli, the active filtration unit of the kidney, is the mesh-like collagen type IV which is part of the glomerular filtration barrier (Lemley et al. 1991; Jarad et al. 2009). Type V collagen, a fibril-forming form, is found alongside type IV collagen in the glomerular basement membrane (GBM). The interstitium consists of the fibrillar collagens of type I and III as well as the filamentous collagen type VI (Lemley et al. 1991).

Fibrillar types I and III collagen represent the vast majority of that in the heart, comprising ~80% and ~11%, respectively (Bornstein et al. 1980; de Souza 2002). The basement membrane collagens, type IV and V are also present as well as type VI collagen (Bishop et al. 1995; Bailey et al. 1998). Most of the different types of collagen have several different isoforms encoded by individual genes. For example, collagen type I is encoded by two genes in humans, which translate into 2 alpha chains (Col1a1 and Col1a2).

Other major components of the ECM include elastin, which provides for elasticity, fibronectin - essential for cell matrix interaction and ECM structure, and laminin which is part of the structural scaffolding of the basement membrane (Colognato et al. 2000; Alberts et al. 2002; Singh et al. 2010). Integrins are trans-membrane receptors that bind to collagen, fibronectin and laminin amongst others and serve as an anchor for the ECM while they also act as a bidirectional signal transducers across the plasma membrane (Hynes 2002). These additional proteins are all essential for ECM function; however the focus of this study was on the analysis of collagen which is the most abundant ECM protein and thus well suited for post-translational analysis (Lodish et al. 2000).

1.2.2 Altered mRNA- and protein levels of collagen and the involvement of growth factors

1.2.2.1 Changes in the diabetic kidney

The thickening of the glomerular basement membrane is a frequently-observed event in the more advanced stages of clinical DN (Fioretto et al. 1996). Expression and protein levels of collagen type IV, the main component of the basement membrane, and the ECM-inducing transforming growth factor beta (TGFβ) have also been shown to be elevated in kidneys of STZ-diabetic rats as early as 3, 7 and 14 days after induction of diabetes (Park et al. 1997). Increased immunoreactive collagen type III and IV in kidneys of STZ-diabetic rats was reported by Liu et al. after 24 weeks of diabetes whereas no fibrosis was detected in the
Chapter 1: Introduction

Acute phase (Liu et al. 2001). An increase in kidney collagen level by hyp measurement in STZ-diabetic rats after 8 months is reported by Forbes et al. (Forbes et al. 2003). Huang et al. also report an increase in collagen levels as determined by hyp measurements after 8 months of diabetes alongside increased collagen protein levels of type I, III and IV in STZ-diabetic rats following unilateral nephrectomy (Huang et al. 2009). Collagen mRNA levels were reported to be increased in parallel for type I, III and type IV collagen after 8 months. No changes of type I, type III and type IV collagen mRNA levels were reported in these rats after 4 months of diabetes while protein levels of type IV but not of type I and type III collagen were increased. In contrast to this, a study by Poulsom et al. showed mRNA levels of collagen type IV isoform α1 to be decreased or unchanged in STZ-diabetic rats with 2 to 4 months of diabetes, possibly due to the presence of both kidneys (Poulsom et al. 1988).

Regarding the fibrillar collagen types I and III, Abrass et al. report unaltered protein levels after 6 weeks of diabetes by immunohistochemistry, while Fukui et al. report increased mRNA levels of collagen type I and III isoform α1 after 1, 3 and 6 months of diabetes in isolated glomeruli of STZ-diabetic rats (Abrass et al. 1988; Fukui et al. 1992).

Studies in humans have found altered mRNA levels of different isoforms of type IV collagen. While the isoforms α1 and α2 reportedly disappeared at advanced stages of DN, isoforms α3 and α4 appeared, according to an immunohistochemical study of glomeruli by Kim et al. (Kim et al. 1991). A decrease of type IV collagen immunostaining in kidney nodules was reported by Tamsma et al. and Nerlich et al. (Nerlich et al. 1991; Tamsma et al. 1994). In particular Nerlich et al. showed that type IV collagen immunostaining was increased at early stages of nodule formation while it decreased during more advanced stages. Studies of tissue from diabetic humans have reported hyp levels to be either slightly increased or unaltered in DN (Reddi et al. 1990).

ECM-inducing growth factors such as transcription growth factor beta (TGFβ) and connective tissue growth factor (CTGF) have been implicated in the formation of fibrosis in diabetic kidneys. Mesangial cells cultured under high glucose conditions in vitro displayed increased TGFβ mRNA and protein levels (Ziyadeh et al. 1994; Di Paolo et al. 1996). A connection between renal hypertrophy and increased Tgfb expression was also made in the insulin-dependent diabetic Biobreeding Wistar (BBW) rat and the non-obese diabetic (NOD) mouse (Sharma et al. 1995). CTGF was up-regulated in the kidney of mice models of T1D and T2D (Ban et al. 2008). CTGF acts as a downstream mediator of TGFβ although it can also be regulated independently. It may exert fibrotic effects via stimulation of ECM production or the increase of inhibitors of ECM degradation (Lam et al. 2003; McLennan et al. 2004). However, while McLennan et al. have suggested that CTGF induces the increase of tissue inhibitor of
metalloproteinase 1 (Timp1) mRNA, and that this in turn results in a decreased degradation capability, they do not supply evidence of collagen accumulation in the STZ-diabetic rat model.

The inconsistent reports particularly regarding type IV collagen have been attributed to the different collagen isoforms by Costigan et al. (Costigan et al. 1995). Other contributing factors may be the different methods and models applied as well as the different time frames of the individual studies. Tissue culture studies run for days; animal studies usually run for weeks or months while DN in humans takes years to develop. Furthermore, some studies look at isolated glomeruli while others determine levels in whole cortices of kidneys thus including the tubulointerstitium. Differences between such studies can be explained by the fact that the glomerulus makes up only around 2% - 6% of the volume of the cortex (Pfaller 1982). Overall, the opinion prevails that type IV collagen accumulates in the glomerulus in diabetes while fibrillar type collagens are said to appear at later stages of the disease (Ban et al. 2008). Furthermore there is an increasing awareness that fibrosis of the tubulointerstitium is an important contributor to proteinuria (Jefferson et al. 2008). Increased expression of growth factors as mentioned above may play a role in the accumulation of collagen. Additional factors that may influence collagen turnover in diabetes are changes in protease activity in combination with altered post-translational modifications which are discussed in separate sections (see page 14 ff. and 25 ff.).

1.2.2.2 Changes in the diabetic heart

Growth factors and their putative roles in the formation of DCM have been the target of extensive research. TGFβ levels were reported to be unaltered by one group while others reported them to be increased in hearts of STZ-diabetic mice and rats (Way et al. 2002; Roestenberg et al. 2006; Westermann et al. 2007). Levels of CTGF were reportedly increased in STZ-diabetic rat hearts (Candido et al. 2003; Daniels et al. 2009). Protein levels of another growth factor, the vascular endothelial growth factor alpha (VEGFA) were decreased in the hearts of STZ-diabetic mice after 4 and 8 weeks of diabetes while this was accompanied by fibrosis detected by Sirius red staining (Messaoudi et al. 2009). Evidence from the analysis of DN of humans supports the importance of increased CTGF levels with a concomitant decrease of VEGFA levels as an increase of the CTGF/VEGFA ratio was shown to be the strongest predictor of fibrosis (Kuiper et al. 2008). Another factor affecting the regulation of ECM synthesis in the heart is the mechanical load (Bishop et al. 1999; Chan et al. 2010).
Chapter 1: Introduction

Regarding transcription levels of collagen and the effect on fibrosis, one group reported apparently contradicting results in STZ-diabetic rats 6 weeks after induction of diabetes; collagen type I and III mRNA synthesis in the left ventricle (LV) of the STZ-diabetic rat heart were decreased while collagen protein levels, taken as evidence for fibrosis, were increased as evidenced by immunohistochemistry (Van Linthout et al. 2008). The increased collagen protein levels were explained via reduced matrix metalloproteinase 2 (MMP2) levels and consequential decreased degradation of collagen which is supported by the results of a previous study by Li et al. (Li et al. 2007; Van Linthout et al. 2008). A decrease of collagenase MMP2 activity alongside an accumulation of collagen was also reported in the LV of a mouse model of diet-induced metabolic syndrome, characterised by high blood glucose levels, high triglyceride levels and high total cholesterol levels in fasting serum, resulting in cardiac remodelling (Zibadi et al. 2009). The role of proteases in the formation of fibrosis is here discussed in detail in the following section starting on page 14. As opposed to the report by Van Linthout et al., other groups reported an increase in mRNA levels of collagen types I and III in the LV (Candido et al. 2003; Gong et al. 2006). A decrease of collagen synthesis in the LV of STZ-diabetic rats was reported by Reddi et al. and was measured by L-[2,3-3H]-proline incorporation while an accumulation of collagen by hyp measurement was reported at the same time after a total of 4 weeks of diabetes (Reddi 1988).

Regarding long-term diabetes, an increase of collagen levels by hyp measurement was described in the myocardium of diabetic dogs and monkeys after 12 months and 18 months of diabetes, respectively (Haider et al. 1978; Regan et al. 1981). In contrast to the findings for short to mid-term STZ-diabetic rats, Norton et al. found no increase of collagen content by hyp measurement in the LV of STZ-diabetic rats after a total of 4 months of untreated diabetes while the stiffness of the heart was increased (Norton et al. 1996). Another study comes from Modrak who reported unaltered hyp levels in STZ-diabetic rats at 24 weeks while levels were increased after 6 weeks of diabetes (Modrak 1980). Furthermore, Modrak et al. reported the unaltered incorporation of 14C-proline into collagen in these diabetic rats. However, the study design was different from that by Reddi et al. as the incubation with the isotopically-labelled proline was carried out ex vivo for a relatively short time span rather than in vivo (Reddi 1988). Unaltered levels of hyp in the LV of STZ-diabetic rat hearts were also reported by Candido et al. despite an increase of collagen type III mRNA and protein levels after 32 weeks of diabetes (Candido et al. 2003).

In summary, an altered collagen transcription in the LV of the diabetic heart is constantly reported which may lead to the accumulation of collagen and fibrosis. Regarding STZ-
diabetic rats, there appears to be a transient increase of collagen levels after 4 to 8 weeks of diabetes, with a normalisation of collagen levels after 16 weeks and up to 32 weeks of diabetes (Modrak 1980; Reddi 1988; Norton et al. 1996; Candido et al. 2003; Van Linthout et al. 2008). Increased cardiac collagen levels have been reported in long-term diabetic dogs and monkeys (Haider et al. 1978; Regan et al. 1981). Considering that the increase in collagen hyp levels in STZ-diabetic rats occurs quite early and is transient, it is possible that this could be due to a mechanism distinct from the one seen in long-term diabetic dogs, monkeys and possibly in diabetic humans.

1.2.3 Degradation of the ECM and changes present in diabetes mellitus

Degradation of the ECM is catalysed at least in part by certain matrix metalloproteinases (MMPs), cysteine proteases of the cathepsin family (CTS), as well as by some of the serine proteases (e.g. plasmin and plasminogen activator), while the exact process of degradation of different ECM components is not known (Everts et al. 1996; Yan et al. 2003). Regarding the absolute turnover of collagen, the major protein of the ECM, the half-life has been reported to be several days to hundreds of years depending on the method employed, the organism and the tissue that was studied (Gerber et al. 1960; Verzijl et al. 2000). The initial view that collagen is an inert protein with a very slow turnover has thus been somewhat refined. It is now clear that different tissues display different turnover behaviour while each tissue also contains collagen pools with different accessibility for proteinases, resulting in the rapid degradation of some collagen while other collagen is being degraded very slowly, or even not at all (Laurent 1987). In general soft tissues like kidney and heart have a faster collagen turnover than bone, tendon or skin (Gineyts et al. 2000).

Collagen degradation in soft tissues involves the extracellular degradation by MMP2 after the activation by the membrane-bound matrix metalloproteinase MMP14 (also called membrane type 1 metalloproteinase, MT1-MMP) and is thought to be followed by the phagocytic uptake by fibroblasts and a subsequent cleavage of collagens by cysteine cathepsins in the lysosome (Everts et al. 1996; Creemers et al. 1998; Kerkvliet et al. 1999; Hernandez-Barrantes et al. 2000; Osenkowski et al. 2004; Arora et al. 2005). In this regard, it is important to note that MMP2 but not MMP9 is able to act as an interstitial collagenase and to cleave intact fibrillar type 1 collagen, despite its initial classification as a gelatinase (Aimes et al. 1995). In line with this observation, MMP9 is only present in the glomerulus in kidneys where it may act to degrade the mesh-like type 4 collagen present in the glomerular basement membrane (Catania et al. 2007).
Chapter 1: Introduction

The phagocytic uptake of collagen was shown to be mediated by MMP14 (Lee et al. 2006). While different cathepsins are present in the heart and kidney and may be involved in the subsequent lysosomal degradation, CTSB and CTSL are the most abundant: of these, CTSL displays much higher activity towards collagen (Soderstrom et al. 1999; Li et al. 2004). It is worth noting that the mechanism of collagen degradation in bone is distinct from that in soft tissues like kidney and heart. CTSK, which has the highest collagenase activity of all the cysteine proteases, is abundant in bone, where it has been shown to be responsible for the degradation of collagen; by contrast, it is only expressed in very small amounts in soft tissue (Drake et al. 1996; Garnero et al. 1998; Soderstrom et al. 1999).

Investigating the role of MMPs as well as cathepsins in collagen degradation has been difficult, probably due to redundancy (Ronco et al. 2007). For example, the knockout of MMP2 in mice did not lead to a serious phenotype while MMP9 was upregulated concomitantly, suggesting a partial redundancy of MMP2 and MMP9 (Klein et al. 2011). In addition, there are several regulatory mechanisms, including activation of the pro-enzymes as well as physiologic inhibitors and pH, which can influence the activity of these proteases in vivo making the actual activity and the potential contribution to pathogenesis hard to predict (Osenkowski et al. 2004). However, Stypmann et al. showed that CTSL knockout mice display dilated cardiomyopathy, while 25% displayed a severely-enlarged LV, underlining the importance of the ubiquitously expressed CTSL for the normal function of the heart (Stypmann et al. 2002; Petermann et al. 2006). This finding in the CTSL -/- mouse model was accompanied by cardiac fibrosis. Selective expression of CTSL in the heart employing an α-myosin heavy chain promoter attenuated several but not all of the observed pathologic features (Spira et al. 2007). Further data on the involvement of CTSB and CTSL in the physiological function of the heart comes from correlative studies, where mRNA and protein levels of CTSB and Ctsl mRNA levels were inversely correlated with ejection fraction (Müller et al. 2012).

The role of MMPs in DN as well as DCM has been studied in great detail. MMP2 and MMP9 are among the metalloproteinases that have reported to be altered in DN as well as DCM (Catania et al. 2007; Li et al. 2007; Lu et al. 2008; Van Linthout et al. 2008). Other deregulated proteins in diabetic kidneys linked to ECM degradation include the tissue inhibitors of metalloproteinase-1 and -2 (Li et al. 2007; Van Linthout et al. 2008). CTSB and L activity has been shown to be decreased or unaltered in glomeruli as well as the proximal tubule of kidneys of STZ-diabetic rats (Olbricht et al. 1992; Reckelhoff et al. 1993; Schaefer et al. 1994). This is in contrast to data reported by Leber et al. who showed that the collagenolytic activity determined by hyp release measurement is significantly increased in STZ-diabetic rat
Chapter 1: Introduction

glomeruli after 4 and 10 weeks of diabetes compared to normal glomeruli (Leber et al. 1987). In an independent study, the same group reported that less collagenous and non-collagenous amino acids are released from diabetic glomeruli than healthy glomeruli (Knecht et al. 1987). It was concluded that diabetic glomeruli are less susceptible to degradation and that this property is offset by an increase of proteases in diabetic glomeruli. The main difference in these two contrasting studies was the use of artificial substrates by Olbricht et al., Reckelhoff et al., and Schaefer et al. while Leber et al. and Knecht et al. measured the collagenolytic activity towards basement membrane collagen.

Evidence for a role of CTSB and CTSL in the aetiopathology of non-diabetic kidney disease comes from a rat model of proteinuria where an increase in activity of these proteases in proximal tubules was reported (Olbricht et al. 1986). Furthermore, Baricos et al. showed that a CTSL inhibitor reduces proteinuria in a rat model of glomerulonephritis (Baricos et al. 1991). Increased CTSL activity may be deleterious in kidneys via the cleavage of dynamin. This connection was made by Sever et al. who showed that an increased CTSL activity can lead to proteinuria through dynamin cleavage while CTSL-resistant dynamin prevented this (Sever et al. 2007).

Another link between DN and CTSL is suggested by the finding that CTSL has been shown to activate heparanase, which cleaves heparan sulphate, a glycosaminoglycan thought to contribute to the glomerular filtration barrier (Abboud-Jarrous et al. 2008). CTSL is also present in large amounts in proximal tubular cells, whose importance in the development of proteinuria has only been recognised recently (Yokota et al. 1988; Jefferson et al. 2008).

1.2.4 Antioxidant defence in the extracellular matrix - Relation to diabetic complications of the kidney and the heart

While Wells-Knecht et al. and Baynes et al. argue against a generalised increase of oxidative stress in diabetes, they do acknowledge that there is substantial clinical and experimental evidence for a tissue-specific increase of oxidative stress and that it may also play a role in the development of diabetic complications (Wells-Knecht et al. 1997; Baynes et al. 1999). Antioxidant enzymes present in the extracellular space include glutathione peroxidase 3 (GPX3), superoxide dismutase 3 (SOD3), as well as catalase (CAT).

While the majority of CAT resides in peroxisomes, it can also be secreted, and it plays an important role in the detoxification of H\(_2\)O\(_2\) produced in the mitochondria (Nohl et al. 1979;
Yokota et al. 1992; Sandstrom et al. 1993). CAT activity is increased in the heart of STZ-diabetic rats as well as rats with T2D (Wohaieb et al. 1987; Wohaieb et al. 1987). Further evidence for the importance of catalase in diabetic hearts comes from over-expression studies where catalase has been shown to protect cardiomyocytes in models of T1D and T2D by two independent groups (Ye et al. 2004; Turdi et al. 2007). Also, humans with catalase deficiency are more susceptible to develop T2D and onset of T2D started more than 10 years earlier in subjects with catalase deficiency compared to normo-catalasaemic subjects (Goth 2008).

GPX3 catalyses the detoxification of hydrogen peroxides. The enzyme binds to the basement membrane adjacent to renal cortical cells, as well as to the basement membranes of other tissues including the gastrointestinal tract (Olson et al. 2010; Burk et al. 2011). GPX3 is mainly synthesised in the kidney and plasma levels were shown to be increased at early stages of DN, while urinary levels of GPX3 increased at later stages of DN (Nishioka et al. 2001). In contrast, Chiu et al. reported plasma and urinary GPX3 levels to be decreased in diabetic patients with glomerulosclerosis (Chiu et al. 2005). They also reported a decrease in GPX3 immunostaining in the glomeruli of STZ-diabetic rats. In other studies, unaltered or slightly decreased levels of GPX in the serum were measured (Walter et al. 1991; Akkus et al. 1996; Hartnett et al. 2000). Activity of glutathione reductase (GSR), the glutathione-reducing and thus regenerating enzyme, was increased in the diabetic rat heart in two studies (Wohaieb et al. 1987; Uriu-Adams et al. 2005). Levels in the kidneys of STZ-diabetic rats and plasma of diabetic humans are unaltered (Walter et al. 1991).

SOD3 catalyses the dismutation of superoxide to oxygen and hydrogen peroxide and is mainly located in the extracellular matrix where it is bound to collagen I as well as heparan sulphate proteoglycans (Petersen et al. 2004). SOD3 serum levels have been reported to be increased, decreased or unaltered in diabetic patients (Walter et al. 1991; Akkus et al. 1996; Hartnett et al. 2000; Kimura et al. 2003). Decreased SOD1 and SOD3 protein levels, both of which are Cu/Zn SODs, were reported in kidneys of diabetic mice and rats, while they were reported to be unaltered or increased in hearts of STZ-diabetic rats (Wohaieb et al. 1987; Uriu-Adams et al. 2005; Fujita et al. 2009).

In summary, there are several deregulations of enzymes involved in the detoxification of ROS in diabetes. Depending on the enzyme and the tissue, levels are reported to be increased, decreased or unaltered. Increased tissue levels of enzymes may possibly reflect an adaptive effort to compensate for increased oxidative stress in a particular tissue while a
decrease may also contribute to the increase in oxidative stress seen in diabetes (Wohaieb et al. 1987).

1.3 **Advanced glycation end-products and diabetic complications**

1.3.1 Advanced glycation end-product formation and structure

AGEs are non-enzymatic post-translational modifications of proteins which form *in vitro* and *in vivo* while the term was first introduced in 1984 by Vlassara *et al.* (Vlassara *et al.* 1984; Monnier 2003). Preliminary data on the formation of AGEs stems from work carried out by Maillard who investigated the non-enzymatic reaction of sugars like glucose and fructose with amino acids *in vitro* (Maillard 1912). This initial, reversible step in the formation of AGEs was termed the "Maillard reaction" and leads to the subsequent formation of the AGE precursors, a "Schiff Base" and the "Amadori compound", with the subsequent irreversible formation of AGEs. The formation of AGEs *in vivo* is thought to be very slow, taking weeks or longer, but their formation *in vitro* has been shown to take place within days at a temperature of 37 °C and pH 7.4 (Brownlee *et al.* 1988; Booth *et al.* 1997; Valencia *et al.* 2004). Also, a recently published *in vivo* time course study, showed that levels of the AGE N$\varepsilon$-carboxymethyllysine (CML) were already increased, by over ~2-fold after 3 weeks of diabetes with no further increases up to 12 weeks and only slightly higher CML levels after 24 weeks compared to 3 weeks (Duran-Jimenez *et al.* 2009). Thus, the formation of AGEs *in vivo* may be faster than it was thought initially.

In contrast to most AGEs, the formation of the Amadori compound is not inhibited in the absence of oxygen or by the addition of metal chelators, meaning that autoxidative processes are not involved in the process (Fu *et al.* 1994). CML is a major AGE and has been studied in great detail (Reddy *et al.* 1995). It was shown to form by the Maillard reaction, via the Amadori compound or the Schiff Base as well as via autoxidation from glucose (Glomb *et al.* 1995). A summary of the different formation pathways is shown in Figure 1-1 for CML as well as for another AGE, glucosepane. The reaction mechanism via glucose and the Schiff Base involves the formation of reactive intermediates, glyoxal and glycolaldehyde that can lead to the formation of CML (Hayashi *et al.* 1980; Wolff *et al.* 1987; Wells-Knecht *et al.* 1995). In addition, Requena *et al.* found evidence for CML formation via
Figure 1-1: Schematic overview of the pathways of formation of CML and glucosepane
The different pathways are explained in detail in the accompanying text.
copper-catalysed peroxidation of polyunsaturated fatty acids (PUFA) so that CML is now considered to be an AGE as well as an advanced lipoxidation end-product (ALE) (Fu et al. 1996). The autoxidation of glucose can also lead to the formation of two other reactive compounds, 3-deoxyglucosone and methylglyoxal which may lead in turn to the formation of other AGES (Konishi et al. 1994; Degenhardt et al. 1998). Methylglyoxal is interesting in that it may also be formed enzymatically, while the enzymes glyoxalase 1 and 2 are involved in the degradation and detoxification of methylglyoxal (see page 30 f.). Glucosepane, the major cross-linking AGE in collagen, has been shown to form via the Amadori compound on lysine, and the final cross-link is formed with the ε-amino group of arginine (Biemel et al. 2002; Sell et al. 2005). The AGE precursor, Amadori compound can also form at NH2-terminal α-amino groups like that of valine of haemoglobin in erythrocytes (Allen et al. 1958; Bucala et al. 1992). The resulting glycated haemoglobin, HbA1c, consists of the glycated NH2-terminal valine as well as glycated ε-amino groups of lysine and is used in the clinic as a long-term marker of blood glucose levels (Roberts et al. 2001).

The importance of the different pathways for the formation of AGEs in vivo is uncertain. Wells-Knecht et al. and Fu et al. have argued that autoxidation of glucose is of high importance for the formation of CML (Fu et al. 1994; Wells-Knecht et al. 1995). In contrast, Glomb et al. and Monnier et al. have shown evidence that CML forms mainly via the Amadori compound and the Schiff Base, and argued that autoxidation of glucose as well as peroxidation of PUFAs are of low importance for the formation of CML in vivo (Glomb et al. 1995; Monnier et al. 2005). A common characteristic of CML formation via the Amadori compound, PUFA peroxidation as well as glucose autoxidation is the involvement of copper- or transition metal-catalysed oxidative reactions. Consistent with this finding, metal chelators in general and copper chelators in particular inhibit the formation of several AGES in vitro and in one case in vivo (Ahmed et al. 1986; Wolff et al. 1987; Fu et al. 1994; Wells-Knecht et al. 1995; Fu et al. 1996; Hamada et al. 2005). These findings and their implications for the treatment of diabetic complications are discussed in more detail on page 34 ff. AGES can also form via non-oxidative mechanisms from the α-dicarbonyl compounds 3-deoxyglucosone, and methylglyoxal and glyoxal in a process termed “carbonyl stress” (Baynes et al. 1999). The role of sugars other than glucose in the formation of AGES has also been investigated in vitro. A comparative study of glucose and fructose showed a higher reactivity of fructose with haemoglobin (Bunn et al. 1981). Fructose was also found to non-enzymatically modify ribonuclease A and bovine serum albumin (BSA) at a 10- to 15-fold increased rate compared to glucose (McPherson et al. 1988; Suarez et al. 1989; Takagi et al. 1995). Such a comparatively low reactivity of glucose may be due to the high stability of the ring structure (Bunn et al. 1981). Considering the high reactivity of fructose it is possible that
those sugars play a role in the formation of AGEs but it is not known to what extent this is the case in vivo.

The resulting AGEs are manifold and in addition to CML and glucosepane, numerous other AGEs have been described. They can be classified according to their cross-linking behaviour, meaning if they connect two proteins covalently as well as according to their fluorescence behaviour. The structure of some AGEs was elucidated in the late 1960’s although they were not recognised as AGEs at that time (Kalan et al. 1965; Ubuka et al. 1967; Ahmed et al. 1986). A summary of cross-linked and non-cross-linked AGEs is shown in Figure 1-2 and Figure 1-3, respectively. It was proposed early on that proteins with a long turnover time, such as collagen or lens crystallins, would be particularly prone to the accumulation of AGEs (Monnier et al. 1981). This idea was corroborated in a later study by Verzijl et al. who compared AGE levels in collagen from cartilage, which has a relatively low turnover rate, to those in skin collagen which has a higher turnover (Verzijl et al. 2000). It was found that while the rates of increase of the AGEs, CML, \(N\)-carboxyethyllysine (CEL) and pentosidine were higher in cartilage collagen, the rates were equivalent in cartilage and skin collagen after correction for the turnover of the protein. Other AGEs such as hydroimidazolones have a relatively short half-life themselves so that the absolute level of any one AGE depends on the half-life of the AGE and the protein half-life as well as the rate of formation of the particular AGE (Ahmed et al. 2007).
Figure 1-2: Structure of AGEs that form protein cross-links
AGEs are grouped by their fluorescence behaviour. References are given for the first report of each compound and those that have only been detected in vitro so far are indicated.
Chapter 1: Introduction

Non-cross-linked AGEs

Non-fluorescent compounds

\[ \text{Non-fluorescent compound 1} \]

\[ \text{MG-Hydroimidazolone 1} \]

\[ \text{MG-Hydroimidazolone 2} \]

\[ \text{MG-Hydroimidazolone 3} \]

\[ \text{3DG-Hydroimidazolone 1} \]

\[ \text{3DG-Hydroimidazolone 2} \]

\[ \text{3DG-Hydroimidazolone 3} \]

\[ \text{N\textsuperscript{\(\alpha\)}}\text{-Carboxy methylarginine} \]

\[ \text{S-Carboxy methylcysteine} \]

\[ \text{S-Carboxy ethylcysteine} \]

\[ \text{S-Carboxy ethyllysine} \]

\[ \text{S-Carboxy methyllysine} \]

\[ \text{Op-Lysine} \]

\[ \text{Pyrraline} \]

\[ \text{GLAP} \]

Fluorescent compounds

\[ \text{Argpyrimidine} \]

\[ \text{LW1} \]

**Figure 1-3: Structure of AGEs that do not form protein cross-links**

AGEs are grouped by their fluorescence behaviour and references are given for the first report of each compound.
1.3.2 AGE levels in diabetes mellitus

As mentioned in the previous section AGEs form \textit{in vitro} and \textit{in vivo}. While the focus of this thesis lies in the analysis of AGEs formed \textit{in vivo} rather than those formed \textit{in vitro}, both types may be relevant to the aetiopathology of diabetic complications. The equivalent of AGE formation \textit{in vitro} can take place during food processing, particularly at high temperatures, and an increased uptake of exogenous AGEs has been associated with diabetes mellitus and fibrosis (Zhou et al. 2004; Vlassara et al. 2011).

A comprehensive study regarding the quantity of AGEs in different tissues of healthy humans, and changes in STZ-diabetic rats stems from Thornalley et al. (Thornalley et al. 2003). CML amongst other AGEs was found to be increased in glomeruli, retina and sciatic nerve as well as plasma proteins of diabetic rats, compared to their sham-treated counterparts. The most abundant AGEs in humans as well as in rats were hydroimidazolones (see page 23 for structures). It was found that approximately 0.1–0.2\% of lysine and arginine residues in proteins are glycated, and that this may be increased by a factor of 10 in lens fibre cells with a slow turnover of proteins (Ahmed et al. 2007). A study by Sell et al. found the cross-link, glucosepane to be increased in skin collagen and the glomerular basement membrane of diabetic humans compared to healthy controls (Sell et al. 2005). The maximum portion of glucosepane-modified lysine and arginine residues was 1 and 1.2\%, respectively. This was calculated to be 1 cross-link per 2 and 5 collagen molecules in healthy participants and diabetic patients, respectively, and makes it the major cross-link in collagen found to date. An LC-MS/MS based study by Ahmed et al. looked at plasma concentrations of AGEs as well as urinary levels and renal clearance rates of healthy humans compared to diabetic counterparts (Ahmed et al. 2005). The level of free CML (CML-free adduct, meaning not bound to proteins), was increased in the plasma of diabetic patients along with increased urinary levels, while the renal clearance rate of CML was decreased. CML-containing serum protein adducts in general and CML haemoglobin levels were slightly but not significantly increased in those patients. Again hydroimidazolones were among the most abundant AGEs. The AGE-free adducts measured in this context were thought to be mainly endogenous but could also be partially derived from foodstuffs. Other studies based on antibodies by Schleicher et al. and Kilhovd et al. found increased levels of CML in plasma proteins of diabetic patients (Schleicher et al. 1997; Kilhovd et al. 1999).

While AGEs have been shown to be increased in tissues and the plasma in diabetes, the question remains as to whether the levels seen \textit{in vivo} contribute to the formation of diabetic complications or whether they are merely another indicator of high blood glucose level and oxidative stress seen in diabetes (Baynes et al. 1999). Regarding this question, it has been
proposed that the rate of accumulation of AGEs rather than the absolute level of the resulting AGEs is correlated with the development of diabetic complications (Requena et al. 2000). The observation that older non-diabetic people who do not develop complications have similar CML levels to those of young diabetic patients with complications has been interpreted to support this hypothesis. The effect of AGEs on proteins and their potential role in the formation of diabetic complications is discussed in the following section.

1.3.3 The role of AGEs in the development of diabetic complications

1.3.3.1 Physicochemical and biological properties of glycated proteins

Proteins with a slow turnover rate such as collagen are particularly prone to the accumulation of AGEs such as CML (Monnier et al. 1981; Verzijl et al. 2000). This observation taken together with the fact that fibrosis is observed in diabetic complications of the kidney and the heart (see page 3 ff.) as well as its easy accessibility, made collagen a popular target for the analysis of glycation-induced physical, chemical and biological changes (Monnier et al. 1996). Regarding the structure of collagen, it was later reported that the inside of tightly packed collagen is protected from glycation which is important to consider when comparing it to the glycation of globular proteins like albumin (Slatter et al. 2008). Lens crystallins also have a slow turnover and are related to diabetic complications of the eye which made them another target for AGE-related analysis (Stevens et al. 1978).

Early studies regarding the solubility of collagen incubated with glucose showed a decrease of solubility in 6 M urea (Bensusan 1965). The attachment of glucose to ε-amino groups of lysine from collagen, the initial stage in the formation of AGEs such as CML and glucosepane (see page 19), was shown by Robins et al. (Robins et al. 1972). Functional studies on the digestibility of collagen by pepsin or collagenases and solubility studies in acetic acid, showed that incubation of collagen with glucose in vitro as well as diabetes decreased collagen digestibility and solubility (Hamlin et al. 1975; Golub et al. 1978; Schnider et al. 1981; Brownlee et al. 1986; Salmela et al. 1989; Fu et al. 1992; Fu et al. 1994). Furthermore, it was found that fluorescence and absorbance are increased in skin collagen from diabetic patients compared to healthy counterparts of the same age (Monnier et al. 1984). An increased amount of chromophores and an increased breaking time were also reported after in vitro incubation of collagen-rich tendon with glucose (Kohn et al. 1984). Regarding the effect of glycation on collagen structure, the molecular packing of type 1
collagen was found to be expanded after *in vitro* incubation with ribose and glucose (Tanaka *et al.* 1988). Reiser *et al.* determined whether certain lysine residues of type 1 collagen are particularly susceptible to glycation, while they did not measure AGE formation (Reiser *et al.* 1992). They found that certain lysine residues were more susceptible than others while they did not find an increased randomness in glycation with age. Thus they concluded that primary structure is more important for the glycation of collagen, than the higher order structure which is disturbed with age. However, no common pattern in the primary structure of collagen lysine residues susceptible for glycation could be identified.

It was inferred from early studies that the decreased solubility and digestibility of collagen reflects an increase of non-enzymatic cross-links in diabetic collagen (Schnider *et al.* 1981; Brownlee *et al.* 1986). Brennan investigated in two studies how the different parameters in collagen from rat tail tendon of diabetic and healthy rats correlated with cross-linking behaviour (Brennan 1989; Brennan 1989). Interestingly, no change in pepsin digestibility was found while acid solubility of collagen was decreased in diabetic rats. Also, no change in collagen fluorescence was reported in diabetic rats, and acid-insoluble collagen displayed no different fluorescent behaviour from acid soluble collagen. Non-enzymatic glycation was increased in diabetic animals while cross-links were not increased, as was judged by cyanogen bromide digests. At the same time, a control incubation of rat-tail tendon collagen with fructose *in vitro* showed an increase of cross-links. Taken together these experiments showed that the data from collagen studies *in vitro* need to be interpreted carefully and that there is not necessarily a correlation between fluorescence behaviour, solubility, digestibility, cross-links and non-enzymatic glycation of collagen. Verzijl *et al.* analysed glycated collagen from cartilage and found that CML levels, but not the levels of the fluorescent AGE pentosidine correlated with collagenase digestibility and collagen fluorescence (Verzijl *et al.* 2002). One potential explanation of this finding is that AGEs other than pentosidine contribute to the fluorescence and one additional major fluorescent non-cross-linking AGE was discovered recently whose structure has not yet been determined (Sell *et al.* 2010).

In addition to changes in physicochemical characteristics, glycation can also alter the biological properties of proteins. It can affect the function of enzymes as well as signalling pathways via altered ligand-receptor interaction (Brownlee *et al.* 1984). An altered signalling upon glycation has been shown for collagen in regard to the interaction with integrins (Avery *et al.* 2006).

Other effects that have been proposed for AGE-modified proteins were the interactions with so-called AGE receptors, the most prominent of which is named advanced glycosylation end
product-specific receptor (RAGE) (Tanji et al. 2000). Studies regarding Rage included diabetic mice overexpressing RAGE where an increase in renal lesions was found while other studies investigated the effect of an RAGE-neutralising antibody (Tanji et al. 2000). Further studies found that binding of CML modified proteins to RAGE are able to activate endothelial cells, smooth muscle cells and macrophages in vitro (Ramasamy et al. 2007) and the interaction has also been linked to a nuclear factor kappa B (NF-κB) dependent pro-inflammatory response (Morcos et al. 2002). Pro-inflammatory responses like increased levels of TGFβ and CTGF as well as altered levels of MMP's have been linked to renal fibrosis (Liu 2011). However, some doubt has been cast on the concept of AGE-RAGE interaction recently. Proteins modified with AGEs at a physiological level were shown not to interact with RAGE while the CML specific interaction with RAGE has also been disputed (Baynes et al. 1999; Ahmed et al. 2007; Thornalley et al. 2009). While food derived proteins may be modified with AGEs to a higher extent if cooked at high temperatures these are degraded prior to internalisation and such AGE-containing peptides or free AGEs are unable to trigger the receptor (Heizmann 2007).

Other consequences of AGE formation on proteins include an altered protein turnover and structure (Coussons et al. 1997; Thornalley et al. 2009). Coussons et al. showed that the initial glycation reaction, meaning the formation of the Amadori compound (see page 19) does not significantly affect the structure of human serum albumin (HSA). At the same time, the addition of copper, which catalyses the subsequent formation of stable AGEs was shown to exert a marked effect on HSA conformation. Two major findings were the discovery of the abundant AGE, CML, in the mid 80’s (Ahmed et al. 1986) and later identification of the cross-linking AGE glucosepane (Sell et al. 2005). The increased levels of glucosepane in diabetic patients were thought to partially explain the decreased solubility and digestibility of glycated collagen (Sell et al. 2005). One interesting property of AGE-modified proteins is the increased binding of redox-active Cu(II) which has been shown for CML in particular. This characteristic and its consequences are discussed in greater detail in section 1.5.2.2.

It can be concluded that three major hypotheses have evolved over time in regard to how AGEs may contribute to diabetic complications (Brownlee 2001; Thomas et al. 2005; Ramasamy et al. 2007; Forbes et al. 2012):
- Altered proteolytic and functional and mechanical properties of the extracellular matrix
- Altered proteolytic and functional properties of intracellular proteins and enzymes
- An interaction of AGEs with RAGE
Evidence in support of the hypothesis concerning the extracellular matrix (ECM) is discussed in the following section.

1.3.3.2 AGEs in the development of complications in the diabetic kidney and heart with a focus on the ECM

AGE associated fluorescence in skin collagen has been shown to correlate with the severity of nephropathy, arterial stiffness and diabetic complications in general (Monnier et al. 1986). Further measurements revealed a correlation between skin collagen AGE levels and DN (McCance et al. 1993). Data supporting the role of AGEs in the structural deformation of collagen in the diabetic kidney come from a study by Makino et al. (Makino et al. 1995; Makino et al. 1996). Nodular lesions in the glomerulus were found to display increased immunohistochemical staining for AGEs while the meshwork of collagen, visualised by electron microscopy, displayed significantly enlarged pores. Increased AGE staining in the glomerulus of diabetic kidneys and its co-localization with glomerular lesions has also been reported in numerous subsequent studies (Nishino et al. 1995; Horie et al. 1997; Niwa et al. 1997; Schleicher et al. 1997; Suzuki et al. 1999; Tanji et al. 2000; Forbes et al. 2003). Evidence that AGEs may contribute to diabetic complications of the kidney due to a decreased digestibility of type IV basement collagen comes from an in vitro glycation study by Mott et al. (Mott et al. 1997). One obvious limitation of such an in vitro study is that a potential adaptive response of the body in vivo, for example through increased expression of protease levels is not taken into account (see page 14). Vlassara et al. showed that rat serum albumin (RSA) modified with AGEs in vitro administered by tail vein injection induces glomerulosclerosis and glomerular hypertrophy in rats (Vlassara et al. 1994). This finding was interpreted to provide evidence that AGEs can induce pathologic features found in diabetic kidneys in the absence of high blood glucose levels. However it must be noted that the relevance of such in vitro modified albumin has been disputed recently due to the large, non-physiologic, number of modifications that are formed (Heizmann 2007; Ramasamy et al. 2007; Thornalley 2007).

AGEs have also been reported to play a role in the aetiopathogenesis of diabetic heart failure. Deposition of CML and fibrosis were found to contribute to left-ventricular stiffness in heart failure of diabetic patients with a reduced ejection fraction while heart failure in patients with a normal ejection fraction was associated with myocyte hypertrophy and myocyte stiffening but not CML levels (van Heerebeek et al. 2008; van Heerebeek et al. 2009). The excessive accumulation of CML and other AGEs, including cross-links, on collagen can lead to altered physicochemical behaviour (see paragraph 1.3.3.1) and may provide a molecular explanation for this observed correlation in patients with a reduced ejection fraction.
Chapter 1: Introduction

Considering that the synthesis of collagen in the heart is regulated by mechanical load, this physicochemical alteration may also have an effect on collagen transcription levels (Bishop et al. 1999). Further evidence for an involvement of AGEs in heart failure comes from a study by Koyama et al. in which high pentosidine levels were an independent risk factor of cardiac events (Koyama et al. 2007). Another study in T2D rats found a linear correlation between myocardial CML levels and the left-ventricular relaxation constant \( \pi \) (Schafer et al. 2006). Also, serum AGE levels have been associated with left-ventricular diastolic dysfunction (Berg et al. 1999). In addition, CML levels of skin collagen have been shown to predict the progression of DR and DN (Genuth et al. 2005). However, while urine CML levels correlated well with albuminuria, CML plasma levels were not predictive of DCM or DN (Morcos et al. 2002; Busch et al. 2006).

Based on the findings of increased collagen AGE levels and the decreased pepsin digestibility of collagen in vitro (see paragraph 1.3.3.1), a connection between AGEs and fibrosis due to a decreased susceptibility towards proteolysis of collagen in vivo has been frequently implied although no substantive direct experimental evidence exists (Bell 2003; Asbun et al. 2006). In fact, there is a surprising lack of in vivo data regarding collagen AGE formation and its relation to proteases. One observation that casts doubt on the proposed connection between increased AGE levels, a subsequent decrease in pepsin digestibility in vitro and a resulting pathologic accumulation of collagen resulting in fibrosis, comes from Saito et al. (Saito et al. 2010). While they show a constant increase of the cross-linking AGE pentosidine in collagen along with a decreased pepsin digestibility of bone collagen, no parallel increase in collagen levels were observed. In fact, collagen levels constantly decreased starting from 10 years of age until 80 years of age. There is also some evidence that the body is able to adapt to an altered digestibility of the extracellular matrix in diabetic kidneys which was discussed in a previous section (see page 14 ff.)

Taken together there is evidence for the involvement of AGEs in the development of diabetic complications although the extent to which they contribute to the pathogenesis as well as the exact molecular mechanism that could be relevant in vivo are currently unknown. Consequently, several compounds have been tested to prevent or reverse the formation of AGEs in diabetes. This area is discussed in more detail on page 34 ff. The intrinsic defence system of the body against AGE effects is summarised in the following section.
1.3.4 Cellular defences against AGE formation

1.3.4.1 The Glyoxalase system and its alterations in diabetes

Methylglyoxal, glyoxal and 3-deoxyglucosone are reactive α-dicarbonyl intermediates in the formation of AGEs (see page 19). Methylglyoxal is not only formed non-enzymatically from glucose and triose-phosphates but can also be formed enzymatically through cytochrome P450, semicarbazide sensitive amine oxidase (SSAO), myeloperoxidase and triose phosphate isomerase (Vander Jagt 2008). The glyoxalase system (Figure 1-4) catalyses the conversion of methylglyoxal and glyoxal to less reactive α-hydroxyacids (Thornalley 2003). The third α-dicarbonyl, 3-deoxyglucosone, is processed as well, albeit at a much slower rate (Inagi et al. 2002). The first step in the glyoxalase system is the non-enzymatic reaction of reduced glutathione (GSH) with the α-dicarbonyl compound to form a hemithioacetal followed by a glyoxalase 1 catalysed isomerisation reaction to yield S-2 hydroxyacylglutathione derivatives (Thornalley 2003). The S-2 hydroxyacylglutathione derivatives are subsequently converted to α-hydroxyacids and GSH by glyoxalase 2.

![Reaction scheme of the glyoxalase system](image)

**Figure 1-4: The glyoxalase system**
The reaction scheme was adapted from Thornalley et al. (Thornalley 2003). See text for a detailed description of the reaction.
Chapter 1: Introduction

Glyoxalase 1 and 2 activity have been detected in the cytosol of cells and can be found in all prokaryotic as well as eukaryotic organisms (Thornalley 1993). Glyoxalase 2 activity has been detected in mitochondria in addition. However, the mitochondrial glyoxalase 2 enzyme is kinetically as well as immunologically distinct from the cytosolic one. Functional glyoxalase 1 is a zinc metalloenzyme. Glyoxalase 2 is also a metalloenzyme containing two bivalent metals, one of which is zinc while the second one can be either zinc or iron (Mannervik 2008).

Glyoxal- and methylglyoxal-derived AGEs such as CML, CEL and hydroimidazolones belong to the most abundant class of AGEs and their enhanced formation has been shown in diabetes, which makes the glyoxalase system an interesting research target (Degenhardt et al. 1998; Thornalley et al. 2003). The results regarding the activity of the glyoxalase system in diabetes have been mixed depending on the organ as well as the particular enzyme that was studied. Glyoxalase 1 activity was found to be unchanged in the kidneys of STZ-diabetic rats while the activity of glyoxalase 2 was significantly decreased in the medulla as well as in the cortex (Phillips et al. 1993). A decrease in activity was found in the liver while activity in the skeletal muscle was increased for both glyoxalase 1 and 2 in the STZ-diabetic rat. No differences were detected in lenses, red blood cells or in the sciatic nerve of these rats.

Regarding clinical diabetes mellitus, it was shown that insulin-dependent diabetic patients with retinopathy have a significantly higher activity of glyoxalase 1 in red blood cells than patients without retinopathy, while glyoxalase 2 activity is decreased in those patients with retinopathy (Thornalley 1993). In another study it was shown that glyoxalase 1 activity is elevated in T1D and T2D compared to healthy patients while glyoxalase 2 activity was elevated only in T2D (McLellan et al. 1993; McLellan et al. 1994). At the same time, levels of reduced glutathione were shown not to be altered in whole blood samples of T1D and T2D patients.

1.3.4.2 Other potential enzymatic defence systems

Aldose reductase (AKR1B1), 2-oxoaldehyde dehydrogenase (ALD1A1) and betaine aldehyde dehydrogenase represent additional enzyme systems that can metabolise methylglyoxal (Collard et al. 2007; Vander Jagt 2008). AKR1B1 and ALDH1A1 have also been shown to act on 3-deoxyglucosone in addition to the alcohol dehydrogenase (AKR1A1) and the dihydrodiol dehydrogenase (AKR1C1) (Fujii et al. 1995; Suzuki et al. 1998; Collard et al. 2007). The product of Aldh1a1, 3-deoxy-2-ketogluconic acid, was shown to be elevated in serum of diabetic patients as well as in their erythrocytes. This could mean that this process is a physiologically relevant protection mechanism in diabetes (Fujii et al. 1995).
1.4 Further post-translational modifications

1.4.1 Carbonyl formation and oxidative stress in diabetic complications

Carbonyl formation is a non-enzymatic modification of proteins that is frequently reported as a marker of oxidative stress (Berlett et al. 1997; Stadtman 2004). Carbonyl groups can be found in the Amadori compound or in the AGE pyrraline as non-enzymatic adducts on the ε-amino group of lysine (see pages 19 and 23). Carbonyl levels are mostly detected using the aldehyde- and ketone-specific derivatisation of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) (Buss et al. 1997; Dalle-Donne et al. 2003). Importantly, the ketone-containing Amadori compound was reported not to react with DNPH which means that the marker is not affected by the initial glycation reaction (Liggins et al. 1997; Stefek et al. 1999). Lipid peroxidation reactions may also lead to the formation of protein carbonyl groups (Berlett et al. 1997).

Carbonyl levels have been reportedly increased in kidneys of diabetic rats (Chang et al. 2011). This is in contrast to another report by Portero-Otin et al. who described unaltered and even decreased carbonyl formation in kidney and liver of STZ-diabetic rats respectively (Portero-Otin et al. 1999). One major disadvantage of the procedure employed by Chang et al. was the use of tissue lysates, as DNA is known to interfere with the assay while Portero-Otin et al. employed an antibody-based approach which would be expected to eliminate such interference (Cao et al. 1995). Formation of carbonyls in vitro was shown to be enhanced on BSA incubated with glucose and fructose (Takagi et al. 1995; Liggins et al. 1997). Telci et al. reported on the enhanced formation of protein carbonyl levels in blood plasma of T1D patients (Telci et al. 2000; Telci et al. 2000). A direct connection between AGEs and carbonyl formation in vitro has been shown by Requena et al. who reported an enhanced formation of carbonyls in BSA modified with increasing levels of CML (Requena et al. 1999).

Wells-Knecht et al. measured other markers of oxidative stress in skin collagen of diabetic patients (Wells-Knecht et al. 1997). The oxidative markers ortho-tyrosine as well as methionine sulphoxide were found to not be increased in diabetic patients. Thus the authors argued that there is no generalised increase of oxidative stress in diabetes while they did not exclude a tissue specific increase of oxidative stress.
Chapter 1: Introduction

1.4.2 Enzymatic cross-linking of the extracellular matrix via lysyl oxidase

1.4.2.1 Lysyl oxidase: Properties, functions and regulation

Lysyl oxidase (LOX) is a copper-metalloenzyme which catalyses the cross-linking of collagen as well as elastin in the extracellular matrix (Kagan et al. 1984; Gacheru et al. 1990). Lysyl oxidase-dependent covalent cross-linking takes place between lysine and/or hydroxylysine residues in the non-helical telopeptide region of the two extracellular matrix proteins (Reiser et al. 1992; Robins 2007; Saito et al. 2010). Initial reducible cross-links mature over time to form non-reducible stable trivalent cross-links. These mature cross-links form on collagen in parallel to the non-enzymatically cross-linked AGEs described previously on page 18ff (Bailey et al. 1998). The characteristic of the mature cross-links depends on the amount of hydroxylated lysine residues which is catalysed by a different enzyme, lysyl hydroxylase, and a high activity of lysyl hydroxylase can be found in bone collagen (Saito et al. 2010).

LOX is secreted as a pro-enzyme into the extracellular space where it is processed by the bone morphogenetic protein 1 (BMP1) to yield the active enzyme and the pro-peptide (Uzel et al. 2001). The activity of LOX is also influenced by dietary copper levels and copper deficiency can affect the cross-linking of the ECM (Chou et al. 1968). Interestingly, dietary copper levels affect LOX activity beyond the amount needed for the formation of cross-links in tendon and bone collagen. While cross-link formation of collagen appeared saturated, at dietary copper levels of less than 1 μg/g activity increased linearly to levels higher than 15 μg/g of dietary copper, with no effect on protein or mRNA levels (Opsahl et al. 1982; Rucker et al. 1996; Rucker et al. 1998). Due to the fact that LOX has a high affinity for copper, it can bind up to 5-7 mol of copper per mol of enzyme, and that it can account for 10% or more of tissue copper, a role in general copper metabolism has also been proposed for the enzyme (Rucker et al. 1996; Rucker et al. 1998). LOX transcription is regulated by growth factors including TGFβ, as well as conditions of hypoxia (Feres-Filho et al. 1995; Hong et al. 1999).

1.4.2.2 Alterations of LOX and the resulting enzymatic cross-links in diabetes

Madia et al. showed that LOX activity is increased in lungs of STZ-diabetic rats while activity was reported to be decreased in the diabetic eye of humans (Madia et al. 1979; Coral et al. 2008). It was also reported that the LOX inhibitors β-aminopropionitrile (BAPN) and D-penicillamine are able to reverse the decreased acid solubility of collagen in STZ-diabetic
rats (Chang et al. 1980). BAPN is an irreversible inhibitor which is postulated to covalently bind to the active site of the enzyme (Wilmarth et al. 1992). D-Penicillamine is thought to reversibly inhibit LOX dependent cross-link formation through interaction with the immature precursors of the cross-links, while the exact mechanism may depend on the type of tissue (Deshmukh et al. 1969; Nimni 1977; Robins 2007). While D-penicillamine is approved as a copper chelator for the treatment of Wilson’s disease, copper chelation is thought not to be a mode of action for the inhibition of LOX activity in vivo (Deshmukh et al. 1969; Walshe 1982).

The resulting enzymatic collagen cross-links were analysed in STZ-induced diabetes and in rats fed with a high galactose diet and the divalent crosslink dihydroxy-lysinonorleucine (DHLNL) was shown to be increased (Reiser et al. 1991). The level of the trivalent cross-link hydroxypyridinium (OHP) was found to be unaltered or decreased in the STZ-induced diabetic and high galactose diet fed-rats, respectively. This occurred along with a decrease of collagen acid solubility in the STZ-induced diabetic rats and an increase in collagen acid solubility in the rats fed with the high galactose diet. The decreased acid solubility could be explained by the increase in DHLNL cross-links in the STZ-induced diabetic rats while the increase of acid solubility in the galactose fed rats may be due to the decreased mature OHP cross-links, despite an increase in DHLNL cross-links. In a separate study, it was reported that the LOX-dependent cross-link hydroxyl-lysinonorleucine (HLNL) is not altered in rat tail tendon collagen from STZ-induced diabetic rats (Le Pape et al. 1981). An increase of DHLNL as well as OHP was also measured in skin collagen from diabetic patients and the changes correlated with the duration of diabetes (Buckingham et al. 1990). Considering that LOX activity may be altered in diabetes and that the resulting enzymatic cross-links influence the solubility of collagen, LOX should be included in a comprehensive analysis of the ECM of diabetic tissue.

1.5 The role of copper in the development of diabetic complications and potential treatments

1.5.1 Copper metabolism in healthy mammals

Most of the copper is absorbed in the small intestine via copper transporter 1 (CTR1), while the molecular mechanism is currently not well understood (Arredondo et al. 2005; Lönnerdal 2008). Absorbed copper is transported via the blood mainly to the liver but also the kidney, bound to albumin and transcuprein (Linder et al. 1998). Both, the kidney and the liver synthesise caeruloplasmin and excrete the protein in tight association with up to six
molecules of copper, and this represents the largest copper pool in the blood (Hellman et al. 2002). However, it is under dispute whether caeruloplasmin plays a role in the distribution of copper in the body (Linder et al. 1998; Choi et al. 2009). The finding of normal copper transport in caeruloplasmin knockout mice was taken as evidence that such a function, if present, must be redundant possibly due to albumin and transcuprein (Meyer et al. 2001; Moriya et al. 2008).

Uptake of copper into the cells takes place via CTR1 or the divalent metal transporter 1 (DMT1) followed by chaperone-mediated delivery to the trans-Golgi network (via antioxidant protein 1, ATOX1), cytosolic Cu/Zn SOD (via copper chaperone for SOD, CCS) or the mitochondrial protein cytochrome c oxidase (via cytochrome c oxidase chaperone, COX17) (Arredondo et al. 2005). The P-type ATPases ATP7A (also designated MNK due to causative genetic defect in Menkes disease) and ATP7B (also designated WND due to causative genetic defect in Wilson’s disease) located in the trans-Golgi network are responsible for the excretion of cell copper when cellular levels become too high. While ATP7A is constitutively expressed in most cells, ATP7B is currently considered to be expressed only in hepatocytes and is responsible for the excretion of excessive amounts of copper into the bile as well as for the distribution of caeruloplasmin-bound copper to other organs. Another potential mechanism for copper excretion via the bile is thought to involve the lysosomes (Gross et al. 1989; Wijmenga et al. 2004).

1.5.2 Altered copper metabolism in diabetes mellitus

1.5.2.1 Changes of tissue copper levels in diabetes

Levels of copper were shown to be increased in plasma of diabetic patients by several independent groups while Sjögren et al. also demonstrated a correlation with HbA1c levels (Mateo et al. 1978; Sjögren et al. 1986; Walter et al. 1991; Zargar et al. 1998). Sjögren et al. measured copper levels in striated muscle as well, and reported a decrease of copper in T1D patients. Increased copper levels were reported in the kidney and liver of the STZ-diabetic rat (Failla et al. 1981). This was further supported by a study using \(^{67}\)Cu where 4-fold increased copper levels were found in diabetic rat kidneys along with an increase in serum copper levels compared to healthy rats (Uriu-Adams et al. 2005). At the same time, the heart and muscle tissue displayed a tendency for lower copper levels which is consistent with the results in type 1 diabetic patients of Sjögren et al. (Sjögren et al. 1986). This effect became significant when looking at the uptake of \(^{67}\)Cu into the muscle which was lower in diabetic animals than in healthy animals. No change of copper level in skeletal muscle in STZ-diabetic rats was reported by Aguilar et al. while liver copper levels were strongly
increased (Aguilar et al. 1998). Thus overall, the current findings support an increase of copper levels in the serum, kidney and liver of diabetic patients, while there tends to be a decrease in copper levels in the heart and muscle. The main site of copper accumulation in diabetic kidneys is thought to be the lysosomes, although these data need to be interpreted with caution as doubt has been cast on the specificity of some of the applied techniques (Gassman et al. 1983; Bremner 1998). Despite the decreased overall copper levels in the diabetic heart, Cooper et al. found an increase in the amount of chelatable copper in the STZ-diabetic rat heart suggesting that there may be an accumulation of extracellular loosely bound copper in the ECM of the diabetic heart (Cooper et al. 2004).

1.5.2.2 Potential causes and consequences of perturbed copper metabolism

As described above, there is considerable evidence for disturbed copper metabolism in diabetes. Considering that copper is a cofactor for essential enzymes such as Cu/Zn SOD, cytochrome c oxidase and lysyl oxidase, and also the redox activity of copper, this could have deleterious consequences which may contribute to the formation of diabetic complications. The diabetic rats from the study by Uriu-Adams et al. mentioned in the previous section not only displayed higher serum copper levels, but also lower Cu/Zn SOD activity (Uriu-Adams et al. 2005). The observed changes in activity of lysyl oxidase in diabetes as well as the resulting cross-links summarised on page 33 may also be partially due to altered copper metabolism in diabetes, as the enzyme is highly sensitive to copper, beyond the amount needed as a co-factor at the active site (see page 33).

Due to the redox activity of copper, this transition metal could contribute to the enhanced formation of AGEs via several pathways as described on page 18 ff., as well as to an increased oxidative stress in certain tissues in diabetes (Chevion 1988; Baynes et al. 1999). In line with this theory, the application of copper chelators as well as metal chelators have been shown to decrease AGE formation, as well as associated measures like collagen pepsin solubility and collagen fluorescence in vitro and in vivo (Ahmed et al. 1986; Chace et al. 1991; Fu et al. 1992; Fu et al. 1994; Wells-Knecht et al. 1995; Fu et al. 1996; Loske et al. 2000; Cameron et al. 2001). The addition of copper to incubations of proteins with glucose had the opposite effect, namely the enhanced formation of AGEs (Wolff et al. 1987; Wolff et al. 1991; Sajithlal et al. 1998; Sajithlal et al. 1999). There is also in vivo evidence that copper as opposed to iron is a major factor contributing to the enhanced AGE formation in diabetes, independent from blood glucose levels (Elgawish et al. 1996).
Further findings come from glucose pre-incubation experiments where the formation of AGEs from glycated proteins or amino acids is observed, as opposed to the formation after incubation with free glucose. In such incubations, the rate of CML formation from fructoselysine was inhibited by metal chelators in combination with a N₂ atmosphere in vitro (Zyzak et al. 1995). In another experiment, the incubation of collagen with glycated BSA induced collagen cross-linking (Sajithlal et al. 1998). This was inhibited by aminoguanidine (50 mM), which acts by the direct inactivation of carbonyl compounds in vitro, but not by the metal chelator EDTA (5 mM). This finding contradicts the theory that the collagen cross-linking in this setting is based on a transition metal-catalysed fragmentation of the Amadori compound to reactive carbonyl intermediates described by Glomb et al. (Glomb et al. 1995).

It is difficult to draw conclusions for the treatment of AGE formation in vivo from this one contradictory in vitro experiment by Sajithlal et al. as the fragmentation of the Amadori compound is only one of several pathways that could lead to the formation of AGEs (see page 18 ff.). The successful use of metal chelators for the inhibition of AGE formation in vivo and in vitro mentioned in the previous paragraph also argues against the relative importance of the interesting in vivo finding by Sajithlal et al. (Sajithlal et al. 1998).

There is also evidence that the enhanced AGE formation can in turn contribute to a disturbed copper metabolism. An increased binding of redox active copper was found in tendons from diabetic rats alongside an increased amount of serum chelatable copper (Eaton et al. 2002). Increased amounts of copper bind to albumin, gelatine and elastin after glycation in vitro (Qian et al. 1998). It was shown that CML-modified poly-L-lysine as well as albumin are able to bind transition metals, with significantly enhanced affinity compared to unmodified lysine and albumin while also being redox active (Saxena et al. 1999). This was confirmed in another study employing peptide-bound CML (Seifert et al. 2004). Thus, once enhanced AGE formation takes place in diabetes initiated by high blood glucose levels, this may lead to a vicious cycle as the AGEs may subsequently bind larger amounts of copper leading to an enhanced oxidative stress (Saxena et al. 1999). Other potential sources of redox active copper independent from AGE formation include the copper carrier caeruloplasmin (Ehrenwald et al. 1994).

There is always the question whether in vitro experiments and findings from animal models are applicable to the deregulations found in humans. Some doubt has been cast on the direct applicability towards human studies as it was shown that copper binding to albumin from diabetic humans was decreased rather than increased (Guerin-Dubourg et al. 2012). This was also the case for albumin that was glycated for a short time in vitro suggesting that extensive glycation of albumin in vitro may not be an appropriate model (Argirova et al.
However, increased serum copper levels were found in STZ-diabetic rats as well as diabetic humans discussed in the previous section. Thus, while the exact molecular mechanism of copper dysregulation in diabetes still needs to be unravelled, it is clear that such a perturbation exists. It is possible that peptide-bound AGEs, like CML, and non-protein bound AGEs, so called AGE-free adducts which are increased in serum of diabetic humans as well as accumulation of certain AGEs in long-lived extracellular proteins like collagen, contribute to an altered copper metabolism in diabetic humans as well as the development of diabetic complications (Kilhovd et al. 1999; Monnier 2001; Thornalley et al. 2003).

1.5.3 Copper chelation as a treatment for diabetic complications

1.5.3.1 Treatment of diabetic complications with AGE-inhibitors and AGE-breakers - relation to copper chelation

Several advanced glycation end-product inhibitors as well as AGE-breakers have been studied for the treatment of diabetic complications. The first compound introduced as an AGE-inhibitor was the dicarbonyl scavenger, aminoguanidine. Early experiments revealed that aminoguanidine restores aortic collagen digestibility by pepsin, solubility by acetic acid and cyanogen bromide-cleavage of collagen in diabetic rats (Brownlee et al. 1986; Soulis-Liparota et al. 1991). In another study, Norton et al. showed that collagen fluorescence is increased in diabetes and that aminoguanidine inhibits this increase (Norton et al. 1996). Contrary to this finding, while pentosidine and CML were increased by 150% and 30%, respectively in diabetic skin collagen, aminoguanidine treatment did not affect this increase, while it did inhibit albuminuria (Degenhardt et al. 1999). In contrast to AGE-inhibitors, AGE-breakers were designed to cleave already existing cross-links (Yang et al. 2003).

The mechanism of action of both, AGE-inhibitors and AGE-breakers, has been questioned after it became known that they have transition metal-chelating properties (Price et al. 2001; Yang et al. 2003). This property was also shown for a more recent AGE-inhibitor, pyridoxamine, belonging to the vitamin B6 family (Voziyan et al. 2003; Adrover et al. 2008). It was also discovered that AGE-breakers, while cleaving model compounds in vitro, fail to break collagen cross-links from skin and tendon collagen in vivo (Yang et al. 2003). Thus, it was concluded that the decreased formation of AGEs in vitro and the associated improvements in collagen solubility and digestibility is due to the chelating activity of these compounds (Monnier et al. 2006; Nagai et al. 2012). The initial AGE-inhibitory compound aminoguanidine, as well as the compound pyridoxamine displayed adverse effects in clinical
trials while other AGE inhibitors, thiamine and benfotiamine did not show any improvement (Forbes et al. 2012). Considering that the alleviating effect of the AGE-inhibitors and AGE-breakers on the development of diabetic complications is now ascribed mainly to their chelating activity, the application of a copper chelator like triethylenetetramine (TETA), whose favourable safety profile is known from the application in the treatment in Wilson’s disease (Sarkar et al. 1977; Williams 1983; Walshe 1996), holds promise for the treatment of diabetic complications.

1.5.3.2 The Cu(II) chelator triethylenetetramine as a treatment for diabetic complications

TETA in the dihydrochloride form (triente, see Figure 1-5), is currently used for the removal of excess copper from liver and brain in patients with Wilson disease, an autosomal recessive disorder caused by a genetic defect in the copper-transporting P-ATPase ATB7B (Walshe 1982; Walshe 1996; Roberts et al. 2003). It has been experimentally tested for the treatment of diabetic complications as early as 1995 by Cameron et al. who reported an improved neurovascular function in diabetic rats after TETA treatment (Cameron et al. 1995; Love et al. 1996). In subsequent studies, the improvement of the relaxation of the aorta and the mesenteric vascular bed after TETA treatment was shown (Keegan et al. 1999; Inkster et al. 2002). A study by Nakamura et al. confirmed the beneficial effects of TETA on diabetic neuropathy in rats while Hamada et al. reported a beneficial effect of TETA on carbonyl stress in diabetic rat lenses (Nakamura et al. 2002; Hamada et al. 2005).

Studies from our group showed an improved function of the heart as well as the kidney in diabetic humans and STZ-diabetic rats after treatment with TETA (Cooper et al. 2004; Gong et al. 2008; Cooper et al. 2009; Cooper 2012). TETA treatment led to an increased output of copper in the perfused diabetic rat heart compared to sham hearts, while it also reversed elevated copper levels in the diabetic kidney. Improvement of measures of the heart and kidney after TETA treatment in diabetic rats were confirmed by Baynes et al. as well as Moya-Olano et al. (Baynes et al. 2009; Moya-Olano et al. 2011).
Molecular targets that were shown to be affected by TETA treatment include the enzyme amine oxidase copper, containing 3 (AOC3), also known as semicarbazide-sensitive amine oxidase which is reportedly increased in STZ-diabetic rat kidneys as well as in serum and the aorta (Hayes et al. 1990; Hamada et al. 2005; Jüllig et al. 2010). Growth factors were also altered by TETA treatment in STZ-diabetic rats (Gong et al. 2006; Gong et al. 2008). The aim of this thesis was to expand on the previous work based on the hypothesis outlined in the next section.

1.6 Hypothesis and experimental approach

1.6.1 Hypothesis

Considering the current evidence for:

- Transition metal-catalysed AGE formation in vivo and in vitro (see pages 18 ff.);
- Altered copper metabolism in diabetes (see pages 35 ff.);
- The beneficial effects of copper chelators in the treatment of diabetic humans and rats (see pages 38 ff.),

it was of interest to look at AGE formation and other copper-associated metabolic dysregulation in diabetic kidneys and hearts and compare these to tissues from healthy counterparts, as well as to compare them to tissues after treatment with the Cu(II) chelator TETA. As discussed previously (pages 25 ff.) collagen, the main constituent of the extracellular matrix, is particularly prone to AGE accumulation due to its long half-life, and dysregulation of collagen is a prominent pathogenic feature of diabetic complications of the heart and kidney (pages 3 ff.). Thus it was hypothesised that treatment with the Cu(II) chelator TETA reverses the enhanced formation of AGEs in the diabetic kidney and heart, and thereby protects the ECM from an AGE-mediated deterioration that otherwise occurs in diabetes mellitus.

1.6.2 Experimental approach

Methods from biochemistry and molecular biology were employed to detect alterations of kidney and heart collagen in diabetes, and to find possible changes after treatment of animals with the Cu(II)-chelator, TETA with a focus on post-translational modifications of collagen. The analysis was to be extended towards proteins associated with the turnover and
enzymatic cross-linking of collagen including the copper-containing cross-linking enzyme lysyl oxidase and collagen-degrading proteases.

The model of choice for the studies was the well-established STZ-diabetic rat model (Wilson et al. 1990). STZ is a naturally occurring antibiotic from Streptomyces achromogenes which selectively induces toxicity in the insulin-producing pancreatic islet beta-cells (Lenzen 2008). It is transported into the cell via the glucose transporter 2 (GLUT2) and subsequently induces necrosis via alkylation of DNA and proteins. The STZ-induced diabetic rat model has been shown to develop typical chronic complications of diabetes including DN and DCM which were the desired targets of analysis in this thesis (Tomlinson et al. 1992; Wei et al. 2003; Tesch et al. 2007). Another advantage of the model is the good comparability due to the vast amount of data that has been accumulated so far, including data from our own group (Cooper et al. 2004; Gong et al. 2006; Gong et al. 2008).

Limitations of the STZ-induced diabetic model are the severity of the disease, with a very poor glycaemic control concomitant with weight loss and a high mortality rate (Requena et al. 2000). However, the experience from previous experiments with the STZ-induced diabetic rat model in our group would make it possible to carry out the study with a relatively low mortality rate of animals and a stably high blood glucose level is desired in this study. Another criticism is that the STZ-induced diabetic model resembles T1D, a less prevalent form compared to the much more prevalent T2D. However, the employment of a T2D model may introduce confounding factors like hypertension, independent of the diabetic phenotype (Srinivasan et al. 2007). Hypertension for example, could well lead to an altered extracellular matrix turnover due to an increase in mechanical stress, which would in turn have an effect on post-translational modifications of collagen independent of diabetes (Bishop et al. 1999; Chan et al. 2010).

The fact that a stable high blood glucose level can be achieved in combination with development of diabetic complications of the kidney and the heart make the STZ-induced diabetic model attractive for my thesis. In addition, the mortality rate can be kept at a reasonable level due to previous experience with the model by our group. This combined with the good comparability to previous data from our lab as well as other labs makes the STZ-induced diabetic rat model the model of choice for my studies. Importantly, treatment of the animals with the Cu(II) chelator or placebo treatment was to be started 8 weeks after induction of diabetes rather than at the time of onset of the disease. This allowed for a more realistic outcome of the study as treatment in diabetes usually starts after varying durations of the disease.
Chapter 2 - Materials and methods

Materials and buffers specific to a specific method are mentioned directly in the methods section. A list of standard materials, reagents and equipment used can be found at the end of this chapter (see page 73). All dilutions referred to are dilution factors, meaning for example that a 1:2 dilution is a 50:50 mixture of solution A and B while a 1:1 dilution is an undiluted solution.

2.1 STZ induced diabetes model and treatment regime

All animal protocols were approved by the University of Auckland Animal Ethics Committee. Diabetes was induced by injection of STZ into the tail vein of male Wistar rats which are considered to be more susceptible to the STZ-induced destruction of the pancreatic islet β-cells and the subsequent increase in blood glucose levels, than females (Tesch et al. 2007). Rats of 300 g ± 30 g (approximately 8 weeks of age) were obtained from the Integrated Physiology Unit of the School of Biological Sciences, the University of Auckland. They were anaesthetised using isoflurane (Medsource, Lunan Better Pharmaceuticals, China) in an anaesthetic induction box and kept anaesthetised throughout

\[n=12 \text{ per group}\]

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<th>0 weeks</th>
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<td>Sham/Placebo</td>
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Sham injection / STZ injection | TETA / Placebo administration | Collection of heart and kidney

Figure 2-1: Setup and timeline of the animal trial
Shown is the setup of the animal trial with the time frame shown alongside the procedures that were undertaken as well as the resulting four groups of animal. Rats were injected with saline or STZ (n=24 for both disease groups), at a time point defined as "0 weeks" and TETA- or placebo treatment was started 8 weeks later each in half of the animals of the two disease groups resulting in 12 animals of each of the four final groups. A total of 16 weeks after injection of the animals and 8 weeks after the start of the treatment animals were culled and the heart and the kidneys of the animals were collected.
Chapter 2: Materials and methods

the procedure at 2-3% isoflurane/O2 at 2 L/min via a nose cone. Rats to be made diabetic were injected with STZ (S0130, Lot No. 119K1591, Sigma-Aldrich, USA; see page 173 for certificate of analysis) in 0.90 % w/v sterile sodium chloride solution (saline solution; 55.0 mg/mL) at 55 mg STZ per kg of body weight using a syringe fitted to a catheter (Insite 24 GA 0.75 ln with dim 0.7x19 mm, BD, USA). As the α-anomer has been shown to be more active than the β-anomer in the induction of diabetes and STZ is only commercially available as a mixture of the two anomers, it was ensured that the same lot number was used throughout the study (Rossini et al. 1977). The catheter was then flushed with an equivalent volume of saline solution in order to completely administer the remainder of the STZ-solution and to prevent infection at the site of injection. A sham-group was injected with 0.90% saline solution only (see Figure 2-1 for setup).

A total of 48 male Wistar rats were injected; 24 with STZ and 24 with a saline solution (sham). Rats were fed rat chow (Teklad TB2018, Harlan, UK) and tap water ad libitum. Body weight of animals was measured daily during the first week followed by weekly measurements thereafter. Pre-specified criteria were applied so that undue suffering of experimental animals was minimised. For example, if the body weight of an animal dropped 10% below the starting weight, it was monitored daily thereafter and was culled if the weight dropped 15% below the starting weight. Blood glucose levels were measured 3 times in the first week followed by fortnightly measurements using a blood sample from the tail vein (Advantage II System, Roche Diagnostics, Germany). Rats were considered to be diabetic if the blood glucose level was above 15 mmol/L (Tesch et al. 2007). It was standard practice to measure both the body weight and blood glucose levels at around 10.00 am. Treatment of rats with a placebo or TETA dihydrochloride (T5033, Sigma-Aldrich, USA) was started 8 weeks after injection in half of the STZ- or Sham-injected animals. As TETA was administered in the drinking water and diabetic rats drink approximately 200 mL/day while non-diabetic rats drink around 20 mL/day, the concentration of the drug in the drinking water was adjusted accordingly. Sham-treated rats injected with saline received drinking water without (Placebo) or with (TETA) drug at a concentration of 1 g/L. STZ-induced diabetic rats received water without (Placebo) or with (TETA) drug at a concentration of 0.1 g/L. This resulted in an uptake of 20 mg of TETA per day in both the Sham/TETA and the STZ/TETA treatment groups. A total of 12 animals were present in each of the four groups and tissue was collected 8 weeks after start of the treatment.

Rats were anaesthetised with isoflurane and dissected on the ventral side for tissue collection. Heparin (DBL Heparin Sodium solution, Hospira, Australia) at 1 I.U. per g of body weight was injected into the inferior vena cava. The animal was culled by removal of the
Chapter 2: Materials and methods

heart which was perfused with DEPC (diethylpyrocarbonate) treated saline solution and the LV was collected. DEPC treatment of the solution was necessary to inactivate RNA degrading ribonucleases in the water as messenger RNA levels were to be measured in these samples. The kidneys were perfused with DEPC \textit{in situ} via the abdominal aorta. Kidneys were removed and cut in two halves alongside the anterior-posterior axis. Small pieces of the LV of the heart and the kidney cortex were excised and stored in RNAlater at 4°C overnight to allow for perfusion of the tissue with the solution. The rest of the LV and kidneys were frozen in liquid N\textsubscript{2} immediately. The RNAlater-treated samples were frozen in liquid N\textsubscript{2} the following day and stored at -80°C.

Animals injected with saline solution are designated “Sham” while STZ-induced diabetic animals are designated “STZ” in the subsequent results section. Animals treated with a placebo were designated “Plac” while TETA treated animals were designated “TETA”. The effects are presumed to be those of diabetes as discussed in section 1.6.2 although possible contributions by direct STZ-mediated toxicity cannot be completely excluded.

2.2 Collagen preparation from kidney and heart

2.2.1 Hydroxyproline assay

The collagen content was determined via the measurement of the hydroxyproline (hyp) content using the method by Bergman and Loxley with minor modifications (Bergman et al. 1963). Tissues was hydrolysed for the measurement according to the method of Stegemann and Stalder (Stegemann et al. 1967).

Tissue from the kidney cortex and LV was freeze-dried and weighed prior to the assay. Collagen extracts were also freeze-dried before the hyp measurement was carried out. For digestibility measurements of collagen extracts, a portion of the supernatant and the pellet were hydrolysed for hyp measurement without prior freeze-drying. Samples were hydrolysed by incubation with an excess of 6 M HCl in Pyrex tubes with a teflon membrane-lined lid (Corning, USA) for 16 h at 110°C. A hyp standard (150 μg) was treated accordingly. Acid was evaporated and samples were reconstituted in water and adjusted to approximately pH 8 using NaOH. The standard was reconstituted in water and a 7 point ½ dilution standard curve, ranging from 0-150 μg/mL was prepared. Standard and samples were diluted with isopropyl alcohol (IPA) at a factor of 1:2 and 440 μL of Chloramine T reagent was added and sample mixtures were incubated at RT for 5 min. A total of 1.3 mL of Ehrlich’s reagent was added and tubes were incubated at 60°C for 25 min. Samples were chilled in an ice bath to
Chapter 2: Materials and methods

stop colour development, transferred to 96-well plates in triplicate and absorbance was measured at 558 nm.

Hyp concentration was calculated from the standard curve and the amount of collagen was estimated assuming that 12.5% of collagen consists of hyp.

Buffers and reagents:

- Acetate Citrate buffer
  - 0.7 M Sodium acetate
  - 0.2 M trisodium citrate/sodium citrate tribasic
  - 0.045 M citric acid
  → Store at 4 °C.
  → Dilute 6 parts into 4 parts IPA before use

- Chloramine T reagent
  - Dissolve 300 mM Chloramine T in acetate citrate buffer/IPA
  - Add acetate citrate buffer/IPA to a final concentration of 70 mM Chloramine T
  → Prepare freshly each time before use

- Ehrlich’s reagent
  - 3.5 M 4-(dimethylamino) benzaldehyde in 72% perchloric acid
  Dilute 1 part in 4 parts IPA
  → Prepare freshly each time before use

2.2.2 Collagen extraction

Fibrillar collagen was extracted from the kidney and heart using a method adapted from Avery and Bailey (Avery et al. 1995). The renal pelvis as well as the major calyx of the kidney were removed and discarded while only the LV of the heart was used. Kidney and heart tissue was roughly chopped and approximately 250 mg or 160 mg, respectively were added to 2 mL microtubes containing 0.5 mL Hasselbach-Schneider buffer (H-S buffer) and a steel bead, on ice. Tissue was lysed using a steel bead homogeniser (TissueLyser, Qiagen, Germany) at 30 Hz for 4 min, while rearranging the adapter plates after 2 min. After lysis, 750 mg of kidney tissue or 500 mg of LV tissue was pooled and topped up to 5 mL with ice cold H-S buffer in 15 mL centrifuge tubes. Lysates were sonicated for 1 min at an amplitude of 15 μm in an ice bath with the sonication probe submerged into the lysates (Soniprep 150, MSE-Sanyo, UK). The precipitate was filtered over a 500 μm sieve (aperture between 500
Chapter 2: Materials and methods

μm and 355 μm was used) and added to another 5 mL of fresh H-S buffer. Two shorter sonication cycles of 30 s at an amplitude of 10 μm followed by sieving were carried out. The precipitate was resuspended in 1 mL of water and dialysed against water using a 3500 Da cut-off membrane for 24 h, with at least 3 changes of water. The dialysed extracts were frozen in liquid N₂, freeze-dried and stored at -30 °C. Collagen extracts were characterised quantitatively by hyp assay (see previous section) and the amount of collagen in the extracts was given in collagen per mg dry weight. In addition the extracts were analysed qualitatively by LC-MS/MS analysis as is described on page 58. The amount of collagen recovered from the kidney and heart was approximately 80-90 % which is in good agreement with the values reported by Avery and Bailey (Avery et al. 1995).

Buffers and reagents:

Hasselbach-Schneider buffer
- 0.6 M KCl
- 0.1 M Na₂HPO₄
- 0.01 M Na₄P₂O₇ x10 H₂O
- 0.001 M MgCl₂ x6 H₂O
→ Adjust pH 7.9. Store at 4 °C.
→ Add dithiothreitol (DTT) to 0.005 M from 1 M stock just prior to use.

2.2.3 Collagen solubilisation

Solubilisation of collagen was performed by pepsin or Liberase DH digestion. The advantage of pepsin digestion is that it yields collagen molecules with intact helices while it does not solubilise all of the collagen. Liberase DH is a commercial collagenase preparation which digests collagen non-specifically and thus yields many small peptides. The advantage of Liberase DH digestion is the almost complete solubilisation of the collagen extracts.

2.2.3.1 Solubilisation by pepsin digestion

Freeze-dried collagen extracts were digested with pepsin/acetic acid at a concentration of 1 mg of collagen per mL of buffer except for LC-MS/MS analysis, for which collagen was digested at a concentration of 2 mg of collagen per ml. Digestion was carried out for 24 h at 4 °C on a vertical rotor. Samples were centrifuged at 13,000g for 30min at 4 °C. The supernatant was carefully collected and the hyp (see page 44) content was determined as a measure of collagen content.
Chapter 2: Materials and methods

Pepsin/acetic acid digestion buffer
- Add 1 mg Pepsin (P6887, Sigma-Aldrich, USA) to 0.5 M acetic acid and rotate or stir at 4 °C until solubilised completely (approx. 15min-30min)
- Prepare fresh immediately before digestion

2.2.3.2 Solubilisation by Liberase DH digestion

Freeze-dried collagen extracts were digested with Liberase DH (Roche Diagnostics, Germany) at a concentration of 1 mg of collagen per mL of buffer for 4 h at 37 °C while rotating slowly. Digests were spun at 13,000g for 30 min at 4 °C. The supernatant was collected on ice and a small aliquot was used to determine the hyp content (see page 44). The hyp content in the pellet was determined in parallel for solubility studies. The remainder of the supernatant was stored at -30 °C for subsequent analysis. No protease inhibitors were added for carbonyl analysis as they interfere with the assay resulting in a massive increase in the background reading. This was found to be the case for the complete mini protease inhibitor (Roche, Germany) in particular, but appears to be the case for other protease inhibitors as well. One possible explanation for this could be the presence of carbonyl groups in the protease inhibitor compounds.

Buffers and reagents:

Liberase DH/TES buffer
- 0.05 M TES
- 0.36 M CaCl₂
→ Adjust pH 7.5. Store at 4 °C.
→ Add Liberase DH immediately prior to digestion. Optimal concentration of Liberase DH was determined to be 0.05 mg/mL (see page 78 for details).

2.3 Measurement of mRNA levels by RT-qPCR

2.3.1 RNA isolation and cDNA synthesis

The process of RNA isolation, detection of RNA quality, and measurement of quantity as well as cDNA synthesis is best carried out in one day without freezing the RNA sample. If this cannot be done RNA may be frozen at -80 °C after the isolation.
Chapter 2: Materials and methods

2.3.1.1 Column based RNA isolation

Both RNA from the kidney (RNeasy Mini Kit, Qiagen, Germany) as well as from the LV of the heart (RNeasy fibrous tissue Mini Kit, Qiagen, Germany) was extracted using kits. The protocol and reagents were the same unless indicated. All kidney samples or heart samples were treated on the same day in order to minimise the variations in extraction.

A piece of tissue stored in RNAlater (15 mg for LV, 10 mg for kidney) was lysed using a steel bead homogeniser with DEPC-treated and autoclaved beads, and ice-cold RLT lysis buffer with β-mercaptoethanol (300 μL for kidney; 600 μL for heart). The homogeniser was operated at 20 Hz for 4 min with rearrangement of the tubes after 2 min. Heart tissue was subsequently treated with 600 μL of proteinase K solution for 10 min at 55 °C and centrifuged at 16,100g for 3 min at RT. Kidney samples were centrifuged without prior proteinase K treatment. The supernatant was collected.

To heart supernatants, 450 μL (0.5 volumes) of 100% ethanol were added while 600 μL of 70% ethanol (1 volume) were added to the supernatant from the kidney. Samples were mixed well and 700 μL was transferred to the RNA binding columns, and spun at 16,100g for 15 s. The flow-through was discarded and the procedure was repeated for the remaining supernatant/ethanol mixture.

Washing buffer RW1 (350 μL) was added to the column, centrifuged for 15 s at 161000g and the flow-through was discarded. DNase in RDD buffer (80 μL) was added onto the column and incubated for 15 min at RT. The column was washed again with 350 μL of RW1 and centrifuged for 15 s at 16,100g.

RPE washing buffer (500 μL) reconstituted with ethanol was added to the column, centrifuged at 16,100g for 15 s and the flow-through was discarded. Columns were centrifuged again at 16,100g for 2 min. Columns were placed into fresh collection tubes and centrifuged for 1 min at 16,100g to completely remove the washing buffer.

Columns were placed into fresh 1.5 mL tubes and 40 μL of RNase-free water was added. Tubes were centrifuged for 1 min at 16,100g and the eluent was loaded onto the column again and centrifuged for another 1 min at 16,100g. The eluted RNA was stored at -80 °C or kept on ice to continue RNA quality measurements and cDNA synthesis.
2.3.1.2 Detection of RNA quality and quantity

RNA was run on a 1% agarose/TBE gel made with DEPC-treated water containing 50 μL/L SYBR Safe gel stain (Life Technologies, USA). RNA was mixed with the loading buffer (1 μL RNA + 4 μL 5 x Loading buffer) and run for 40 min at 100 V. Bands were visualised under UV light and a photograph was taken. RNA quality was determined to be good if the higher running 28S rRNA band was more intense than the 18S rRNA band. It was also checked whether DNA contamination was present, which is visible by an intense staining close to the loading pockets as or a visible band larger than the 28 S rRNA band.

In addition to agarose gel electrophoresis, RNA was analysed by applying 1 μL to the NanoDrop (Thermo Scientific, USA) spectrophotometer. RNA quantity was determined using the absorbance at 260 nm. Protein contamination was determined by looking at the 260/280 ratio. Only samples with a ratio of 2.0 or above were used. Solvent contamination was detected by measuring the 260/230 ratio. Samples were used for cDNA synthesis if the 260/230 ratio was >1.5.

Buffers and reagents:

- **DEPC-treated water**
  - Add 1 mL Diethyl pyrocarbonate (DEPC) to 1 L of water and shake vigorously
  - Leave standing at RT for six hours shaking the bottle repeatedly.
  - Autoclave the solution to remove remaining DEPC.

- **RNase-free TBE buffer**
  - 0.1 M Trisbase
  - 0.09 M boric acid
  - 1 mM EDTA
  - Make up to 1 L with DEPC-treated water.

2.3.1.3 Synthesis and quantification of cDNA

Synthesis of cDNA was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Sciences, Germany). In most cases, batches of cDNA were prepared using up to 4x of the described single set of reaction per sample as cDNA is more stable than RNA and can thus be stored better. OligoDT (oDT) primer was used as they yielded much more reproducible results than the random hexamer primer which was also supplied with the kit. RNA was adjusted to 0.5 μg/11 μL on ice. A master mix of
Chapter 2: Materials and methods

4.0 μL 5 x Transcriptor reverse transcriptase reaction buffer
1.0 μL water
1.0 μL oDT primer
0.5 μL RNase inhibitor,
2.0 μL Deoxynucleotide mix
0.5 μL Transcriptor reverse transcriptase

was pipetted per reaction to a PCR tube containing 0.5 μg RNA/11 μL on ice. The reverse transcription reaction was left to proceed for 1 h at 50 °C followed by an inactivation step for 5 min at 85 °C.

The cDNA was diluted in water at a dilution factor of 1:2. Concentration of cDNA was measured using the Quant-It Oligreen ssDNA Kit (Life Technologies, USA). A six point ½-dilution standard curve ranging from 0 ng/mL – 500 ng/mL (final concentration 0 ng/mL – 250 ng/mL) was pipetted and 100 μL was added to a black 96-well plate in duplicate. The diluted cDNA was added to the plate in duplicate with 1 μL added/well. TE buffer, supplied with the kit was added to the cDNA containing wells to 100 μL. Oligreen reagent/TE buffer was added at a volume of 100 μL/well. Fluorescence was measured after 2 min of incubation at an excitation wavelength of 480 nm and an emission wavelength of 520 nm, with a 515 nm cut-off filter (Spectra Max Gemini XS, Molecular Devices, USA). Equal concentrations of cDNA, between 5 ng/μL and 20 ng/μL depending on the set of cDNA, were adjusted.

2.3.2 Measurement of relative mRNA levels by RT-qPCR

2.3.2.1 Primer design

Primers were designed using the primer-blast programme (NCBI). The salt correction formula by Schildkraut and Lifson and the table of thermodynamic parameters by Breslauer et al. were used (Schildkraut 1965; Breslauer et al. 1986). Primer pairs were designed to be intron-spanning in order to allow for the selective amplification of mRNA. Primers were ordered from Life Technologies (USA) and Integrated DNA Technologies (USA). Primer sequences can be found in the appendix on page 174.

2.3.2.2 Reference genes

Relative levels of mRNA of target genes were to be measured by reverse transcription quantitative real-time PCR (RT-qPCR). The choice of stably-expressed reference genes for the normalisation of the desired target gene is important. In particular reference genes need
to be stably expressed in the tissue of choice and expression levels should not be affected by either the disease state or the treatment. Three appropriate reference genes, each for the kidney and the heart of sham or TETA-treated healthy or diabetic animals were previously determined by Hogl and were used in the current study (Hogl 2009).

Reference genes for kidney:
- Ribosomal protein L13a (\textit{Rpl13a})
- Tata box binding protein (\textit{Tbp})
- U2 auxiliary factor 35kDa subunit (\textit{U2af})

Reference genes for heart:
- Ribosomal protein L13a (\textit{Rpl13a})
- Tata box binding protein (\textit{Tbp})
- Nucleoporin 1 (\textit{Ndc1})

2.3.2.3 RT-qPCR

Primers were reconstituted with water to yield a 100 $\mu$M stock solution. They were then diluted with water to a 10 $\mu$M working solution. The primer sets for the target genes as well as the reference genes to be analysed, were run on a standard curve of pooled cDNA to determine the suitable amount of cDNA to be used for analysis. Results can only be used if the calculated crossing point (cp) value is reached before the 32\textsuperscript{nd} cycle of the PCR. Cp values higher than that indicate that the copy number of the cDNA for the gene is too low or that the amplification efficiency is much lower than 2, both of which leads to unreliable results. The amounts of cDNA used per well ranged from 0.5 ng – 20.0 ng depending on the gene analysed.

Three genes per 384-well plate were analysed. A total of 36 samples were analysed per gene with 9 samples analysed for each of the four groups. Each sample was analysed in triplicate. Also, a standard curve using pooled cDNA was run for each gene on the same plate to determine the amplification efficiency for the gene. The three reference genes were always run on the first plate and target genes run on subsequent plates were normalised to the appropriate reference gene values.

The appropriate amount of cDNA, as determined previously, for an equivalent of 3.3 x runs was added to microtubes and adjusted with water to 19.8 $\mu$L. A six point $\frac{1}{2}$-dilution standard curve using pooled cDNA from all the samples was also pipetted for each gene with a total volume of 19.8 $\mu$L per concentration.
A master mix was pipetted for each of the three genes which contained the following amount of reagents per reaction:

- 7.5 μL SYBR Green I Master (Roche Applied Sciences, Germany)
- 0.5 μL Fwd primer (at 10 μM)
- 0.5 μL Rev primer (at 10 μM)
- 0.5 μL water

A total of 29.7 μL, equivalent to 3.3 reactions, of master mix was added to each standard curve and sample tube. In addition, one water negative control was run for each primer set. Tubes were spun quickly and 20 μL of cDNA/master mix was loaded per well. Each sample and standard curve concentration was loaded in triplicate. The plate was sealed with a plastic lid. The qPCR was performed on a Light Cycler 480 instrument (Roche Applied Sciences, Germany) with the Software version 1.5.0 SP1 using the setting shown below (Table 2-1).

<table>
<thead>
<tr>
<th>Programme</th>
<th># of Cycles</th>
<th>Temp [ °C]</th>
<th>Time</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Pre-Incubation</td>
<td>1</td>
<td>95</td>
<td>5 min</td>
<td>Activation of polymerase and DNA denaturing</td>
</tr>
<tr>
<td>2 Amplification</td>
<td>45</td>
<td>95</td>
<td>10 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>15 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>72</td>
<td>20 s</td>
<td>Fluorescence acquisition</td>
</tr>
<tr>
<td>3 Melting</td>
<td>1</td>
<td>95</td>
<td>5 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>65</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>65-97</td>
<td>Varies</td>
<td>Continuous fl. acquisition</td>
</tr>
<tr>
<td>4 Cooling</td>
<td>1</td>
<td>37</td>
<td>∞</td>
<td></td>
</tr>
</tbody>
</table>

Total time ~1h 15 min

The melting curve of the samples was scanned for unspecific melting peaks which could be due to off-target PCR products or primer dimers. Such primer pairs were not used for analysis. Samples were analysed employing the advanced relative quantification method. The crossing point (Cp) value was calculated using the Light Cycler 480 software. It is defined as the point of the 2nd derivative maximum of a curve fitted through the fluorescence values of the amplification cycles of each sample (see Figure 2-2 for a detailed description).
The concentration of reference- or target cDNA in each sample was calculated using the efficiency value, determined via the standard curve of pooled cDNA. The concentration of cDNA of each target was normalised to the geometric mean of the reference gene sample (see Figure 2-3). This average relative value for each sample was then normalised to the arithmetic mean of the Sham/Placebo treated group.

\[
\text{conc. target} = \frac{\text{conc. target}}{\text{geom. mean (conc. reference)}} \quad \text{Sample}
\]

\[
\text{conc. target} = \frac{1}{9} \sum_{i=1}^{9} \frac{\text{conc. target}}{\text{geom. mean (conc. reference)}} \quad \text{Sham/Plac}
\]

Roche LC 480 Software
Version 1.5.0 SP1

Microsoft Excel

Figure 2-2: Representative amplification curve of an RT-qPCR measurement
Shown is the amplification curve of a single Col4a1 RT-qPCR measurement with a calculated Cp=26.01. The cycle number is shown on the x-axis and the fluorescence intensity on the y-axis. The second derivative maximum is reached for cycle x=26.01 indicated by the dashed line. While the first derivative maximum gives the point of the curve where the slope is the steepest (approximately x=30.0), the second derivative maximum is the point of the curve where the slope increases the most.

The concentration of reference- or target cDNA in each sample was calculated using the efficiency value, determined via the standard curve of pooled cDNA. The concentration of cDNA of each target was normalised to the geometric mean of the reference gene sample (see Figure 2-3). This average relative value for each sample was then normalised to the arithmetic mean of the Sham/Placebo treated group.

Figure 2-3: Normalisation procedure for RT-qPCR
Shown is a summary of the normalisation procedure, including the software used for the advanced relative quantification analysis of the target genes.
2.4 Protein analysis

2.4.1 Determination of protein concentration by BCA assay

Protein concentration of lysates was determined using a bicinchoninic acid (BCA) based assay kit (Thermo Scientific/Pierce, USA). This assay does not detect or grossly underestimates the collagen content of a sample so that purified collagen samples were always analysed using the hyp method described previously.

The BCA assay reagents A and B were diluted at a factor of 1:50. A 7 point 1:2 dilution standard curve of BSA was prepared with the highest concentration being 2 mg/mL. Samples were diluted 1:20 in water and 25 μL of samples and the standard curve were loaded to a clear 96-well plate in duplicates. The diluted BCA assay reagent (200 μL) was added to the plate using an 8-channel pipette. The plate was incubated at 37 °C for approximately 30 min. Absorbance was read at 562 nm and the protein concentration of the samples was calculated based on the standard curve.

2.4.2 Western Blotting

2.4.2.1 Tissue lysates for SDS-PAGE followed by Immunoblotting

Kidney or heart tissue that had been stored at -80 °C was kept on ice. Pieces of approximately 10 mg were excised and added to 2 mL microtubes containing a steal bead on ice, and 200 μL ice-cold RIPA buffer with protease inhibitor. Samples were lysed using a steel bead homogeniser for 6 min at 25 Hz, rearranging the samples after the 3 min. Samples were freeze-thawed once using liquid N₂ and then incubated for 2 h at 4 °C with shaking. Insoluble tissue was pelleted at 13000 g for 30 min at 4 °C and. The supernatant was collected and frozen at -80 °C.

Buffers and reagents:

RIPA (Radioimmunoprecipitation assay) buffer
- 150 mM sodium chloride
- 50 mM Tris
→ Adjust pH 8.0
Chapter 2: Materials and methods

- 1.0% Triton X-100 (v/v)
- 0.5% sodium deoxycholate (w/v)
- 0.1% SDS (w/v)

→ Store at 4 °C and add protease inhibitor complete mini (Roche Applied Sciences, Germany) immediately before lysis of the tissue

2.4.2.2 SDS-PAGE

Protein concentration of tissue lysates was determined by BCA assay as described previously (see page 54). Collagen extracts were solubilised using the pepsin/acetic acid based method. The concentration of collagen in the supernatant was determined by hyp assay as the BCA assay does not detect collagen reliably. Equal protein concentrations or collagen concentrations were adjusted and samples were mixed with 4 x concentrated Laemmli buffer and 10 x concentrated DTT. Samples were heated to 70 °C for 10 min and run on pre-cast 4-12% Bis-Tris gels (Life Technologies, USA) for 1-1.5 h at 100 V in MES buffer until the desired separation was achieved. A marker was run for molecular mass estimation of the proteins. Markers used were Precision Plus Protein Dual Color Standards (BioRad, USA) or HiMark Pre-Stained HMW (Invitrogen, USA). Collagen samples for LC-MS/MS analysis were stained with colloidal Coomassie. Tissue lysate samples were immunoblotted for relative quantification of protein levels.

Buffers and reagents:

Laemmli buffer 4 x conc
- 0.312 M Tris
- 50% Glycerol (v/v)
- 0.01% bromophenol blue and adjust pH 6.8 with HCl

→ Adjust pH 6.8 and add 10% SDS (w/v) afterwards.

DTT 10x conc
- 1 M DTT in water

MES buffer 10 x conc
- 0.5 M MES
- 0.5 M Tris
- 10 mM EDTA

→ Adjust pH 7.2 and add 1% SDS (w/v) afterwards.
Chapter 2: Materials and methods

2.4.2.3 Blotting

Proteins were separated by SDS-PAGE. The gel was then placed onto a pre-wetted 0.45 μm nitrocellulose membrane (BioRad, USA) in a tray containing transfer buffer sandwiched between filter paper and the transfer cassette. Transfer of the proteins was carried out using a wet transfer tank by Amersham Bioscience (Hoefer TE22, Amersham Bioscience, USA) 100 V for 1 h at RT with the gel and the membrane being fully submerged in transfer buffer.

Buffers and reagents:

Transfer buffer 10 x conc
- 30.1 g Tris
- 144 g glycine
→ To make 1 x TB add 10% 10 x TB, 20% methanol and fill up with water.

2.4.2.4 Immunodetection

The membrane was blocked with blocking buffer for 2 h at RT and incubated with the primary antibody, diluted in fresh blocking buffer overnight at 4 °C on a shaker. The blot was washed for 3 x 10 min with TTBS and incubated with a horseradish peroxidase-coupled secondary antibody was added in 5% milk powder in TTBS. The blot was incubated for 2 h at RT followed by a for 3 x 10 min wash with TTBS. TTBS was discarded and the blot was layered between two foils to remove the remaining TTBS. The top foil was removed and enhanced chemiluminescence reagent (ECL Prime, GE healthcare, UK) was added onto the blot and left to incubate for 5 min at RT. The reagent was removed by reapplying the top foil to the blot and chemiluminescence signal was detected using a CCD camera based digital imaging system (LAS 4000, Fujifilm, Japan). Band intensity was measured using the software MultiGauge (Fujifilm, Japan). Band intensity was normalised to the calibrator sample that was run on each blot. Intensity was then normalised for the corresponding normaliser band. Normalisers used were beta actin for kidney and GAPDH for heart samples. Finally the band intensity of each sample was normalised for the average intensity value of the Sham/Plac samples.

Buffers and reagents:

TBS (Tris buffered saline) buffer 10 x conc
- 50 mM Tris
- 1.5 M NaCl
→ Adjust pH to 7.6.
Chapter 2: Materials and methods

**TTBS**
- To make 1 x TTBS (Tween-20 containing Tris-buffered saline) dilute 10 X TBS at a factor of 1:10 with water and add Tween-20 to 0.05% (v/v).

**Blocking buffer**
- 5% skim milk powder (w/v) in TTBS

### Table 2-2: List of antibodies used for Western Blotting

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution factor</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary</strong></td>
<td>Kidney Heart</td>
</tr>
<tr>
<td>Anti-Lysyl oxidase (E19), sc-32410 (Santa Cruz, USA), goat polyclonal</td>
<td>1/4000 -</td>
</tr>
<tr>
<td>Anti-Cathepsin B, ab 33538 (abcam, UK), rabbit polyclonal</td>
<td>1/1000 1/1000</td>
</tr>
<tr>
<td>Anti-Cathepsin L, ab 6314 (abcam, UK), mouse monoclonal</td>
<td>1/2000 1/2000</td>
</tr>
<tr>
<td>Anti-Glutathione reductase, ab 16801 (abcam, UK), rabbit polyclonal</td>
<td>1/4000 1/3000</td>
</tr>
<tr>
<td>Anti-beta Actin (C4), sc-47778 (Santa Cruz, USA), mouse monoclonal</td>
<td>1/2000 -</td>
</tr>
<tr>
<td>Anti-Gapdh, ab 9485 (abcam, UK), rabbit polyclonal</td>
<td>- 1/3000</td>
</tr>
<tr>
<td>Anti-Catalase, ab 1877 (abcam, UK), rabbit polyclonal</td>
<td>- 1/4000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Secondary (all HRP coupled IgG antibodies)</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti rabbit, sc-2004 (Santa Cruz, USA)</td>
<td>1/10000</td>
</tr>
<tr>
<td>Goat anti mouse, sc-2005 (Santa Cruz, USA)</td>
<td>1/10000</td>
</tr>
<tr>
<td>Donkey anti goat, sc-2020 (Santa Cruz, USA)</td>
<td>1/10000</td>
</tr>
</tbody>
</table>

### 2.4.3 Staining of SDS-PAGE and mPAGE gels with colloidal Coomassie

Collagen extracts separated on the SDS page gel were visualised by Coomassie staining and bands were excised for LC-MS/MS analysis. Gels based on mPAGE (see page 66 ff.) were also stained with colloidal Coomassie. The Coomassie staining method by Neuhoff *et al.* was adapted (Neuhoff *et al.* 1988). The gel was fixed for around 20 min – 1 h with 1 change of fixing solution. The gel was quickly rinsed with water and the freshly prepared staining solution was added; the gels were incubated in the solution overnight on a shaker at RT.

The gel was de-stained for several hours on a shaker while addition of a tissue removes excessive dye. It is important not to de-stain gels containing collagen for too long as the affinity of the dye for collagen is rather weak and excessive de-staining may remove the dye completely.

**Buffers and reagents:**
Chapter 2: Materials and methods

Fixation solution
- 40% methanol
- 10% acetic acid

Stock solution A
- 2% (w/v) phosphoric acid
- 10% (w/v) ammonium sulphate

Stock solution B
- 5% of CBB G250 in water

Staining solution
- Dilute stock solution B 1:50 with diluent solution A
  ➔ Add methanol to a final concentration of 20%

De-staining solution
- 5% acetic acid in water

2.4.4 LC-MS/MS based identification of proteins

Collagen was digested with pepsin/acetic acid as described on page 46 and the soluble collagen present in the supernatant was mixed with 4 x concentrated Laemmli buffer and 10x concentrated DTT, heated to 70 °C for 10 min and run on a 3-8% NuPAGE Tris-Acetate gradient gel (Life Technologies, USA) for 1.5 h at 150 V. Gels were stained with colloidal Coomassie blue as was described on page 57. Care was taken not to contaminate the gel with dust or other particles that can interfere with subsequent LC-MS/MS analysis. Thus solutions were sterile-filtered wherever possible (the final staining solution including the Coomassie stain and stock solution B, cannot be sterile-filtered but the other stock solutions can be). Bands of interest were excised using a fresh scalpel blade.

The subsequent sample preparation and LC-MS/MS analysis was carried out by Martin Middleditch from the Proteomics Facility of the University of Auckland. Gel bands were washed with 50 mM NH₄HCO₃ and cut into small pieces (1 x 1 mm). The Coomassie dye bound to the protein was removed by washing the gel pieces in a 1:2 dilution of acetonitrile in 50 mM NH₄HCO₃ and shrunk with pure acetonitrile. Gel pieces were then dehydrated completely by incubating them in a heat block at 56 °C. The gel pieces were then rehydrated in 10 mM DTT and incubated for 30 min in a CEM Discover temperature controlled microwave (CEM Corporation, USA) using 50W of power at a maximum temperature of 56
Chapter 2: Materials and methods

°C. The supernatant was removed and the gel pieces were then incubated with 50 mM iodoacetamide (GE Healthcare, UK) in 50 mM NH₄HCO₃ at room temperature in the dark for another 1 h. The supernatant was removed and the gel pieces then dehydrated with pure acetonitrile, dried in a heat block, then rehydrated and covered with 12.5 ng/μl trypsin solution (Promega, USA) in 50 mM NH₄HCO₃. Samples were incubated for 30 min in the temperature controlled microwave using 50 W of power at a maximum temperature of 40 °C to allow for a speedy but controlled digestion. Digests were acidified to pH 3 by the addition of 10% formic acid (Scharlau, Spain).

For LC-MS/MS analysis, samples were injected onto a C18 trap cartridge (LC Packings, Netherlands) for desalting prior to chromatographic separation on a capillary-scale Zorbax 300SB C18 Stablebond column (Agilent Technologies, USA). Column eluates were ionized in the electrospray source of a QSTAR-XL Quadrupole Time-of-Flight mass spectrometer (Applied Biosystems, USA). A TOF-MS scan from 300-1600 m/z was performed, followed by three rounds of MS/MS on the three most intense multiply-charged precursors in each cycle. The resulting data was searched against the Mammalia or Rattus subsets of NCBI’s non redundant protein sequence database (NCBI, USA) using Mascot software (Matrix Science, London, UK).

The following search parameters were used:

- Type of search: MS/MS ion search
- Enzyme: Trypsin
- Fixed modifications: Carbamidomethyl (C)
- Variable modifications: Deamidated (NQ), oxidation (P), oxidation (M), oxidation (K);
- Peptide mass tolerance: ± 0.15 Da
- Fragment mass tolerance: ± 0.15 Da;
- Maximum number of missed cleavages: 3
- Instrument type: ESI-QUAD-TOF

Buffers and reagents:

- Laemmli buffer 4 x conc
  - 0.312 M Tris
  - 50% Glycerol (v/v)
  - 0.01% bromophenol blue and adjust pH 6.8 with HCl
  - Adjust pH 6.8 and add 10% SDS (w/v) afterwards.
DTT 10x conc
- 1 M DTT in water

Tris-Acetate SDS Running Buffer (10X)
- 0.5 M Tricine
- 0.5 M Tris base
→ Adjust pH 8.24
- add 1% SDS (w/v)

2.4.5 Detection of protease activity by gelatine zymography

The activity of gelatinases in the kidney cortex was detected using commercially available 10% SDS-PAGE gels containing 0.1% of gelatine as an enzyme substrate (EC 61752, Life Technologies, USA). Kidney cortex was lysed using ice-cold lysis buffer at a concentration of 50 mg/ml in 2 ml round bottom microtubes in a steel bead beater at 25 Hz for 5 min rearranging the tubes after 2.5 min (TissueLyser, Qiagen, Germany). Lysates were spun for 5 min at 9,500 g at 4 °C. The supernatant was collected on ice and the protein concentration was determined using a BCA-based assay as was described on page 54. Equal protein concentrations were adjusted and loading buffer was added on ice. Equal amounts of protein (approximately 40 μg) were loaded onto the gel. A molecular mass marker (Precision Plus Dual Color, BioRad, USA) was run alongside the samples and one calibrator sample was run on all gels. As it was found that the marker affects the activity of the sample in the adjacent well, most likely due to diffusion of reducing agent, this well was not used for analysis. Gels were run for 90 min-120 min at 125 V and then incubated in renaturation buffer (LC2670, Life Technologies, USA) for 30 min at RT followed by incubation in developing buffer (LC2671, Life Technologies, USA) for 30 min at RT on a shaker. The final developing/gelatine degrading step was carried out for approx. 72 h at 37 °C in developing buffer. In order to classify the proteases involved, all incubations were carried out in parallel with buffers containing 10 Mm EDTA in preliminary experiments. As it was found that this inhibits protease activity completely for all bands in all groups, this was not done for subsequent experiments thereafter. Gels were stained using the colloidal Coomassie method as described on page 57 and bands of protease activity appeared clear on a blue background. Gels were scanned and the inverse colour image was analysed by densitometry using the software MultiGauge (Fujifilm, Japan). Intensity of the bands on each gel was normalised for the calibrator sample on each gel followed by normalisation for the average value of the Sham/Placebo group.
Buffers and reagents:

Lysis buffer
- 0.25% Triton X-100
- 10 mM CaCl₂

2.5 x Loading buffer
- 160 mM Tris-Cl (157.60 g/mol), pH 6.8
- 5% SDS (w/v)
- 0.015% bromophenol blue (w/v)
- 25% glycerol (v/v)

0.5 M EDTA solution
- add EDTA to 0.5 M and adjust pH 8.0 in order for the EDTA to dissolve completely
→ 0.5 M EDTA solution was added to commercial renaturation and developing buffer at a dilution factor of 1:50 in order to yield buffer with 10 mM EDTA

General Tris/Glycine Running buffer (10X)
- 0.25 M Tris
- 1.92 M glycine
→ Check that pH is around 8.3
- 1.0% SDS (w/v)

2.4.6 Cathepsin L activity assay

The cathepsin L activity assay was run using a peptide substrate, Ac-HRYR-ACC (Cat # 219497, Merck Millipore, Germany), coupled to a probe, ACC (7-amino-4-carbamoylmethylcoumarin), which fluoresces upon cleavage of the substrate. The substrate was first described by Choe et al. and was shown to be cleaved by CTSL with a 270-fold and 2500-fold higher activity over CTSB and CTSK, respectively (Choe et al. 2006). This is an improvement over the previously employed substrate Z-FR-AMC with the fluorescent leaving group 7-amino-4-methylcoumarin (AMC) for which CTSL had only an 18-fold and 77-fold higher activity over CTSB and K, respectively.

The assay was carried out as described by Choe et al. with some modifications. Different concentrations of substrate and different amounts of lysate were tested for the suitability of the assay initially. The optimal substrate concentration used for the final assay was 5 μM which is the same concentration previously used by Barrett et al. to determine CTSL activity.
in tissue lysates employing a different substrate (Barrett et al. 1981). Approximately 10 mg of tissue was used and homogenised in 250 μL of ice-cold lysis buffer in 2 mL microtubes using a steel bead TissueLyser (Qiagen, Germany). It was operated for 2 x 3 min at 25 Hz, repositioning the tubes in between. Tubes were centrifuged for 15 min at 16100g at 4 °C. The supernatant was collected on ice and 10 μL of the supernatant were loaded to a black 96-well plate in triplicate on ice. The activity assay buffer, pre-warmed to 30 °C, was mixed with the 1 mM fluorogenic substrate stock at a dilution factor of 1:190 to yield a final substrate assay concentration of 5 μM in the well. An AMC fluorescence standard curve (200 μl of 0.1 μM to 50.0 μM final concentration) was loaded per well containing 10 μl of lysis buffer. The substrate/activity assay buffer solution (200 μl) was loaded to the wells containing the lysates. A negative control was run in parallel with the substrate/activity assay buffer added to lysis buffer-containing wells without tissue. Measurements were carried out in a fluorescent plate reader at 30 °C every 5 min for 30 min at an excitation wavelength of 380 nm and an emission wavelength of 460 nm with a 435 nm cut-off filter initially. As the increase in fluorescence was not linear over 30 min, only values from the first 10 min were used for analysis while the values obtained at 0 min were subtracted. Protein concentration was determined using the BCA assay as described (see page 54). Samples were diluted at a factor of 1:20 for the protein assay to yield a reasonable protein concentration and a low DTT concentration as DTT can interfere with the assay.

The amount of cleaved substrate was calculated from the AMC standard curve and was corrected for the 2.8-fold increased fluorescence intensity of ACC at the measured wavelength (Harris et al. 2000). Data was normalised for the protein content and the duration of the assay (10 min) and was given as [nm/min*mg].

For the relative activity assay CTSL activity in kidney lysates was measured as described above with the exception that 25 μM CuCl₂, 25μM TETA or 25 μM CuCl₂ and 25 μM TETA (all final concentration) in lysis buffer was added to the assay. The activity was then measured for each combination of the sample and was given relative to the activity of the sample with no additive.

Buffers and reagents:

Lysis buffer
30 mM Tris-HCl
→ Adjust pH 7.2 and add 0.1% Brij35 (v/v).
→ Add DTT to 0.1 mM from 1 M stock at -30 °C prior to usage
Chapter 2: Materials and methods

Activity assay buffer
100 mM Sodium acetate
→ Adjust pH 5.5.
100 mM NaCl
1 mM EDTA
0.01% Brij-35 (v/v)
→ Add DTT to 10 mM from 1 M stock at -30 °C prior to usage.

Substrate Ac-HRYR-ACC (Cat # 219497, Merck Millipore, Germany)
Dissolve in DMSO to yield a concentration of 1 mM and add to activity assay buffer at a dilution factor of 1:200 to yield a final substrate concentration of 5 µM

Standard 7-Amino-4-methylcoumarin (Cat # A9891, Sigma, USA)
Dissolve in DMSO to yield a concentration of 10 mM

2.5 Analysis of collagen extracts for post-translational modifications

2.5.1 Synthesis of artificially glycated BSA as a standard

BSA for the standard curve was incubated with sterile-filtered PBS (BSA-PBS), 0.5 M fructose/PBS (BSA-Frc) or 0.5 M glucose/PBS (BSA-Glc) for 8 weeks at 37 °C at a concentration of 6 mg/mL. Na azide was added to 0.015% (w/v) to prevent bacterial growth. After 8 weeks BSA was dialysed against water for 48 h at 4 °C with several changes of water using a dialysis membrane with a MW cut off of 3500. BSA was aliquoted and stored at -30 °C. Protein concentration of BSA-PBS and BSA-Frc and BSA-Glc was determined by BCA assay as was described previously (see page 54).

Buffers and reagents:

PBS
- 137 mM NaCl
- 2.7 mM KCl
- 10 mM Na$_2$HPO$_4$
- 2.0 mM KH$_2$PO$_4$
→ Adjust pH 7.4
2.5.2 Detection of CML levels via dot blot

The AGE product CML was quantified in collagen extracts from kidneys using an antibody-based dot blot method. Collagen signals for CML were normalised using a standard curve of *in vitro* glycated BSA (see previous section) run on each dot blot. As AGEs are generated more quickly upon incubation with fructose compared to glucose, BSA-Frc was used as a standard rather than BSA-Glc as it already yielded high amounts of CML after 8 weeks of incubation (McPherson *et al.* 1988).

Collagen extracts were solubilised by Liberase DH digestion and hyp concentration of the supernatant was measured as an estimate of collagen content as described previously (see page 44 ff.). A dry nitrocellulose membrane of the size 6 cm x 7 cm and a 0.45 μm pore size (BioRad, USA) was separated into squares of 1 cm edge length using a soft pencil. Collagen concentrations were adjusted to 3 μg/6 μL using Liberase DH-containing digestion buffer to correct for potential interferences of Liberase DH with the assay. Collagen samples, the sample standards and the negative controls were all loaded in a total volume of 6 μL in triplicates. The BSA-AGE standard curve ranged from 0.08 μg – 1.25 μg. Negative controls were BSA-PBS (1.25 μg) and Liberase DH control digests. Only 2 μL was added per square at a time and the membrane was left to dry before repeating the procedure twice.

After adding all the samples the membrane was left to dry completely and was then blocked for 2 h with 5% skim milk powder (MP)/TTBS at RT. The blocked blot was then incubated overnight with a monoclonal goat anti-CML antibody (KAL-KH024 clone no. NF-1G, Cosmo Bio, Japan) diluted 1/1000 in 5% MP/TTBS. The antibody was raised against CML modified human serum albumin (CML-HSA). The blot was washed with TTBS for 6 x 10 min and incubated with the HRP coupled secondary donkey anti goat antibody (sc-2033, Santa Cruz, USA) diluted 1/10000 in 5% MP/TTBS for 2 h at room temperature. The blot was washed again for 6 x 10 min, TTBS removed and enhanced chemiluminescence reagent (ECL Prime, GE healthcare, UK) was added for 5 min. Reagent was removed and signal intensity was captured using a digital imaging system (Las 4000 Fujifilm, Japan).

The signal intensity for CML was normalised to the intensity of the BSA-AGE standard curve and to the amount of collagen added which was the same (3 μg) for all samples as mentioned above. As the BSA-AGE standard curve did not give absolute levels of CML, but rather CML levels on collagen relative to CML levels of known amounts of BSA-AGE the values were finally normalised to the average intensity values of the Sham/Plac group.

Buffers and reagents:
For TTBS and MP/TTBS blocking buffer recipes see the immunodetection section on page 56.

2.5.3 Measurement of carbonyl levels

Collagen carbonyl levels were determined using an ELISA kit (BioCell Corp, New Zealand) based on a method reported by Buss et al. (Buss et al. 1997). Protein carboxyls are reacted with 2,4-dinitrophenylhydrazine (DNPH) to form a stable dinitrophenylhydrazone (DNP) product which absorbs at 370 nm (Dalle-Donne et al. 2003). The resulting product DNP may be detected by spectrophotometry. The disadvantages of the spectrophotometric method are the relatively low sensitivity and the need for large amounts of proteins. For these reasons DNP was detected using the ELISA procedure which is described in detail below.

Collagen extracts were solubilised with Liberase DH as described on page 47 and the collagen content in the supernatant was determined by hyp measurement described on page 44. The carbonyl standards and the control sample were resuspended in 25 μL of water vortexed, quickly centrifuged and left to stand at RT overnight, vortexed and centrifuged again and then diluted 1:100 in EIA buffer. Solubilised collagen was adjusted to a concentration of 0.4 mg/mL using Liberase DH containing digestion buffer. A total of 50 μL of each sample, standard, control sample and one Liberase DH empty digest sample (equivalent to 20 μg of collagen per sample) were derivatised with 200 μL of diluted DNPH solution for 45 min at RT. A portion of the derivatives (50 μL) was mixed with 1 mL of EIA buffer. The diluted derivatives were added to the ELISA plate in triplicate with 200 μL added per well. The plate was sealed and incubated at 4 °C on a plate shaker overnight. All buffers and reagents were added to the ELISA plate using an 8-channel multi-pipettor in the following steps. The plate was washed 5 times with 300 μL EIA buffer added per well each time, discarding the buffer by inverting the plate each time. All subsequent washing steps were carried out the same way. After the last washing step, 250 μL of blocking solution was added per well. The plate was sealed and incubated on a shaker for 30 min at RT. The plate was washed again and 200 μL of the anti-DNP-biotin-antibody diluted in blocking solution was added per well. The sealed plate was incubated for 1 h at 37 °C. The plate was washed again and 200 μL of the streptavidin coupled HRP diluted in blocking solution was added per well. The sealed plate was incubated for 1 h at RT. After washing the plate, 200 μL of chromatin reagent were added per well. Colour development was followed at 650 nm and stopped after approximately 10-20 min when the highest standard reached an absorbance value of around 0.7. The reaction was stopped by addition of 100 μL of stopping reagent which causes the colour to change from blue to yellow and the absorbance was measured at 450 nm.
Chapter 2: Materials and methods

The carbonyl levels in the samples were determined based on the standard curve. The amount of carbonyl was normalised for the amount of collagen added to each well and is given as [nmol/mg].

Buffers and reagents:

All buffers and reagents including the ELISA plate were part of the “Biocell Protein Carbonyl Assay Kit” (Biocell Corp, New Zealand).

2.5.4 Measurement of the digestibility of collagen

The pepsin digestibility of collagen extracts was measured as an indicator of post-translational modifications (Nagaraj et al. 1996). Collagen extracts were digested with pepsin. The digests were centrifuged and the hyp content in the supernatant and the pellet was determined. The individual procedures have been described previously in detail on page 44 ff. The solubility was calculated and given in [%].

2.5.5 Mpba based gel electrophoresis for the analysis of glycated proteins

Methacrylamido-phenylboronic acid gel electrophoresis (mPAGE) was described by Morais et al. as a tool for the detection of glycated proteins (Pereira Morais et al. 2010). Such proteins displayed an altered retardation compared to unmodified proteins.

2.5.5.1 Synthesis of 3-methacrylamido phenylboronic acid

Methacrylamido-phenylboronic acid (mpba) was synthesised under supervision of Tom Woods according to the method described by D’Hooge et al. (D’Hooge et al. 2008). Starting material 3-aminophenylboronic acid monohydrate ($M_{(3-apba)}=154.96$ g/mol) was reacted with 1 eq. of pinacol ($M_{(pinacol)}=118.17$ g/mol) in chloroform at a concentration of 0.2 M overnight at RT while stirring (see Figure 2-4). Insoluble material was removed by filtration. Water that had formed during the reaction was removed by addition of magnesium sulphate, and the hydrated solid was removed by another filtration step. Product formation was checked by TLC employing a silica plate as the polar stationary phase and 25% ethyl acetate/hexane as a solvent (non-polar mobile phase). Under UV light it could be seen that the polar starting material (3-apba) did not move with the non-polar solvent. In contrast almost the entire product moved up around a quarter of the plate which confirmed that the more polar 3-aminophenylboronic acid ester ($M_{(3-apbe)}=219.09$ g/mol) was formed. Only small amounts
remained stationary in the product lane. This was confirmed by ninhydrin reaction (reacts with primary amines to form a purple product) of the applied materials. The chloroform was removed by rotary evaporation under vacuum, followed by another stronger vacuum step to remove any potentially remaining water which would have been detrimental to the subsequent steps. The resulting solid formed nice crystals which again suggested a pure product of 3-apbe and was kept under argon.

Next, methacryloyl chloride was synthesised which will then be reacted with 3-apbe. Methacrylic acid (1.1 eq., $M_{\text{methacrylic acid}}=86.1 \text{ g/mol}$) was added to dichloromethane under argon (as water in the air reacts with the product) at a concentration of 1 M, and was reacted with 1.1 eq. oxalyl chloride ($M_{\text{oxalyl chloride}}=126.99 \text{ g/mol}$, see Figure 2-5). The solution was kept under argon while stirring constantly and 0.25% (v/v) dimethylformamide (DMF) was added as a catalyst to start the reaction. The reaction was left to proceed for 2 h at RT when no gas formation was observed anymore. Solvents were not removed on a rotary evaporator as the resulting product methacryloyl chloride is volatile.

The two products 3-apbe ($M_{\text{3-apbe}}=219.09 \text{ g/mol}$) and methacryloyl chloride ($M_{\text{methacryloyl chloride}}=104.54 \text{ g/mol}$) were reacted with each other to form methacrylamido-phenylboronic ester (mpbe, see Figure 2-6). One eq. of 3-apbe was dissolved in THF, 1.3 eq. of triethylamine was added and the mixture was kept stirring under an atmosphere of argon on

Figure 2-4: 3-mpba synthesis reaction 1
Shown is the first reaction of 3-amino phenylboronic acid with pinacol to form 3-amino phenylboronic acid ester.

Figure 2-5: 3-mpba synthesis reaction 2
The reaction of methacrylic acid with oxalyl chloride to form methacryloyl chloride is shown. The product methacryloyl chloride is then reacted with 3-apbe in the next reaction.
ice. Methacryloyl chloride (1.1 eq.) in dichloromethane/DMF from the previous reaction was added dropwise to the mixture stirring on ice. The reaction was left to proceed for 20 min on ice followed by 1 h at RT. Formation of the product was checked by TLC (silica plates, Kieselgel F254 0.2 mm, Merck, Germany) using 1% methanol/dichloromethane (medium to strong eluent) as a solvent. Plates were checked under UV light (254 nm) and stained with KMnO₄. KMnO₄ stains for alkenes and thus the desired product mpbe but not the starting material 3-apbe. Only small amounts of 3-apbe were left and most of the starting materials had reacted to form mpbe. An additional TLC was run with weaker eluents as the retardation factor, Rᵣ, of the eluent 1% methanol/dichloromethane (Rᵣ=0.8; meaning mpbe ran at 80% of the solvent front) was too high for column chromatography where a Rᵣ of 0.2 is desired. This was achieved with the weaker eluent 15% ethyl acetate/hexane. The product was dried overnight and purified using flash chromatography with silica gel (Kieselgel S 63–100 µm, Riedel-de Haen, Germany) as a stationary phase and 15% ethyl acetate/hexane as the mobile phase. As the compound was insoluble in the mobile phase, the product was applied to the column in dichloromethane. Three column volumes of 15% ethyl acetate/hexane, equivalent to 400 mL each, were added and collected separately followed by one column volume of 25% ethyl acetate/hexane which was collected. After this eluent, 25% ethyl acetate/hexane was used and 20 mL fractions, 31 in total, were collected. The first four 400 mL fractions as well as the 31 x 20 mL fractions were analysed for presence of the product mpbe by TLC. It was found in the 3rd 400 mL fraction and the subsequent 8 x 20 mL fractions, and those fractions were pooled. Small amounts were also seen in fractions 9-24 and these fractions were pooled as well. Both pools were dried and stored under argon. The product was analysed by liquid chromatography mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) spectroscopy and found to be pure (see appendix on page 177). The yield of 3-mpbe from the first reaction starting with 3-apbe was 62.5% compared to 66.5% given by D’Hooge et al. (D’Hooge et al. 2008).
In order to de-protect the product 3-mpbe it was resuspended in a 1:1 mixture of methanol/water at 0.05 M (see Figure 2-7). The mixture looked like a slurry but became clear after addition of 6 eq. of KHF₂ and was kept stirring overnight at RT. Methanol was removed using a rotary evaporator while not heating the solution above 30 °C. Remaining water was evaporated using a freeze drier. The dried product was re-dissolved in hot acetone at a concentration of 0.15 M. Some slurry remained even after heating the acetone to the boiling point at 56 °C. The hot mixture was filtered and the filtrate was removed by rotary evaporation. Some liquid (most likely residual water) remained. A further 20 mL of water was added and the solution was extracted with three times with 20 mL of ether. After the third extraction with ether, crystals started to form in the aqueous phase which suggested that a pure product was obtained. The water phase was collected and analysed by LC MS and NMR spectroscopy (see appendix on page 178). It was confirmed that the product was 3-methacrylamido phenyl trifluoroborate \((\text{M}_{\text{3-mptfb}})=267.10 \text{ g/mol})\). The yield of reaction 4 was 80% compared to the 60% reported in the literature.

For the last reaction, 3-mptfb was dissolved in a 2:1 mixture of acetonitrile/water at 0.05 M. LiOH dissolved in a small portion of the solvent and was added in excess (8 eq.) to the 3-mptfb mixture. The reaction was kept stirring overnight at RT. The solution appeared to have two phases the next day, with some precipitation present in the bottom layer. Saturated ammonium hydrochloride (30 mL/mmol) and 1 M hydrochloric acid (8 mL/mmol) were added to the mixture and it was kept stirring at RT for 5-10 min. The solution was extracted four times with ethyl acetate. The suggested extraction volume was four times 40 mL/mmol but we found four times 15 mL/mmol to be sufficient. The bottom aqueous phase was collected each time and mixed with ethyl acetate again. The combined organic extracts were dried over magnesium sulphate until the solution appeared clear. Magnesium sulphate was removed by filtration and the solvent was removed by rotary evaporation followed by a hi-vac
Chapter 2: Materials and methods

pump. The resulting solid was not easy to weigh and handle. Thus it was re-dissolved in water and freeze-dried, which resulted in a fluffy white powder which could be handled and weighed easily. The product was analysed by $^1$H and $^{13}$C-NMR spectrometry (400 MHZ and 100 MHZ Bruker spectrometer, respectively) and was shown to be pure 3-methacrylamido phenylboronic acid (see appendix on page 179). The yield of reaction 5 was 78%, while a yield of 77% is reported in the literature.

![Figure 2-8: 3-mpba synthesis reaction 5](image)

The final reaction from 3-methacrylamido phenyl trifluoroborate (MW=267.10 g/mol) which yielded 3-methacrylamido phenylboronic acid (MW=205.02 g/mol).

Reagents:

All chemicals were obtained from Sigma-Aldrich (USA) while solvents were obtained from Merck Millipore (Germany)

2.5.5.2 Analysis of glycated proteins by mPAGE

The mpba containing SDS-PAGE gels were prepared as described by Morais et al. (Pereira Morais et al. 2010). Gels of 1 mm thickness were poured using disposable plastic gel cassettes (Cat # 2010, Life Technologies, USA). All solutions were bought sterile or were sterile-filtered before use, particularly for gels that were intended for subsequent LC-MS/MS analysis. Different concentrations of mpba and polyacrylamide were tested for the separation of collagen. The ideal concentrations were found to be 0.4% of mpba in a 6.25% polyacrylamide gel. Higher concentrations of polyacrylamide did not allow for proper separation of collagen due to its high molecular weight, while lower concentrations would not polymerise properly in the presence of mpba.

For the resolving gel, mpba was dissolved in 1.5 M Tris of pH 8.8 and sterile-filtered as there were small amounts of particulate matter present. All solutions were added and after addition of the polymerising agents ammonium persulphate (APS) and tetramethylethylenediamine (TEMED), the solution was quickly mixed and added to the gel cassettes with the upper level being just above the last division line to leave enough space for the stacking gel. Isopropyl
alcohol (IPA) was added on top of the resolving gel to allow for an even surface and the gel was left to polymerise at RT for 1h. The detailed recipe is shown in Table 2-3.

The IPA was removed and the stacking gel, which did not contain mpba, was poured. All solutions given in Table 2-4 were combined and after the addition of APS and TEMED, the solution was quickly mixed and poured on top of the resolving gel. A 10-well comb was inserted and the gel was left to settle for 1-2 h at RT. Overnight polymerisation at 4 °C was also tested. This had the effect of improving the resolution of proteins in general while it appeared to decrease the desired retarding effect of mpba on glycated proteins. Thus gels were poured on the same day and left to polymerise for 1-2 h before running.

Collagen extracts were digested with pepsin, centrifuged and the concentration of hyp as a measure of soluble collagen was determined in the supernatant (see page 44 ff. for a detailed description of the procedure). Equal collagen concentrations were adjusted and 4-fold concentrated loading buffer and 10-fold concentrated DTT were added. The samples were reduced and denatured for 5 min at 95 °C. Equal amounts of collagen, approximately 2 µg, were loaded per lane. An artificially glycated standard of BSA was also run on some gels (see page 63 for synthesis). The protein standard Precision Plus Protein Dual Color Standards (BioRad, USA) was used. Gels were run for up to 6 h at 100 V until the desired separation was achieved. The gels were stained with colloidal Coomassie blue described on page 57 ff. Gels were scanned and some bands were analysed by LC-MS/MS as was described on page 58 in order to identify the nature of the proteins present.

Buffers and reagents:

- Acrylamide solution
  - 30% (w/v) acrylamide/bis-acrylamide (37.5/1 factor) solution (Cat # A3699, Sigma-Aldrich, USA)

- General Tris/Glycine Running buffer (10X)
  - 0.25 M Tris
  - 1.92 M glycine
  → Check that pH is around 8.3
  - 1.0% SDS (w/v)

Table 2-3: Resolving gel recipe sufficient for 1 gel

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Volume [mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>3.84</td>
</tr>
<tr>
<td>30% acrylamide/bis-acrylamide (37.5/1 factor) solution (w/v)</td>
<td>1.56</td>
</tr>
</tbody>
</table>
Chapter 2: Materials and methods

Tris 1.5 M pH 8.8 with 30 mg 3-mpba (w/v) 1.95
10% SDS (w/v) 0.075

→ APS and TEMED were added, solution was quickly mixed and transferred to gel cassettes
10% APS (w/v); make fresh each time 0.075
TEMED 0.0075

Table 2-4: Stacking gel recipe sufficient for 1 gel

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Volume [mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>1.70</td>
</tr>
<tr>
<td>30% acrylamide/bis-acrylamide (37.5/1 factor) solution (w/v)</td>
<td>0.43</td>
</tr>
<tr>
<td>1.0 M Tris pH 6.8</td>
<td>0.33</td>
</tr>
<tr>
<td>10% SDS (w/v)</td>
<td>0.025</td>
</tr>
</tbody>
</table>

→ APS and TEMED were added, solution was quickly mixed and transferred to gel cassettes
10% APS (w/v); make fresh each time 0.025
TEMED 0.0025

2.6 Statistical analysis

Data involving the two disease groups in combination with the two treatment groups (Sham/Plac, Sham/TETA, STZ/Plac and STZ/TETA) was analysed by two-way ANOVA using SAS version 9.3 (SAS Institute Inc., USA) followed by a Tukey-Kramer post-hoc test. Data obtained from Immunoblotting and RT-qPCR were log transformed prior to analysis in order to yield normally distributed data. This is a commonly employed transformation method for data obtained from these two methods (Sokal et al. 1987; Gallup et al. 2006; Kreutz et al. 2007). Data that was not normally distributed was analysed using the Mann Whitney U-test.

In order to test for correlations between different data sets linear regression analysis was carried out. Data was analysed using Pearson correlation analysis using the software GraphPad Prism 4 (GraphPad Software Inc., USA)
# 2.7 List of standard reagents and equipment

## Table 2-5: List of standard reagents

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Name of manufacturer and country of origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>ICP biologicals NZ</td>
</tr>
<tr>
<td>Dialysis membrane MW cut off 3500</td>
<td>Membrane Filtration products Canada</td>
</tr>
<tr>
<td>Standard chemicals</td>
<td>Sigma USA</td>
</tr>
<tr>
<td>Solvents</td>
<td>Merck Millipore Germany</td>
</tr>
<tr>
<td>Water referred to is Milli-Q water with resistance of 18.2 MΩ/cm</td>
<td>Merck Millipore Germany</td>
</tr>
</tbody>
</table>

## Table 2-6: List of standard equipment

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Name of manufacturer and country of origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance plate reader SpectraMax 340</td>
<td>Molecular Devices USA</td>
</tr>
<tr>
<td>Agarose gel electrophoresis Mini-Sub Cell GT</td>
<td>BioRad USA</td>
</tr>
<tr>
<td>Centrifuge 5810 R and 5415 R</td>
<td>Eppendorf Germany</td>
</tr>
<tr>
<td>Fluorescence plate reader SpectraMax Gemini XS</td>
<td>Molecular Devices USA</td>
</tr>
<tr>
<td>Magnetic stirrer platform IKAMAG RCT</td>
<td>IKA Germany</td>
</tr>
<tr>
<td>Minishaker MSI</td>
<td>Ika Germany</td>
</tr>
<tr>
<td>pH meter seven easy</td>
<td>Mettler Toledo Switzerland</td>
</tr>
<tr>
<td>Pipettes Eppendorf research</td>
<td>Eppendorf Germany</td>
</tr>
<tr>
<td>Power supply PowerPac 3000</td>
<td>BioRad USA</td>
</tr>
<tr>
<td>Scientific balances MT5, AB204-S and PM400</td>
<td>Mettler Toledo Switzerland</td>
</tr>
<tr>
<td>SDS PAGE apparatus Xcell sure lock</td>
<td>Invitrogen USA</td>
</tr>
<tr>
<td>Shaker Orbitec</td>
<td>Infors Switzerland</td>
</tr>
</tbody>
</table>
Chapter 3 – Collagen extraction from kidney and heart of healthy and diabetic rats treated with placebo or TETA and its preliminary characterisation

3.1 Introduction

The STZ-induced diabetic rat model was employed for reasons discussed on page 40 ff. In this chapter, the basic characteristics of the animals employed in the current study are included.

The focus of the current study was the ECM. Collagen is the major ECM protein and has been the focus of extensive research (Bornstein et al. 1980; Bailey et al. 1998; Eyre et al. 2008; Avery et al. 2009). Extraction methods for collagen are numerous and are frequently applied to tissue of high collagen content such as bone or skin (Nagai et al. 2000; Sadowska et al. 2003). They often involve the solubilisation of collagen in acetic acid, sometimes in combination with pepsin followed by the subsequent precipitation of collagen with different concentrations of NaCl (Nalinanon et al. 2007). The extraction of collagen from bone also involves an EDTA step for the removal of calcium prior to the extraction procedure. Many of these methods find application in the preparation of collagen or gelatine for use in food stuff, cosmetics or biomedical material but also in research applications.

The extraction of collagen from kidney and heart tissue differs from abovementioned cases, as the concentration of collagen is much lower in both. In addition, the amount of available tissue is limited in rodents such as rats. Another critical point is that, while acid-insoluble collagen may well be discarded in the preparation of industrial products, it is essential to analyse this portion in research studies such as were undertaken in this thesis. Thus a method was needed to extract collagen, both soluble and insoluble, from the small amounts of heart and kidney tissue available, with high purity. The method described by Avery et al. termed “An efficient method for the isolation of intramuscular collagen” was found to yield these desired qualities (Avery et al. 1995). This was the case for the isolation of heart collagen as well as kidney collagen and the method employed in this thesis has been described in detail on page 45.

The extracted collagen needed to be solubilised for further analysis. Solubilisation of collagen can be achieved by incubation in neutral salt buffers, acidic or basic solvents or with
Chapter 3: Collagen extraction and preliminary characterisation

the help of enzymes (Miller et al. 1982). Neutral salt solvents are only capable of solubilising collagen that is not covalently cross-linked while acidic or basic solvents may solubilise cross-linked collagen only to a slightly higher extent while these methods hydrolyse aspects of the collagen. Solubilisation of collagen with the help of limited pepsin digestion can increase the yield of collagen-derived protein and produces mostly intact collagen molecules: this was therefore the method of choice for the qualitative characterisation of the collagen isoforms in the extracts by SDS-PAGE and LC-MS/MS employed here. The main downside of this method is the partial solubilisation of collagen (~30-40% as reported in sections 4.2.3.1 and 5.2.3.1). Thus another protease, the collagenase mixture Liberase DH (Roche Diagnostics, Germany), was used for the solubilisation of collagen for post-translational analysis.
3.2 Results

3.2.1 Characteristics of the STZ-induced diabetes model

Animals were injected with saline only (Sham), or STZ (STZ) to induce diabetes, at week 0, and treatment with TETA (TETA) or placebo (Plac) was started at week 8 and continued for the following 8 weeks in half of the Sham- and STZ-injected animals each. The procedure is described in detail on page 42 and outcomes in terms of body weight and blood glucose in Figure 3-1 above. Body weight was measured weekly whereas blood glucose measurements were performed fortnightly. Starting body weight and blood glucose levels were not

![Graph A: Body weight (g)](image)

![Graph B: Blood glucose [mmol/L]](image)

Figure 3-1: Blood glucose level and body weight
Shown are the body weights (A) and the blood glucose levels (B) of the four different experimental groups during the 16-week course of the study. The mean ± SEM are shown at the time points of each measurement. Data were analysed by repeated-measures ANOVA and the significance of the comparison of values from the placebo- or TETA-treated diabetic group with those from the placebo-treated healthy group at \( p<0.05 \) is indicated by “a” or “b” as shown. A significant difference in blood glucose levels at \( p<0.05 \) between the placebo-treated and the TETA-treated diabetic group (before the treatment start) is indicated by “\( d_1 \)” and “\( d_2 \)”. The commencement of TETA treatment is as arrowed.
significantly different between the four groups. Animals injected with STZ displayed significantly decreased body weight compared to Sham-injected animals starting from week 2 after injection (Figure 3-1: A; a, *p*<0.05 and b, *p*<0.001 for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). STZ-injected animals displayed higher blood glucose levels compared to Sham-injected animals starting from the first week (Figure 3-1: B; a, *p*<0.001 and b, *p*<0.001 for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). TETA or placebo treatment after 8 weeks after the diagnosis of diabetes (arrowed) did not modify blood glucose levels or body weight in diabetic (STZ) or healthy (Sham) animals. It is noted that the STZ-diabetic group which received placebo treatment starting from week 8 displayed significantly lower blood glucose levels than the group that received TETA treatment in week 1 and 3 after STZ-injection (Figure 3-1: B; d1, *p*<0.001 and d2, *p*<0.05 for STZ/Plac vs. STZ/TETA at week 1 and week 3, respectively). This difference did not persist beyond week 4.

### 3.2.2 Extraction of collagen from rat hearts and kidneys

Collagen was extracted from kidney and heart and the extracts were analysed for purity using the hyp assay. Furthermore the collagen extracts were examined for their susceptibility to solubilisation by Liberase DH (Roche Diagnostics, Germany), a purified collagenase preparation. Also, collagen was partially solubilised with pepsin/acetic acid and the solubilised collagen was run on a SDS-PAGE gel and Coomassie stained. Bands of the different molecular masses were trypsin-digested and analysed by LC-MS/MS to identify which collagen isoforms were present in the extracts.

#### 3.2.2.1 Determination of purity of collagen extracts by hydroxyproline measurement

The extraction of collagen was performed as described on page 44 and the resulting extracts were freeze dried and stored at -30 °C. Parts of the freeze dried collagen samples from the kidney and the cardiac LV were weighed, hydrolysed, and analysed for hyp content. The collagen content of each sample was then calculated as [%] dry weight based on the hyp content. The hyp content of freeze-dried and hydrolysed kidney lysates or cardiac LV lysates were measured in parallel for comparison. The collagen content of both kidney and heart collagen extracts was ~70% - 80% of dry weight for the four treatment groups, as shown in Figure 3-2. No significant difference in collagen content between extracts of the four treatment groups was seen in kidney collagen extracts (Figure 3-2: A). There was no significant difference in the Tukey-Kramer *post-hoc* comparisons of the individual groups for collagen extracts from the LV. However, the disease term was significant in the two-way
Chapter 3: Collagen extraction and preliminary characterisation

ANOVA comparison (Figure 3-2: B; two-way ANOVA Disease term, \( p = 0.020 \) for [Sham/Plac + Sham/TETA] vs. [STZ/Plac + STZ/TETA]) meaning that the overall collagen content in the diabetic group was significantly lower than in the Sham group for collagen extracts from the LV.

3.2.2.2 Solubilisation of collagen extracts employing a collagenase mixture

A collagenase mixture (Liberase DH, Roche Diagnostics, Germany) was tested for its ability to cause complete solubilisation of the extracted collagen. Kidney collagen extracts were incubated with different concentrations of the enzyme at 37 °C for up to 24 h (see Figure 3-3). The aim was to minimise the time that the collagen extract was kept at 37 °C while using the smallest amount of enzyme possible to minimise cost as well as enzyme
contamination of the sample. Once the incubation of the samples was completed, soluble and insoluble collagen peptides were separated by centrifugation and the hyp content in both was determined as a measure of collagen content. All of the collagen was solubilised by Liberase DH digestion after 24 h at 37 °C using as little as 0.25 mg/mL of enzyme and 1 mg of collagen per ml of solution. It was decided that solubilisation of collagen using 0.05 mg/mL of Liberase DH solution for 4 h at 37 °C should be sufficient to dissolve the vast majority of collagen and thus to make it accessible for downstream post-translational analysis.

Collagen extracts from kidneys and cardiac LVs were analysed for Liberase DH digestibility using the previously determined parameters. Liberase DH in solution at a concentration of 0.050 mg/mL of was added to collagen extracts from kidneys and LVs at a concentration of 1 mg/mL and collagen was digested at 37 °C for 4 h. Again, samples were centrifuged to separate digested and undigested collagen and the supernatant and the pellet were analysed for collagen content. The amount of solubilised collagen was measured for each of the four groups. Results are shown in Figure 3-4 where the amount of collagen solubilised from kidney collagen extracts (A) and from left ventricular collagen extracts (B) are shown. It appears as if the amount of collagen solubilised in the kidney (95% - 96%) is slightly lower than in the heart (around 97%). This may be due to inter-assay variation rather than a true difference in solubility as kidney samples were measured separately from heart samples. There were no differences between the individual groups regarding the amount of collagen

![Figure 3-3: Time and concentration dependent collagen digestibility](image)

Time- and concentration-dependent Liberase DH solubility of kidney collagen extracts from healthy rats. Solubilisation was carried out at 37 °C. Solubilisation at a Liberase DH concentration of 0.050 mg/ml for 4 h was found to be sufficient to maximise the amount of collagen solubilised and minimise the time at 37 °C as well as the contamination of the preparation with the enzyme.
Chapter 3: Collagen extraction and preliminary characterisation

3.2.2.3 LC-MS/MS analysis of pepsin/acetic acid digested extracts from the LV and kidneys

After the collagen extracts had been analysed quantitatively, it was of interest to find out which isoforms of collagen were present in the extracts. In order to determine the types of collagen that were extracted from the kidney and the LV, extracts were digested with 1 mg/ml pepsin in 0.5 M acetic acid. Treatment with pepsin leads to the digestion of collagen in the non-helical region and leads to the release of monomeric helical alpha chains of collagen (Worrall et al. 1966). Collagen was added to this solution at a concentration of 2 mg/ml and digestion was carried out for 24 h at 4 °C. The undiluted or 1:2 diluted supernatant was heated with Laemmli buffer and DTT, run on SDS-PAGE gels and stained with Coomassie. Bands were excised, trypsin digested and then analysed using LC-MS/MS for collagen extracts from the LV. The resulting pattern can be seen in Figure 3-5. The different isoforms

Figure 3-4: Liberase DH solubility of collagen extracts from rat kidney and LV

Shown is the Liberase DH solubility collagen extracted from rat kidneys (A) as well as for collagen extracted from the LV of the heart (B). The solubility is shown for the four different groups of animals. Approximately 96% of collagen is solubilised. The amount of solubilised collagen appears to be slightly higher in the extracts from the left ventricle. Shown are the individual data points and the mean ± SEM are indicated.

solubilised in kidney- or left ventricular extracts after analysis with the Tukey-Kramer post-hoc test.
of collagen that were detected by LC-MS/MS analysis for each of the numbered bands are annotated with the lowest molecular mass band \#1 being pepsin which was used for the solubilisation of the collagen. A molecular mass marker was run in the left-hand lane with the size indicated next to each band. Only collagens of the fibrillar type were detected in the pepsin-digested extracts and no type IV collagen was identified. Results were compared with published data and those isoforms that were found to agree with the ones reported by Chace et al. are shown in bold (Chace et al. 1991)
two isoforms in general (Miller et al. 1982). Isoforms of type I and type III collagens are present at different molecular masses (bands # 2, 3, 5, 6, 7, 9 and 10). The lower molecular mass bands, # 2 and 3, are equivalent to the monomeric bands (α component) while bands # 5 and 6 as well as # 7 to 10 are multimers of the different isoforms which are termed β- and γ-components, respectively (Miller et al. 1982; Chace et al. 1991). As mentioned above, only the isoforms shown in bold are equivalent to the ones reported by Chace et al. (Chace et al. 1991). The pattern was further compared to previous analysis particularly regarding the collagen type III isoforms. Miller et al. report on the collagen type III isoform at three molecular masses, equivalent to the mentioned α-, β- and γ-components of collagen type I (Miller et al. 1982). This is in agreement with our findings, where COL3A1 is found alongside collagen of type I in band # 3 (collagen type I α component), band # 6 (collagen type I β component) and band # 8 (close to band # 7 a collagen type I γ-component). Thus, the only isoforms which were unaccounted for in the literature at the detected molecular mass were COL1A1 in band # 2 as well as COL1A2 in band # 3. The most likely explanation for this phenomenon would be cross-contamination of the samples as both isoforms are very abundant in the adjacent bands. It may also be that some of the isoforms did not completely separate from each other, although this would be expected to lead to a shift in molecular mass at the same time.

Kidney collagen extracts were analysed by LC-MS/MS in the same way as was described for the LV collagen extracts. The results were very similar to the results obtained for the LV collagen extracts and are shown in Figure 3-6. The only differences were the presence of COLA1(I) in band # 4 and the presence of COLA1(III) in band # 5 as well as COLA2(I) in band # 10. The presence of COLA1(I) in band # 4 and Cola1(III) in band # 5 may be caused by cross-contamination with the adjacent bands; COLA1(I) is very abundant in band #3 while COLA1(III) can be found in band # 6 where it was detected in the LV extracts previously. Considering that band # 9 and # 10 are well separated, and that COLA2(I) displays a higher abundance than COLA1(I) in band # 10, this may be a true difference compared to the LV extracts of collagen. However, band # 10 in the LV extracts was rather weak so that this needs to be interpreted with caution (see Figure 3-5). Collagens in band # 7 and # 8 could not be identified but can be assumed to be COLA1(I) and COLA1(III), respectively based on comparison to the extracts from the LV.
Collagen was extracted from kidneys of healthy rats. The extracts were then solubilised using pepsin/acetic acid digestion, centrifuged and the supernatant containing soluble collagen was run on a SDS-PAGE gel at a 1:1 and a 1:2 dilution. The gel was stained using colloidal Coomassie. Bands at 1:2 dilution were excised, de-stained, trypsin digested and analysed by LC-MS/MS to identify the proteins present. A molecular mass marker (HiMark PreStained HMW protein marker, Life Technologies, USA) was run in the first lane on the left hand side and the molecular mass of each band is indicated next to the band. Bands that were analysed are numbered starting from the lowest molecular mass band. Bands of the 1:2 dilution were analysed and are numbered starting from the lowest molecular mass band. The proteins detected were collagens of different isoforms except for band # 1 which represented pepsin. The identified proteins are annotated in the order of abundance (Exponentially Modified Protein Abundance Index, emPAI). Agreements with the publication by Chace et al. are printed in bold (Chace et al. 1991). Collagens in band # 7 and # 8 could not be identified by LC-MS/MS but were determined by comparison with the extracts of the LV.
Chapter 3: Collagen extraction and preliminary characterisation

Collagen from the LV and the kidney from healthy and STZ diabetic rats was digested at a concentration of 1 mg/ml in 1 mg/ml pepsin/0.5 M acetic acid for 24 h at 4 °C. The digestes were centrifuged, the undiluted supernatant was heated with Laemmli buffer and DTT, equal volumes were run on a SDS-PAGE in parallel and the gel was stained with Coomassie. This was done to compare the extracts from the different organs directly and to see whether there were any effects of the disease on the collagen pattern seen after SDS-PAGE. The stained gel is shown in Figure 3-7 with the marker in the column on the far left. The pepsin-digested collagen extract from the kidney of healthy rats labelled “Sham” is shown next to the marker lane on the left hand side of the gel. Pepsin-digested collagen extracts from kidneys of diabetic rats labelled “STZ” can be seen in the centre of the gel. Control pepsin digests without substrates which were run as controls are labelled as “-”. Collagen extracts from the LV of hearts from healthy and diabetic rats can are shown in the two right hand columns. The pattern was evidently the same in kidneys and the LV of healthy and diabetic rats.

Figure 3-7: SDS-PAGE of collagen extracts from kidney and the left ventricle (LV) in parallel
Collagen extracted from kidneys or the LV of healthy (Sham) and diabetic (STZ) rats was run on an SDS-PAGE gel in parallel and stained with colloidal Coomassie subsequently. In addition control pepsin digests (without substrates) were also analysed (-).
3.3 Discussion

The aim of this part of the study was in short to characterise the diabetic rat model and to perform a preliminary analysis of collagen extracts from hearts and kidneys of these rats. As expected, the STZ-injected rats became diabetic within a few days while they had a lower body weight than the healthy rats after 2-3 weeks. It was also in line with previous observations that treatment with TETA which was started after 8 weeks does not affect blood glucose levels or the body weight of healthy- or STZ-induced diabetic rats (Cooper et al. 2004; Gong et al. 2006; Gong et al. 2008).

It was shown that collagen extracts from the kidney and heart of the rats could be obtained at good purity. The fact that the collagen content of the dry weight of extracts from the LV of rat hearts in the diabetic group was slightly lower would not affect the subsequent experiments as they will be normalised for the amount of collagen rather than the dry weight of the extracts. Characterisation of the extracts regarding the digestibility with the collagenase preparation Liberase DH was important as this enzyme preparation was to be used to solubilise collagen for analysis of post-translational modifications. Thus large differences between the solubilisation of collagen employing Liberase DH could have led to a bias in the downstream analysis. Importantly, most of the collagen could be solubilised with Liberase DH (96% for kidney and 98% for LV) while no significant differences in solubility were evident between the groups. Thus digestion of collagen with Liberase DH was a suitable tool for the solubilisation of kidney and LV collagen extracts for downstream analysis.

The collagen extracts were characterised further by LC-MS/MS analysis after pepsin digestion and SDS-PAGE. Pepsin digestion was chosen over Liberase DH digestion as it selectively cleaves off the helical domain from the non-helical domain of fibrillar collagens, while the collagenase present in the Liberase DH preparation non-specifically cleaves collagen, precluding subsequent LC-MS/MS analysis. The collagen isoforms that were identified in the heart and kidney belonged to the fibrillar type and these results agreed well with those reported previously (Miller et al. 1982; Chace et al. 1991). While type IV collagen was not identified by using SDS-PAGE, it was later identified after mPAGE in kidneys (described in section 4.2.3.4.). Type IV collagen runs at the same molecular weight as type V collagen on SDS-PAGE (Miller et al. 1982). The running pattern of collagens was altered by mPAGE. Thus while type IV collagen identification may be obscured by the presence of type V collagen in SDS-PAGE, mPAGE facilitated the identification of type IV collagen. No type VI
collagen was detected by SDS-PAGE or mPAGE. This may be due to the relatively low abundance of this type of collagen (Hessle et al. 1984).

It was concluded that the STZ-induced diabetic rat model yielded results comparable to previous trials employing this model, while the effects of TETA on blood glucose levels and the body weight were also in line with previous reports (Cooper et al. 2004; Gong et al. 2006; Gong et al. 2008). Furthermore, the collagen extracts that were obtained from the kidney and the LV of hearts of these rats yielded collagen of a quality that was suitable for downstream analysis. Importantly the solubilisation procedure that was subsequently employed for post-translational analysis of the collagen extracts yielded comparable results in all four groups of rats. The characterisation of the extracts by SDS-PAGE and LC-MS/MS confirmed the purity of these extracts and the identified isoforms of collagen agreed well with previously published data.
Chapter 4 – Diabetes-induced changes in the ECM of the kidney - the effect of TETA

4.1 Introduction

Fibrosis, the pathogenic accumulation of the ECM, is frequently observed in the kidneys of humans with chronic kidney disease (CKD), particularly in the form of DN (Jefferson et al. 2008; Boor et al. 2010). One contributor to fibrosis in CKD is thought to be the activation and proliferation of fibroblasts possibly in combination with inflammation (Simonson 2007; Boor et al. 2010; Liu 2011; Lim et al. 2012). AGEs may also contribute to fibrosis in the case of DN (Cooper 2004). Their formation is thought to be enhanced due to high blood glucose levels, possibly in combination with metal-catalysed oxidative stress (Saxena et al. 1999). While AGEs have been frequently associated with the morphological changes and fibrosis in particular, it is still unclear as to how and to what extent they contribute to the complications (Nishino et al. 1995; Horie et al. 1997; Niwa et al. 1997; Suzuki et al. 1999). One hypothesis regarding AGEs is that they lead to the deterioration and accumulation of the ECM in DN via a decreased susceptibility towards proteases (Mott et al. 1997; Bohlender et al. 2005). This observation is based on the findings that AGE-modified collagen is less susceptible to degradation by pepsin in vitro (see paragraph 1.3.3.1). However, there is currently no clear link between the prominent thickening of the glomerular basement membrane as well as the expansion of the mesangial matrix in diabetes and the development of proteinuria (Jefferson et al. 2008).

One potential connection between enhanced AGE formation and proteinuria may be found in the tubulointerstitium. Interstitial ECM accumulation outside the glomerulus in association with the tubules is another form of fibrosis found in DN and is also associated with AGE formation (Horie et al. 1997; Bohlender et al. 2005). A direct correlation between the extent of tubulointerstitial scarring and the severity of albuminuria has been reported (Gilbert et al. 1999). Scarring of this structure is arguably the best predictor of renal survival in DN as well as other forms of CKD, and tubular cells stain heavily for different AGEs in diabetes even at early stages of the development of nephropathy (Nishino et al. 1995; Horie et al. 1997; Niwa et al. 1997; Morcos et al. 2002; Jefferson et al. 2008). Furthermore the importance of the tubular cells in the development of proteinuria has most likely been underestimated until recently (Jefferson et al. 2008). Available evidence now suggests that a larger amount of albumin than was previously thought is filtered through the glomerulus of the kidney and that
proximal tubular cells play an important role in the retrieval and degradation of this albumin pool (Osicka et al. 2000; Russo et al. 2007).

As mentioned by Boor et al. no single optimal model for the study of fibrosis in CKD exists due to the many different underlying causative diseases (Boor et al. 2010). Diabetes is the most common cause of CKD and the STZ-induced diabetic rat is a well-established model of DN (Pyram et al. 2011). Glomerular basement membrane thickening, mesangial fibrosis as well as interstitial fibrosis have been observed in this model, in combination with a decline in renal function as well as microalbuminuria making it an appropriate model for the study of DN (Tesch et al. 2007). Here, this model was employed to study the molecular processes in the extracellular matrix in kidneys of diabetic rats with a focus on the major constituent, collagen.

In this chapter, changes have been reported in collagen regulation at the mRNA and protein levels in the kidneys of diabetic rats. Perhaps more importantly, these changes have been related to non-enzymatic post-translational changes in collagen-AGE levels as well as carbonyl levels, which are also thought to be altered in diabetes (see pages 24 ff. and 32). Alterations in the level of proteins involved in the defence against AGEs may affect the level of AGE formation and thus these were also determined (see page 30 ff.). As collagen is subject to enzyme catalysed post-translational modification, for example that mediated by lysyl oxidase (see page 33 ff.) in addition to non-enzymatic alterations, the enzymes responsible for enzymatic cross-linking were also analysed. Changes in post-translational modifications of collagen can affect the susceptibility of collagen towards enzyme-catalysed degradation (see page 25 ff. and 33 ff.), which was here analysed by measurement of in vitro pepsin digestibility in tissue collagen extracts. Furthermore, it is known that proteases involved in the degradation of collagen can be altered in the diabetic state, possibly reflecting altered susceptibility of collagen towards degradation (see page 14 ff.), so these represented a further interesting target for analysis. Alterations in signalling pathways evoked by growth factors which may also contribute to the development of fibrosis in DN (Chiarelli et al. 2009) but were not the main focus of this chapter in particular or the thesis in general. They were thus included in the preliminary analysis where their transcription levels were measured by qPCR, but were not followed up further in the subsequent down-stream analysis.

Potential changes measured in diabetic rats promised to give new insights into the development of DN in particular, due to the parallel acquisition of the data sets. The aim was that such analysis would hopefully allow for a more complete picture and to determine which factors play substantive roles in the development of fibrosis in DN. Furthermore, all the factors mentioned above were also measured in animals treated with the Cu(II) chelator
Chapter 4: Diabetes-induced changes in the renal ECM and the effect of TETA

TETA. Intervention by using chelator treatment intervention was started after 8 weeks of established diabetes rather than immediately after its induction. This design was chosen to allow for a more realistic outcome, as treatment in diabetes usually starts after varying duration of elevated blood glucose rather than initiation at the time of onset. This drug has been found not to alter the blood glucose levels or body weight in healthy or diabetic animals (Cooper et al. 2004; Gong et al. 2006) and T2D patients (Cooper et al. 2004; Cooper et al. 2009), and data from the current study are consistent (see page 76 ff.). While the presence of glucose or structurally related sugars is a prerequisite for the formation of AGEs in vitro and in vivo, transition metals are also known to play an essential role in the in vitro process while some data also suggest their importance in vivo (see page 18 ff.). TETA binds Cu(II) in a highly specific manner (Nurchi et al. 2013) but TETA treatment does not affect glucose levels in either the healthy or the diabetic groups, so this study was expected to shed more light on the importance of copper on in vivo AGE formation. Thus an approach comprising an experimental design comparing healthy and diabetic animals as well as the effect of the Cu(II) chelator, was expected to yield new insights into the pathways of AGE formation in vivo, as well as the development of fibrosis in DN in general. In addition the application of a Cu(II) chelator such as TETA may prove to be an option for the treatment of diabetic complications in the future, as was discussed in more detail previously (38 ff.).
4.2 Results

4.2.1 Transcriptional analysis of genes relevant to ECM metabolism and oxidative/glycoxidative stress in the kidney

This study began by looking at changes at the mRNA level in the amounts of transcripts from genes of interest. Subsequently, the initial information regarding the transcriptional level was enhanced for genes of particular interest, and interpreted in light of the evolving literature as well as the results obtained. This detailed analysis was designed to include protein and activity measurements as well as transcript levels. The information obtained at the transcriptional and protein levels was then built upon by measurement of post-translational modifications and pepsin digestibility of kidney collagen extracts.

The relative mRNA levels corresponding to target genes were measured by reverse transcription quantitative real-time PCR (RT-qPCR). For these measurements, RNA was extracted from the renal cortex of the four study groups; mRNA was then transcribed into cDNA which was subsequently analysed by qPCR. The signal for each target gene was normalised to the level of reference genes which need to be measured in parallel for the same set of samples. It is important that the expression levels of those reference genes are stable in the different groups of interest, meaning that it is unaffected by the disease state (Sham/STZ) as well as by the treatment (Placebo/TETA) in this case. Such reference genes were previously determined in our group by Hogl (Hogl 2009). In particular, Rpl13a, Tbp and U2af were found to be stably expressed in the kidney in the disease state, while the treatment did not affect the expression levels either. The mRNA level of each target gene was normalised to the geometric mean of the mRNA level of these three reference genes. In addition, relative mRNA levels were normalised to the average of the Sham/Plac group. Details for the primers of all target and reference genes can be found in the appendix on page 174. Statistical analysis was carried out by two-way ANOVA followed by Tukey-Kramer post-hoc comparison. The purpose of this study was to analyse several pathways of interest for changes at the transcriptional level in the four groups. If changes were found in the diabetic rats or after TETA treatment, such changes were to be analysed in more detail on a protein or activity level. Measurements of mRNA levels were carried out for 9 animals per group. This was enough to yield statistical power for most genes. For genes of particular interest where 9 animals were not enough to yield statistical power despite apparent
Chapter 4: Diabetes-induced changes in the renal ECM and the effect of TETA

differences, a second set of measurements was carried out using mRNA extracted from tissue sourced from a previous animal trial carried out in our group in the same way. This resulted in a total number of 18 animals per group which increased the statistical power. It was not feasible to carry this out for all analysed genes for reasons of cost as well as the limited amount of tissue available from the previous trial.

4.2.1.1 Relative mRNA levels of collagen, genes involved in collagen processing and degradation and growth factors

The major isoforms of collagens found in the kidney are type I, III, IV, V and VI (Lemley et al. 1991). Collagen type IV and V are both constituents of basement membranes in general and the glomerular basement membrane (GBM) in particular. Type IV collagen is a mesh-like collagen while type V collagen found in association with type IV is of the fibrillar kind (Bornstein et al. 1980). The mRNA levels corresponding to the type IV collagen isoforms Col4a1, Col4a4 and Col4a5 were measured and their relative mRNA levels were as shown in Figure 4-1. No changes in mRNA levels between the four groups were found for Col4a1.

Figure 4-1: Relative mRNA levels of basement membrane collagens in cortices of rat kidneys
Shown are the relative mRNA levels of the collagen isoforms found in the basement membrane of the kidneys which were measured by RT-qPCR. Levels of the different collagen isoforms are shown for each of the four groups of rats (Sham/Plac, Sham/TETA, STZ/Plac and STZ/TETA). The different collagen isoforms measured were Col4a1 (A), Col4a4 (B) and Col4a5 (C). Shown are the individual data points (n=9 per group) and the mean ± SEM is indicated. Groups significantly different from the Sham/Plac group at p<0.05 employing the Tukey-Kramer post-hoc comparison are indicated by the letter “a” for the STZ/Plac group or “b” for the STZ/TETA group.
Chapter 4: Diabetes-induced changes in the renal ECM and the effect of TETA

(Figure 4-1: A). A marked decrease of transcripts in the diabetic state was found for the collagen isoform Col4a4 in both the placebo-treated and the TETA-treated groups (Figure 4-1: B; a, $p<0.0001$ and b, $p=0.0004$ for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). Similar changes, albeit less pronounced were found for Col4a5 (Figure 4-1: C; a, $p<0.0036$ and b, $p=0.0011$ for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). Significant treatment effects were not observed according to the treatment term of the two-way ANOVA comparison. This was confirmed when inspecting the individual group comparisons using the post-hoc Tukey-Kramer method, meaning Sham/Plac group vs. Sham/TETA group and STZ/Plac group vs. STZ/TETA group. The mRNA levels for collagen type IV isoforms Col4a2 and Col4a3 as well as the three different collagen type V isoforms Col5a1-3 were below the detection limit.

Collagen isoforms type I, III and VI are the predominant collagens in the tubulointerstitium of the kidney cortex (Lemley et al. 1991). Type I and III are fibrillar collagen types while type VI collagen is of the filamentous type (Bailey et al. 1998). Relative mRNA levels for these collagen isoforms are shown in Figure 4-2. The trend that was observed for the type IV collagen present in the GBM towards a decrease of collagen mRNA levels was also present for several of these interstitial collagen isoforms. No differences between the groups were found for Col1a1 (Figure 4-2: A) but the second isoform Col1a2 displayed a decrease in the disease state (Figure 4-2: B; two-way ANOVA Disease term, $p=0.0007$ for [Sham/Plac + Sham/TETA] vs. [STZ/Plac + STZ/TETA]). The individual group comparison also showed borderline significance, and significance for the comparison of the placebo-treated healthy group with the placebo-treated diabetic group and the TETA-treated diabetic group, respectively (Figure 4-2: B; a, $p=0.053$ and b, $p=0.039$ for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). No significant change for the comparison of the healthy placebo-treated group with the two diabetic groups was found for Col3a1 (Figure 4-2: C). A decrease of mRNA levels in the diabetic group was seen for two of the collagen type VI isoforms. This was significant for Col6a1 for the comparison of Sham/Plac with STZ/Plac (Figure 4-2: D; a, $p=0.049$ for Sham/Plac vs. STZ/Plac). For Col6a2, the comparison of the healthy placebo-treated group with the diabetic placebo- and TETA-treated group was significant (Figure 4-2: E; a, $p=0.023$ and b, $p=0.0092$ for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). The mRNA levels between the healthy placebo-treated group and the two diabetic groups were not significantly different for Col6a3 (Figure 4-2: F). As for the basement membrane type IV collagen, no significant treatment effects were found.
Chapter 4: Diabetes-induced changes in the renal ECM and the effect of TETA

Figure 4-2: Relative mRNA levels of interstitial collagens from cortices of rat kidneys

Shown above are the relative mRNA levels for collagen isoforms found in the interstitium of kidneys which could be detected by RT-qPCR. Levels of the different collagen isoforms are shown for each of the four groups of rats (Sham/Plac, Sham/TETA, STZ/Plac and STZ/TETA). The different collagen isoforms measured were Col1a1 (A), Col1a2 (B), Col3a1 (C), Col6a1 (D), Col6a2 (E) and Col6a3 (F). Shown are the individual data points (n=9 per group) and the mean ± SEM is indicated. Groups significantly different from the Sham/Plac group at p<0.05 employing the Tukey-Kramer post-hoc comparison are indicated by the letter “a” or “b”. One exception is graph B where the p-value for the comparison of STZ/Plac vs. Sham/Plac is 0.053 indicated by an italicised “a”.

93
Chapter 4: Diabetes-induced changes in the renal ECM and the effect of TETA

The mRNA levels of different growth factors are reportedly altered in the kidneys in diabetes and are thought to contribute to fibrosis in DN (Chiarelli et al. 2009). Here, levels of transcripts corresponding to connective tissue growth factor (Ctgf), transforming growth factor beta 1 (Tgfβ1) and vascular endothelial growth factor alpha (Vegfa) were measured (Figure 4-3: A, B and C respectively). No between-group differences were detectable by applying post-hoc Tukey-Kramer comparisons for any of these growth factors. In addition, neither the disease- nor the treatment term of the two-way ANOVA was significant for any of them.

![Figure 4-3: Relative mRNA levels of growth factors in the cortex of rat kidneys](image)

**Figure 4-3: Relative mRNA levels of growth factors in the cortex of rat kidneys**
The mRNA level of the growth factors Ctgf (A), Tgfβ1 (B) and Vegfa (C) for the four different groups is shown. Shown are the individual data points (n=9 per group) and the mean ± SEM is indicated. Data were analysed by two-way ANOVA followed by Tukey-Kramer post-hoc comparison for pairwise between-group comparisons.
Enzymes involved in the processing of pro-collagen as well as the collagen cross-linking enzyme LOX were analysed for transcriptional changes in rat kidney cortices (Uzel et al. 2001). ADAMTS2 (a disintegrin and metalloproteinase with thrombospondin motifs) is a pro-collagen 3 proteinase (Wang et al. 2003). No changes in mRNA levels were detectable for Adamts2 between the four groups (Figure 4-4: A). Bone morphogenetic protein 1 (BMP1) acts as an activator of LOX and is also able to cleave pro-collagens I-III to release the active forms (Uzel et al. 2001). Relative mRNA levels of Bmp1 were increased in the placebo- and TETA-treated diabetic groups compared to the healthy placebo treated group (Figure 4-4: B; a, \( p < 0.0001 \) and b, \( p = 0.0001 \) for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). No TETA-treatment effects were seen for either Adamts2 or Bmp1 (Figure 4-4: A and B; \( p > 0.05 \) for Sham/Plac vs. Sham/TETA and STZ/Plac vs. STZ/TETA). An increase of relative Lox mRNA levels was found in the diabetic group compared to the Sham/Plac group (Figure 4-4: C; a, \( p < 0.0001 \) and b, \( p = 0.0001 \) for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). Furthermore, a significant increase of Lox mRNA levels was found in the Sham/TETA group.

**Figure 4-4:** Relative mRNA levels of pro-collagen proteinases and the collagen cross-linking enzyme Lox in the cortex of rat kidneys

Transcription levels for the pro-collagen proteinase Adamts2 (A), the pro-collagen proteinase and pro-Lox processing enzyme Bmp1 (B) as well as the collagen cross-linking enzyme Lox (C) in the four groups can be seen. Shown are the individual data points (\( n = 9 \) per group except for Lox where \( n = 18 \) per group) and the mean ± SEM is indicated. Groups significantly different from the Sham/Plac group at \( p < 0.05 \) employing the Tukey-Kramer *post-hoc* comparison are indicated by the letter “a” for the STZ/Plac group or “b” for the STZ/TETA group. A significant difference for the Tukey-Kramer *post-hoc* comparison of Sham/Plac vs. Sham/TETA is indicated by the letter “c”.

95
Chapter 4: Diabetes-induced changes in the renal ECM and the effect of TETA

compared to the Sham/Plac group (Figure 4-4: C; c, $p<0.002$ for Sham/Plac vs. Sham/TETA).

The relative mRNA levels of extracellular- ($Mmp2$) as well as lysosomal ($Ctsl$, $Ctsb$ and $Ctsk$) collagen-degrading proteases were measured in the kidney cortices of the four groups of rats (Soderstrom et al. 1999). In addition, the mRNA level of a non-collagenolytic lysosomal protease ($Ctsh$) was measured for comparison. An increase in $Mmp2$ mRNA level in the diabetic group compared to the healthy animals (Figure 4-5: A; two-way ANOVA Disease term, $p=0.0007$ for $[\text{Sham/Plac + Sham/TETA}]$ vs. $[\text{STZ/Plac + STZ/TETA}]$) was found.

Figure 4-5: Relative mRNA levels for extracellular and lysosomal proteases in rat kidney cortices

Relative mRNA levels for the major extracellular collagen-degrading protease $Mmp2$ (A) and the collagen-degrading lysosomal proteases $Ctsl$ (B), $Ctsb$ (C) and $Ctsk$ (D) along with one non-collagenolytic lysosomal protease $Ctsh$ (E) are shown for each of the four groups. Individual data points ($n=9$ per group except for $Mmp2$ where $n=18$ per group) are shown and mean ± SEM values are indicated. Groups significantly different from the Sham/Plac group at $p<0.05$ employing the Tukey-Kramer post-hoc comparison are indicated by the letter “a” for the STZ/Plac group or “b” for the STZ/TETA group.
Chapter 4: Diabetes-induced changes in the renal ECM and the effect of TETA

For Mmp2, the individual post-hoc comparisons between the healthy placebo-treated animals with diabetic placebo- and TETA-treated diabetic groups were not significant. The protease CtsL displayed increased levels of mRNA in the diabetic-placebo and TETA-treated groups compared to the healthy placebo-treated group (Figure 4-5: B; a, \( p=0.013 \) and b, \( p=0.033 \) for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). No significant differences were seen for Ctsk (Figure 4-5: D). Ctsb mRNA levels were decreased rather than increased in the diabetic placebo-treated group compared to the healthy placebo-treated group (Figure 4-5: C; a, \( p=0.018 \) for Sham/Plac vs. STZ/Plac). A similar behaviour was seen for the lysosomal non-collagenolytic protease Ctsh for which both the placebo and the TETA-treated diabetic group displayed decreased mRNA levels compared to the healthy placebo-treated group (Figure 4-5: E; a, \( p<0.0001 \) and b, \( p=0.0002 \) for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively).

4.2.1.2 Relative mRNA levels of genes related to oxidative and glycoxidative stress

Oxidative stress and glycoxidative stress are thought to be involved in the development of the diabetic complications (see pages 32 ff. and 25 ff., respectively). Enzymes involved in the defence against these processes are thus of interest for diabetes research. Decreased synthesis may be involved in the development of the diabetic complications while protective enzymes may also be up-regulated in response to increased pro-oxidant stressors (see pages 16 ff. and 30 ff.). Here levels of transcripts corresponding to relevant genes were analysed by RT-qPCR to determine their potential involvement in the development of diabetic complications, and to investigate potential treatment effects of the Cu(II) chelator TETA.

Relative mRNA levels of genes involved in the defence against oxidative stress caused by ROS are shown in Figure 4-6. GSR contributes to the anti-oxidant properties of cells via the enzymatic reduction of glutathione whereas glutathione peroxidases transform \( \text{H}_2\text{O}_2 \) via the oxidation of glutathione (Meister 1988). Gsr mRNA levels were decreased in the diabetic state (Figure 4-6: A; a, \( p=0.0021 \) and b, \( p<0.0001 \) for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). Relative levels of Gpx3 were measured but significant differences in relative mRNA levels within groups made the interpretation of the data impossible (data not shown). This was found for different sets of cDNA employing two different sets of intron spanning primers. The mRNA levels of Sod1-3 as well as levels of Cat, which catalyse the detoxification of superoxide and hydrogen peroxide, respectively were unaltered in the four different groups (Figure 4-6: B-D for Sod1-3 and E for Cat).
The glyoxalase system consisting of glyoxalase 1 and 2 (GLO1 and GLO2), is involved in the detoxification of the AGE precursors methylglyoxal, glyoxal as well as 3-deoxyglucosone (described in detail on page 30). Messenger RNA levels of Glo1 were lower in the disease state (Figure 4-7: A; two-way ANOVA Disease term, $p=0.0002$ for [Sham/Plac + Sham/TETA] vs. [STZ/Plac + STZ/TETA]). After performing post-hoc between-group Tukey-Kramer comparisons of the individual groups for Glo1, it was found that only the TETA-treated diabetic group was significantly different from the placebo-treated sham group (Figure 4-7: A; b, $p=0.0009$ for Sham/Plac vs. STZ/TETA). The direct comparison of the placebo-treated sham group with the placebo-treated diabetic group was not significant for
Glo1, as the decrease was slightly less than in the TETA-treated diabetic group. A similar, albeit stronger trend was observed for Glo2. Both the placebo- and TETA-treated diabetic groups displayed significantly lower levels than the placebo-treated sham group (Figure 4-7: B; a, \(p=0.0004\) and b, \(p<0.0001\) for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). In addition, the difference between the placebo and the TETA treated diabetic group reached statistical significance for Glo2 where the TETA-treated diabetic group displayed significantly lower levels (Figure 4-7: B; d, \(p=0.0297\) for STZ/Plac vs. STZ/TETA).
Chapter 4: Diabetes-induced changes in the renal ECM and the effect of TETA

Other enzymes can also detoxify methylglyoxal and 3-deoxyglucosone. Aldehyde reductase (AKR1A1), aldose reductase (AKR1B1), dihydrodiol dehydrogenase (AKR1C1) as well as 2-oxoaldehyde dehydrogenase (ALDH1A1) are amongst such enzymes and were analysed for transcriptional changes. Relative mRNA levels are shown in Figure 4-8 where it can be seen that no changes were detectable for *Akr1a1* (A), *Akr1b1* (B) or *Akr1c1* (C) between the four groups of animals. Levels for *Aldh1a1* were decreased in the placebo-treated diabetic group compared to the placebo-treated sham group (Figure 4-8: D; b, \(p=0.030\) for Sham/Plac vs. STZ/Plac). TETA treatment did not significantly modify the mRNA levels of *Aldh1a1* in either the Sham/TETA or diabetic/TETA groups.

![Figure 4-8](image-url)

**Figure 4-8: Relative mRNA levels for additional methylglyoxal and 3-deoxyglucosone detoxification enzymes in rat kidney cortices**

Shown are transcript levels for *Akr1a1* (A), *Akr1b1* (B), *Akr1c1* (C) and *Aldh1a1* (D) in rat kidney cortices from the four treatment groups. These correspond to the genes that encode additional enzymes possibly involved in the detoxification of AGE precursors. Individual data points (\(n=9\) per group) are shown and mean ± SEM values are as indicated. Groups significantly different from the Sham/Plac group at \(p<0.05\) employing Tukey-Kramer post-hoc comparisons are indicated by the letter “a” compared to the corresponding the STZ/Plac group.

The enzyme amine oxidase, copper containing 3 (AOC3), also known as semicarbazide sensitive amine oxidase (SSAO), is a potential contributor to the formation of AGEs as it produces the AGE precursor methylglyoxal (Obata 2006). In addition, the receptor for AGEs (RAGE) has been connected to an increase in renal lesions possibly induced by AGEs and was thus included in the analysis (see page 28). Relative mRNA levels of *Aoc3* were markedly lower in the TETA-treated and placebo-treated diabetic group compared to the
Chapter 4: Diabetes-induced changes in the renal ECM and the effect of TETA

4.2.2 Amounts and enzyme-activity measurements of ECM- and ROS-related proteins in non-diabetic and diabetic rat kidney tissue, and the effects of TETA treatment

Initial measurements of mRNA levels corresponding to genes of interest were made and the results reported in the previous section. Levels of transcripts corresponding to genes related to ECM formation, degradation and cross-linking as well as those of genes related to oxidative and glycoxidative stress that could have potential influence on AGE formation, were analysed in the four different treatment groups of animals.

The goal of the second part of this study, described in this section, was to look more closely at genes emerging from the initial analysis of mRNA levels, by determination of their respective protein and/or activity levels. Collagen protein levels were also determined via the measurement of hyp content. Relative levels of all other proteins shown in the following sections were determined by Western Blotting, for which band intensities were measured by

Figure 4-9: Relative mRNA levels for Aoc3 and Rage in rat kidney cortices

Shown are levels of transcripts corresponding to Aoc3 (A) and Rage (B) in rat kidney cortices from the four experimental groups. Individual data points (n=9/group for Aoc3 and n=18/group for Rage) are shown and mean ± SEM values are as indicated. Groups significantly different from the Sham/Plac group at p<0.05 employing Tukey-Kramer post-hoc comparisons are indicated by the letter “a” for the STZ/Plac group or “b” for the STZ/TETA group.
Chapter 4: Diabetes-induced changes in the renal ECM and the effect of TETA

densitometry and normalised to the respective beta-actin band intensity followed by normalisation for the average value of the Sham placebo-treated group. Activities of MMP2 and CTSL were determined by gelatin zymography and a fluorescent probe-coupled protease substrate, respectively.

Measurements of protein levels were carried out for 11 or 12 animals per group, which was enough to yield statistical power in most cases. For Lox protein-level measurements however, 11-12 animals per group were insufficient to yield statistical power, despite apparent differences of Lox levels between the four groups. As small amounts of kidney tissue were available from a previous animal trial carried out in our group, Lox protein levels were also measured in these tissues. This resulted in a total number of 19 animals per group and increased statistical power. It was not feasible however to carry this out for all analysed proteins as only a limited amount of tissue available from the previous study.

4.2.2.1 Collagen protein levels in rat kidney cortices

Collagen protein levels of the four groups were analysed by measuring hyp levels in acid hydrolysates from freeze-dried rat kidney cortices using a colorimetric assay and a hyp standard curve. The results are shown in Figure 4-10. Consistent with the decrease in mRNA levels corresponding to several collagen isoforms, a significant decrease in collagen protein levels was detected in the placebo-treated diabetic group (Figure 4-10: a, \( p=0.0028 \) for Sham/Plac vs. STZ/Plac). This decrease of collagen protein level in the placebo-treated diabetic group was reversed by treatment with TETA to levels measured in the healthy

![Figure 4-10: Collagen protein levels in rat kidney cortices of the four groups](image)

**Figure 4-10: Collagen protein levels in rat kidney cortices of the four groups**

Collagen content in the four treatment groups was determined by measuring hyp levels while absolute collagen levels were calculated by assuming that 12.5% of collagen consists of hyp. Collagen content is given in µg of collagen per mg of dry tissue. Individual data points (n=11 per group) are shown and mean ± SEM values are indicated. Groups significantly different from the Sham/Plac group at \( p<0.05 \) by the Mann-Whitney-U test are indicated by the letter “a” for the STZ/Plac group. The significant difference between the STZ/Plac and STZ/TETA values is indicated by “d”.

102
4.2.2.2 Protein level and activity measurements of collagen-degrading proteases

Protein levels as well as the activity of collagen-degrading proteases were analysed due to their potential effects on collagen protein levels. Protein levels were estimated by Western Blotting and normalised to beta-actin levels, followed by normalisation for the average values of the corresponding placebo-treated Sham group. Values were subsequently analysed by two-way ANOVA followed by the Tukey-Kramer post-hoc test.

Cathepsin L (CTSL) and cathepsin B (CTSB) are lysosomal proteases that are highly abundant in kidney, and are both capable of degrading collagen (Soderstrom et al. 1999). Changes at the transcriptional level of these proteases have been described in a previous section (see page 95). Both CTSL and CTSB are processed in the lysosome to yield double-chain forms (respectively designated CtsL dc and CtsB dc) and a single chain isoform (CTSL

![Figure 4-11: Relative CTSL protein level in rat kidney cortices](image-url)

Shown are the protein levels of CTSL dc (A) and CTSL sc (B) for the four treatment groups. Individual data points (n=11 per group) are shown and the mean ± SEM is as indicated. Groups significantly different from the Sham/Plac group at p<0.05 by Tukey-Kramer post-hoc comparison are indicated by the letter “a” for the STZ/Plac group or “b” for the STZ/TETA group. A representative blot for CTSL sc and CTSL dc as well as the normaliser beta actin are shown in the bottom panel (C).
Chapter 4: Diabetes-induced changes in the renal ECM and the effect of TETA

sc and CTSB sc); all of these have proteolytic activity (Mach et al. 1992; Mort et al. 1997). Protein levels of CTSL dc were increased in the placebo and TETA-treated diabetic group compared to the healthy placebo-treated group (Figure 4-11: A; a, \( p = 0.0016 \) and b, \( p = 0.0456 \) for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). Similar changes were seen for CTSL sc (Figure 4-11: B; a, \( p = 0.0005 \) and b, \( p = 0.0024 \) for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). No effects of TETA treatment were observed in the healthy or the diabetic groups compared to the placebo-treated group for CTSL dc or –sc. A representative Western Blot for CTSL and the corresponding normaliser beta actin is shown below the two graphs (Figure 4-11: C).

No significant changes between the groups were seen for CTSB dc while a trend for decreased levels of TETA-treated animals compared to placebo-treated animals was apparent in diabetic- and Sham rats (Figure 4-12: A). The same trend was also seen for CTSB sc protein levels where the treatment term by two-way ANOVA comparison became significant (Figure 4-12: B; two-way ANOVA Treatment term, \( p = 0.0009 \) for [Sham/Plac + STZ/Plac] vs. [STZ/Plac + STZ/TETA]). Furthermore, the individual comparison for the

![Figure 4-12: Relative CTSB protein level in rat kidney cortices](image)

Shown are the protein levels of CTSB dc (A) and CTSB sc (B) for the four treatment groups. Individual data points (\( n = 11 \) per group) are shown and the mean ± SEM values are as indicated. Groups significantly different from the Sham/Plac group at \( p < 0.05 \) employing the Tukey-Kramer post-hoc comparison are indicated by the letter “b” for the STZ/TETA group. A difference between the STZ/Plac and the STZ/TETA group is indicated by the letter “d”. A representative blot for CTSB sc and CTSB dc as well as the normaliser beta actin is shown in the bottom panel (C).
Chapter 4: Diabetes-induced changes in the renal ECM and the effect of TETA

healthy and diabetic groups with the diabetic TETA-treated group was significant (Figure 4-12: B; b, \( p=0.017 \) and d, \( p=0.023 \) for STZ/TETA vs. Sham/Plac and STZ/Plac, respectively).

As Ctsl mRNA levels and CTSL sc and CTSL dc protein levels (see previous sections on pages 96 and 103 respectively) appeared to be affected strongly in the diabetic state, CTSL activity was measured to further characterise these changes. CTSL activity was detected in lysates of kidney cortices using a CTSL specific substrate coupled to a probe which fluoresces upon substrate cleavage. A standard curve of free fluorescent substrate was run in parallel. No changes in the TETA- or placebo-treated diabetic groups compared to the placebo-treated healthy group were present using the post-hoc Tukey-Kramer comparison. Both the placebo- and TETA-treated healthy group displayed slightly lower activity levels than the two equivalent diabetic groups. This was reflected by a significant disease term in the two-way ANOVA comparison (Figure 4-13: Two-way ANOVA Disease term, \( p=0.008 \) for [Sham/Plac + Sham/TETA] vs. [STZ/Plac + STZ/TETA]).

As an effect of copper on the activity of purified cathepsins has been described previously, it was investigated whether this could be reproduced in kidney tissue lysates of healthy or diabetic rats treated with a placebo or TETA (Sweeney et al. 2003; Lockwood 2010). The effect of in vitro addition of 25 \( \mu \)M of CuCl\(_2\) on the relative CTSL activity in the four groups of animals is shown in Figure 4-14. Activity of the corresponding group without addition of CuCl\(_2\) was set to 100% and is indicated by a dashed line. As can be seen, activity in all four groups
Chapter 4: Diabetes-induced changes in the renal ECM and the effect of TETA

Figure 4-14: Effect of CuCl₂ on relative CTSL activity in the four groups of rat kidney cortices
Shown is the CtsL activity of the four groups of animals after addition of 25 μM CuCl₂. Activity is given relative to the activity of each group without addition of CuCl₂ which was set to 100% indicated by a dashed line. Individual data points (n=12 per group) are shown as well as the mean +/- SEM. Addition of CuCl₂ significantly decreased activity in each respective group indicated by "a" for Sham/Plac, "b" for Sham/TETA, "c" for STZ/Plac and "d" for STZ/TETA. No significant differences were found using the Tukey-Kramer post-hoc comparison when comparing the four individual groups with each other.

was decreased after addition of 25 μM CuCl₂ (Figure 4-14: a, b, c and d, p<0.001 compared to respective group without addition of CuCl₂). The individual Tukey-Kramer post-hoc comparison did not reveal any significant differences between the four groups. The disease term did become significant in the two-way ANOVA comparison indicating that CTSL activity levels were less strongly decreased in the placebo-treated diabetic and TETA-treated diabetic groups compared to the placebo- and TETA-treated Sham groups (Figure 4-14: Two-way ANOVA Disease term, p<0.05 for [Sham/Plac + Sham/TETA] vs. [STZ/Plac + STZ/TETA]).

Next, the effect of TETA on CuCl₂ addition on CTSL activity in lysates of rat kidney cortices of healthy and STZ-induced diabetic rats was measured in vitro. The results are shown in Figure 4-15 where data of the different additions (25 μM CuCl₂, 25 μM TETA or 25 μM CuCl₂ and 25 μM TETA) in tissues from Sham and diabetic animals is shown relative to respective controls without addition (the 100% value is indicated by a dashed line). As was shown in the previous graph, addition of CuCl₂ significantly decreased CTSL activity in lysates from Sham or diabetic rats compared to those not treated with CuCl₂ (Figure 4-15: a, p<0.001 and b, p<0.001 for CuCl₂ treated Sham and diabetic lysates vs. Sham and STZ control lysates, respectively). Addition of TETA along with CuCl₂ restored activity to normal levels (Figure 4-15: c, p<0.001 and d, p<0.001 for TETA + CuCl₂ treated-Sham and -diabetic lysates vs.
Chapter 4: Diabetes-induced changes in the renal ECM and the effect of TETA

Relative levels of mRNA for Mmp2, a major collagen-degrading protease were shown on page 96. MMP2 activity was now determined using gelatin zymography. Lysates of rat kidney cortices were run on gelatin-containing SDS-PAGE gels and incubated to allow for the degradation of gelatin in relation to protease activity. Gels were subsequently stained with Coomassie brilliant blue so that areas of protease activity appear white on a blue background due to the lack of gelatin in that region. Areas of activity were scanned and analysed by densitometry and are reported relative to the activity of the placebo-treated Sham group. For the initial runs, a second gel was incubated with EDTA as a negative control in parallel, to confirm that observed activity is due to metalloproteases (data not shown). Activities of MMP2 and pro-MMP2, which is activated non-specifically in the course of the assay, are known to occur at 60 kDa and 72 kDa respectively and have been well characterised employing gelatin-zymography (Aimes et al. 1995; Van Linthout et al. 2008). No significant changes in the activity of MMP2 were detectable although a tendency towards increased MMP2 levels was present in placebo-treated diabetic rats but not in the TETA-treated group.
Figure 4-16: Relative gelatinase activity measured in lysates from rat kidney cortices
Shown above is the quantification for relative gelatinase activity for MMP2 (A, 60 kDa), pro-MMP2 (B, 72 kDa) as well as the quantification of the area between 130 kDa and >250 kDa (C). A representative gelatinase gel for two samples from each of the four groups are shown below the two graphs (D) while a sample used for inter-gel calibration is shown in the centre lane. The apparent molecular masses and running fronts of bands of a molecular mass marker are indicated on the left hand side of the gel. The parts that were quantified for graphs A, B and C are indicated on the right hand side of the gel. Individual data points are shown (n=12 per group) for the graphs and mean ± SEM values are as indicated. Groups significantly different from the Sham/Plac group at p<0.05 employing Tukey-Kramer post-hoc comparison are indicated by the letter “a” for the STZ/Plac group or “b” for the STZ/TETA group.
Chapter 4: Diabetes-induced changes in the renal ECM and the effect of TETA

(Figure 4-16: A). The activity of pro-MMP2 was increased in the placebo and TETA-treated STZ-induced diabetic group compared to the healthy placebo-treated group (Figure 4-16: B; a, \( p=0.0073 \) and b, \( p=0.011 \) for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). The activity of a broad area of lysis between 130 kDa and a molecular mass higher than 250 kDa was quantified as well and shown to be decreased in the placebo- and TETA-treated diabetic groups (Figure 4-16: B; a, \( p=0.0007 \) and b, \( p=0.0002 \) for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). No TETA-related effects were seen for pro-MMP2 activity or the gelatinase activity above 130 kDa in healthy or diabetic animals. A representative zymography gel is shown below the graphs (Figure 4-16: D). The bands and the area that were quantified are indicated at the right hand side of the gel.

4.2.2.3 Protein level of the collagen cross-linking enzyme LOX

LOX is the enzyme responsible for the physiological formation of collagen cross-links while changes of LOX have been show in diabetes previously (see page 33). Relative mRNA levels for \( Lox \) were shown to be altered in a previous section (page 95) and it remained to be determined whether these changes were accompanied by increases in LOX protein levels, as this could affect the biochemical properties of collagen. LOX protein levels of the four treatment groups as determined by Western Blotting are shown in Figure 4-17. Values were normalised to beta actin and are given relative to the average of the placebo-treated Sham group. For LOX protein level measurements, 11-12 animals per group were insufficient to yield statistical power despite apparent differences in LOX levels between the four groups. As small amounts of kidney tissue were available from a previous animal trial carried out in our group, LOX protein levels were also measured in these tissues. This resulted in a total number of 19 animals per group and resulted in an increased statistical power. Levels of all samples analysed are shown in the graph (A) while a representative blot for LOX and the normaliser beta actin is shown below (B). LOX levels were increased in the placebo-treated diabetic group compared to the placebo-treated healthy group (Figure 4-17: A; a, \( p=0.0163 \) for Sham/Plac vs. STZ/Plac). Levels in the TETA-treated diabetic group were similar to those in the placebo-treated diabetic group but the difference to the placebo-treated Sham group was not statistically significant, most likely due to an increase in the standard error of the mean in that group. No significant effects of TETA treatment were detectable in the Sham animals or the diabetic animals compared to the placebo-treated animals. A representative blot for LOX protein levels of the four groups as well as for the normaliser beta actin is shown below the graph (Figure 4-17: B).
4.2.2.4 Protein level of glutathione reducing anti-oxidant enzyme GSR

Glutathione reductase (GSR) was the only enzyme involved in the defence against ROS that was shown to be dysregulated at the mRNA level in diabetic rat renal cortices (see page 98). Thus it was to be determined whether this also translated to changes at the protein level. GSR protein levels were determined by Western Blotting and normalised to beta-actin levels as well as the average value of the healthy placebo-treated group. Relative values for the samples of the four groups are shown in the graph (A) seen in Figure 4-18. A representative blot for GSR protein levels and the corresponding beta actin blot are shown below the graph (Figure 4-18: B). Despite the changes of Gsr mRNA levels in diabetic animals no changes of GSR protein level were found between the four groups.

Figure 4-17: Relative LOX protein levels in rat kidney cortices
The relative protein levels of LOX in the four treatment groups are shown in (A) and a representative Western Blot for LOX and the normaliser beta actin in "B". Individual data points (n=19 per group) as well as the mean ± SEM are shown in A. Groups significantly different from the Sham/Plac group at \( p<0.05 \) by Tukey-Kramer post-hoc comparison are indicated by the letter “a” for the STZ/Plac group.
Chapter 4: Diabetes-induced changes in the renal ECM and the effect of TETA

4.2.3 Biochemical analysis of collagen isolated from kidneys of healthy and diabetic rats and the effect of TETA

Collagen was extracted from the kidneys of the four groups of rats in order to analyse it for post-translation modifications as well as the related functional measure, pepsin digestibility (Nagaraj et al. 1996). The aim was to determine whether these properties might correlate with each other. For example, CML-modified BSA was previously reported to enhance the formation of carbonyl levels in vitro (Requena et al. 1999).

4.2.3.1 Changes in pepsin digestibility of collagen extracts

In order to measure pepsin digestibility, freeze-dried collagen extracts were incubated in pepsin/acetic acid, centrifuged and the resulting supernatants and pellets were subsequently analysed for their collagen content. The pepsin digestibility was calculated as [%] of the total amount of collagen. The pepsin digestibility of collagen was decreased in both placebo- and

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Figure 4-18: Relative GSR protein levels in rat kidney cortices
Relative protein levels of GSR in the four groups are shown in (A) and a representative Western Blot for GSR and the normaliser beta actin in “B”. Individual data points (n=11 per group) and mean ± SEM values are presented in (A). Groups significantly different from the Sham/Plac group at p<0.05 by Tukey-Kramer post-hoc comparison are indicated by the letter “a” for the STZ/Plac group.
Chapter 4: Diabetes-induced changes in the renal ECM and the effect of TETA

Figure 4-19: Pepsin digestibility of collagen extracts from rat kidneys
The pepsin digestibility of extracts of collagen from rat kidneys of the four groups is shown. Individual data points (n=11/12 per group) are given and mean ± SEM values are shown. Groups significantly different from the Sham/Plac group at p<0.05 employing Tukey-Kramer post-hoc comparison are indicated by the letter “a” for the STZ/Plac group and the letter “b” for the STZ/TETA group.

4.2.3.2 Collagen CML levels in the four groups of rats
CML levels of collagen extracts were detected by employing a dot blot method. Collagen was solubilised by digestion using a collagenase preparation, the concentration of collagen was determined by hyp assay and equal amounts of collagen were applied onto a raster of a nitrocellulose membrane in triplicates for each animal. A BSA-AGE standard curve and a substrate-free collagenase digest as a negative control were applied in parallel and the CML-specific primary and HRP-coupled secondary antibodies were used for the detection of CML. A graph summarising the results is shown in part “A” of Figure 4-20 while two representative dot blots of the four groups including the BSA-AGE standard curve and a negative control sample are shown in “B”. Levels of CML of collagen extracts were significantly increased in the placebo-treated diabetic group compared to the placebo-treated healthy group (Figure 4-20: A; a, p=0.014 for Sham/Plac vs. STZ/Plac). CML levels in the TETA-treated STZ-induced diabetic group displayed a tendency for decrease compared to the placebo-treated STZ-induced diabetic group. A decrease in CML levels in the TETA-treated diabetic group was supported by the finding that it was not significantly different from the healthy placebo-treated group (Figure 4-20: A; p=0.38 for Sham/Plac vs. STZ/TETA). This lack of statistical significance in the TETA-treated diabetic group compared to the healthy placebo-treated group was seen despite a decrease in the standard error of the mean compared to the TETA-treated diabetic groups compared to the placebo-treated Sham group (Figure 4-19: a, p=0.0446 and b, p=0.0015 for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). No changes after TETA treatment were detected in the healthy or the diabetic group.
Chapter 4: Diabetes-induced changes in the renal ECM and the effect of TETA

Figure 4-20: Relative CML levels of collagen extracts from rat kidneys
CML levels for each of the four treatment groups were measured using a dot blot relative to a BSA-AGE standard and then normalised to the average of the healthy placebo treated group. Data are summarised in “A” where individual points (n=11/12 per group) and the mean ± SEM are shown. Groups significantly different from the Sham/Plac group at \( p<0.05 \) by Tukey-Kramer post-hoc comparison are indicated by the letter “a” for the STZ/Plac group. Two representative dot blots (B) can be seen below the graph with the BSA-AGE standard curve and a negative control blotted in the top three rows with triplicates oriented vertically. Signals for the four groups, with one sample per group and blot, are displayed in the two lower rows and triplicates for each animal are oriented horizontally.

placebo-treated diabetic group (SEM=.58 for STZ/TETA compared to SEM=1.25 for STZ/Plac). The disease term of the two-way ANOVA comparison was also statistically significant whereas the interaction term approached significance (Figure 4-20: A; Two-way ANOVA Disease term, \( p<0.013 \) for [Sham/Plac + Sham/TETA] vs. [STZ/Plac + STZ/TETA]; two-way ANOVA Interaction term, \( p=0.076 \)). When comparing the standard curves of the two dot blots shown in Figure 4-20 (B), it can be seen that the reproducibility for the dot blot measurement was reasonable.
4.2.3.3 Measurement of collagen carbonyl levels in the four groups of rats

Carbonyl levels of collagen extracts were measured as a marker of oxidative stress independent of glycation. As for the detection of CML levels of collagen via dot blot, collagen was solubilised by digestion with collagenase. Carbonyl levels of solubilised collagen were detected using an ELISA kit based on the derivatisation of carbonyls with 2,4-dinitrophenylhydrazine (DNPH) to form dinitrophenylhydrazone (DNP). Derivatised DNP-carbonyl-protein conjugates were applied to an ELISA plate followed by the detection of DNP via a DNPH-specific primary antibody and a HRP-coupled secondary antibody. A chromogenic substrate for HRP was then added and absorbance measured. A standard curve was run in parallel in order to yield absolute levels of carboxyls, and levels are given as nmol of carboxyl per mg of collagen. Carbonyl levels were significantly decreased in the placebo and TETA-treated STZ-induced diabetic group compared to the healthy placebo-treated group (Figure 4-21: a, \( p=0.016 \) and b, \( p=0.039 \) for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). No significant effects of TETA treatment compared to placebo treatment on collagen carbonyl levels were observed in diabetic or healthy animals.

![Figure 4-21: Carbonyl levels of collagen extracts from rat kidneys](image_url)

**Figure 4-21: Carbonyl levels of collagen extracts from rat kidneys**

Carbonyl levels of collagen extracts from rat kidneys belonging to the four treatment groups are shown. Individual data points (\( n=11/12 \) per group) and the corresponding mean ± SEM values are indicated. Groups significantly different from the Sham/Plac group at \( p<0.05 \) by Tukey-Kramer post-hoc comparison are indicated by the letter “a” for the STZ/Plac group and the letter “b” for the STZ/TETA group.
4.2.3.4 Analysis of collagen via mPAGE – Characterisation of one additional collagen band

Collagen was also analysed employing a SDS-PAGE based method, termed mPAGE (or methacrylamido phenylboronic acid PAGE), where methacrylamido phenylboronic acid (mpba) has been added to the gel prior to polymerisation. Glycated proteins are retarded more strongly in mPAGE gels, such that they run at a higher molecular mass compared to their non-glycated counterparts (Pereira Morais et al. 2010). The chemical was synthesised and the gel was poured as was detailed on page 66, according to published methods (D’Hooge et al. 2008; Pereira Morais et al. 2010). The gel contained a final concentration of 6.25% (w/v) polyacrylamide and 0.4% (w/v) mpba. Lower amounts of polyacrylamide as well as higher amounts of mpba did not allow for a proper polymerisation of the gel. Collagen was digested with 1 mg/ml pepsin in 0.5 M acetic acid at a concentration of 1 mg of collagen per ml solution for 24 h at 4 °C. The digests were centrifuged and soluble collagen was mixed with DTT-containing Laemmli buffer, heated, and equal amounts of collagen as previously determined by hyp assay were loaded onto the gel. Gels containing mpba were run very slowly and an acceptable separation could only be achieved after 3 h of electrophoresis at 100 V. Gels were finally stained with Coomassie blue.

An mPAGE gel was run with one collagen extract (20 μl equivalent to 2 μg collagen per lane) from kidneys of each of the four groups of rats (Figure 4-22: Sham/Plac, Sham/TETA, STZ/Plac and STZ/TETA). A molecular mass marker was present in the first lane (Precision Plus Protein Dual Color Standards, BioRad, USA) with the collagen extracts in the following four lanes. BSA (20 μl equivalent to 10 μg of BSA) which was incubated in PBS (BSA/PBS), 0.5 M glucose in PBS (BSA/Glc) or 0.5 M fructose in PBS (BSA/Frc) for 8 weeks at 37 °C was run in parallel in the last three lanes. The collagen extracts from the two diabetic animals (STZ) appeared to display a small shift in the apparent molecular mass compared to the extracts from healthy animals (Sham). In addition, the bands of the diabetic animals were stained less strongly than those from healthy animals while no effect of TETA (TETA) compared to placebo (Plac) treatment animals was observed. It was surprising that the collagen from the diabetic animals stained less strongly than that from healthy animals despite equal loading of all lanes. This effect may be due to a weaker staining of collagen from diabetic animals or a non-specific retardation effect which blurs the bands making them appear fainter. Collagen bands that could be matched to the previously-identified bands from section 3.2.2.3 were numbered accordingly (1-6) while one band could not be matched (x). For all four of the collagen extracts, it was evident that the relative retardation of each band compared to the other collagen bands (running pattern) was different from the SDS-PAGE gels shown in the previous section. In addition, the running behaviour relative to the marker
Chapter 4: Diabetes-induced changes in the renal ECM and the effect of TETA

was altered. Bands # 2 and # 3 ran at approximately 115 kDa and 130 kDa on the SDS-PAGE, while they ran below 100 kDa in mPAGE. Altered running behaviour was also evident for unmodified BSA (BSA/PBS) which was expected to run at 66 kDa but actually ran at an apparent molecular mass of < 50 kDa. The glucose-modified BSA (BSA/Glc) was retarded strongly compared to the PBS-incubated BSA (BSA/PBS) while the effect on fructose-modified BSA (BSA/Frc) was much smaller. However, both BSA/Glc and BSA/Frc stained much less strongly than the control BSA/PBS. The fact that BSA/Frc was retarded only minimally as opposed to BSA/Glc confirms the previous finding by Pereira Morais et al. that mpba interacts strongly with the glucose dependent- but not the fructose dependent precursor of glycated proteins (Pereira Morais et al. 2010).

Collagen extracts from kidneys of two animals of each of the four groups were run on SDS-PAGE and mPAGE in parallel, in order to further elucidate the effect of mPAGE on the

![Figure 4-22: Analysis of collagen and glycated BSA by mPAGE](image)

Collagen extracts from the kidneys of rats from the four treatment groups were run on a 0.4 % mpba-containing 6.25 % (w/v) SDS-PAGE gel. The marker (Precision Plus Protein Dual Color Standards, BioRad, USA) is shown on the left hand side of the gel and the respective molecular masses of each band are as indicated. In the four subsequent lanes collagen extracts from the four different groups of Sham or diabetic rats treated with a placebo or TETA are shown. The bands are numbered according to those identified in section 3.2.2.3 with one band present (x) that was not evident previously. In the last three lanes BSA was run which had previously been incubated with PBS (control) or with glucose (Glc) or fructose (Frc) in order to produce in vitro-glycated BSA.
staining of collagen from diabetic and healthy animals as well as the effect of mPAGE on the retardation of collagen. It was also confirmed that the previously unidentified band seen in Figure 4-22 appears in a reproducible manner. The proteins present in that band were subsequently identified by LC-MS/MS subsequently. Both gels were self-made with a polyacrylamide content of 6.25% (w/v): one without mpba (Figure 4-23: A) and one with 0.4% (w/v) mpba (Figure 4-23: B). Considering that LC-MS/MS analysis of the unknown band was planned, solutions were sterile-filtered before use. Collagen was prepared as described in the introduction of this section and equal amounts of collagen (2 μg in 20 μl per lane) were loaded to both gels. The SDS-PAGE gel was run for 1 h at 100 V while the mPAGE gel was run for 3 h at 100 V and gels were stained with Coomassie blue subsequently. A molecular mass marker was run for both gels in the left-hand lane. Bands of the self-made SDS-PAGE gel could be matched with the previously identified collagens in section 3.2.2.3 and were numbered accordingly (Figure 4-23: A). Considering the staining intensity of the bands, it could be seen that collagen extracted from diabetic animals displayed decreased staining, even in the normal SDS-PAGE (Figure 4-23: A), although the effect appeared to be slightly stronger in the mPAGE gels (Figure 4-23: B). Thus the decrease in the intensity of staining of the bands of collagen extracted from diabetic animals may be due to a combined effect of decreased staining efficiency, in combination with a non-specific retardation effect of the collagen from diabetic animals. However, the specific retardation effect of diabetic collagen which was seen weakly in the previous gel (Figure 4-22) could not be reproduced in this gel. Thus it was concluded that the relative retardation of collagen employing mPAGE could not be used as a reliable measure for the extent of glycation of collagen.

The band (x) that was seen after separation of the collagen extracts via mPAGE gel but not after SDS-PAGE was seen again employing mPAGE (Figure 4-23: B) while it was absent after SDS-PAGE (Figure 4-23: A) This previously unidentified band seen above band # 4 (Cola1(V)) was shown to contain three different isoforms of type IV collagen, Cola1(IV), Cola3(IV) and Cola4(IV) via trypsin digestion followed by LC-MS/MS analysis. The collagen type IV a1 isoform (160.7 kDa) was previously shown to run slightly higher on SDS-PAGE gels than Cola1(V) (184.0 kDa) despite the higher calculated molecular mass of Cola1(V) (Miller et al. 1982). The other two isoforms Cola3(IV) (161.8 kDa) and Cola4(IV) (158.8 kDa) have similar molecular masses as Cola1(IV) (160.7 kDa) which gives a possible explanation for the simultaneous detection of the three type IV collagen isoforms in one band. Thus while mPAGE was not suitable for the analysis of the glycation of collagen, it enabled the identification of the mesh-like type IV collagen in the pepsin digests of my extracts, although it appeared to be present at rather low concentration as judged by the colloidal Coomassie stain.
Figure 4-23: Comparison of SDS-PAGE analysis and mPAGE analysis of collagen extracts – Identification of a previously unrecognised collagen band via LC-MS/MS

Collagen extracts from the kidneys of rats from the four treatment groups were run on 6.25% (w/v) SDS-PAGE gels without (A) or with 0.4% (w/v) mpba (B) added. The marker (Precision Plus Protein Dual Color Standards, BioRad, USA) is shown on the left-hand side of the gels and the respective molecular mass of each band is indicated. In the eight subsequent lanes collagen extracts from the four different groups of healthy or STZ-diabetic rats treated with a placebo or TETA are shown. The bands are numbered according to those identified in section 3.2.2.3. One band (x) is present in the gel containing 0.4% mpba (B) but not in that without mpba (A) and was determined to contain different isoforms of type IV collagen (Cola1(IV), Cola3(IV) and Cola4(IV)) by LC MS/MS. Band 1 resembling pepsin has exited the SDS-PAGE gel (A) and thus cannot be seen.
4.2.4 Correlation analysis of the data

In section 4.2.3 collagen extracts from kidneys of the four treatment groups of animals were analysed for post-translational modifications and associated changes. Changes at the protein level in kidney cortices of the four groups with a potential influence on these post-translational modifications were analysed in section 4.2.2 - partially based on transcriptional changes which were detected in section 4.2.1 of this chapter. In an effort to better understand the interaction between post-translational modifications, protein levels and activity levels of enzymes, all of the data were examined further using linear correlation analysis. Data were analysed by calculation of the Pearson product-moment correlation coefficient which yields a range of correlation ($r$) from -1 to 1, indicating a strong negative or strong positive correlation, respectively, while values approaching 0 indicate an absence of correlation (Taylor 1990; Chinchilli et al. 1995). As the calculation of the correlation coefficient $r$ is sensitive to outliers, a plot of the analysed graph is shown where the fitted line, the 95% confidence interval, as well as the $r$-value are shown along with the fitted data points (Devlin et al. 1975). It was also tested whether the given curve has a slope significantly different from 0, and the $p$-value is displayed for each graph. Whenever a slope was found to be significantly different from 0 at $p<0.05$ the co-variation ($Cv=r^2*100\%$) was calculated and is mentioned in the description. This gives a measure, ranging from 0-100%, of the extent to which the increase or decrease in one variable can be explained by an increase or decrease of the other variable.

Increased mRNA and protein levels of the collagen-degrading protease CTSL were previously found in renal cortices of placebo treated- and TETA-treated diabetic animals compared to placebo-treated healthy animals. Levels of the AGE CML were shown to be increased in kidney collagen extracts from the placebo-treated diabetic group compared to those from the placebo-treated Sham group. As AGES are known to affect the digestibility of collagen in vitro, it would be of interest to know whether CML levels have an effect on the protein level of the collagen-degrading protease CTSL in vivo (Creemers et al. 1998; Li et al. 2004). While correlation analysis cannot provide a definite answer to this question, it can provide some further evidence. Thus correlation analysis for CTSL sc and CTSL dc (both active forms of CTSL) vs. CML was carried out. Data for CTSL and CML were log-transformed before graphing as these sets were obtained using HRP-coupled secondary antibodies in combination with ECL reagent, which leads to a skewing that can be corrected by log transformation (Kreutz et al. 2007). The resulting correlation analysis is shown in Figure 4-24. A positive correlation of moderate strength was observed for the analysis of CTSL sc and CTSL dc vs. CML levels when combining all four groups of rats (Figure 4-24: A; $r=0.38, p=0.011$ and $Cv=14.6\%$ for CTSL sc- vs. CML levels. B; $r=0.49, p=0.0007$ and
Figure 4-24: Linear regression analysis for CTSL protein level vs. CML level
Regression analysis was performed for CTSL sc and CTSL dc protein levels from rat kidney cortices vs. CML levels of collagen extracts from the same kidneys. Analysis is shown for CTSL sc and CTSL dc vs. CML levels for all four groups (A and B) as well as for placebo-treated (C and D) or TETA-treated (E and F) healthy and STZ-diabetic animals separately. Shown are the regression lines (solid) and the 95% confidence intervals (dashed). The correlation coefficients \( r \) are given in the top left -hand corners of each graph. Black arrows indicate healthy samples with particularly high CML levels. The \( p \)-values indicating whether the slope of the regression line is significantly different from 0 are also displayed in the top left -hand corners of each graph. A value of \( p<0.05 \) was considered significant. Black arrows in the analysis separated by treatment groups (C-F) indicate samples from healthy rats with particularly high CML levels.
Cv=24.1% for CTSL dc- vs. CML levels). Differences became apparent when the groups were analysed separately, based on their treatment. A strong positive correlation was observed for the analysis of CTSL sc and CTSL dc vs. CML levels for the placebo-treated Sham and diabetic groups (Figure 4-24: C; \( r=0.62, \ p=0.0023 \) and Cv=38.0% for CTSL sc- vs. CML levels. D; \( r=0.72, \ p=0.0002 \) and Cv=51.9% for CTSL dc- vs. CML levels). This increase in correlation strength was observed despite the decreased sample size (\( n=22 \) for two groups vs. \( n=44 \) for the four groups). The correlation between CTSL sc and CTSL dc and CML levels was completely absent from the two TETA-treated groups (Figure 4-24: E; \( r=0.06 \) and \( p=0.79 \) for CTSL sc vs. CML levels. F; \( r=0.18 \) and \( p=0.43 \) for CTSL dc vs. CML levels). Black arrows in the analysis, separated by treatment groups, indicate samples from healthy rats with particularly high CML levels (see Figure 4-24: C-F). This was associated with high CTSL dc- and CTSL sc protein levels in the placebo- but not the TETA-treated samples from healthy rats which is discussed in the final section of this chapter.
Analysis for CTSL sc- and CTSL dc protein level vs. collagen pepsin digestibility was also carried out. An altered pepsin digestibility of ex vivo collagen extracts from kidney cortices measured in vitro may affect protein levels of the collagen degrading protease CTSL measured in kidney cortices of the same animals. CTSL sc and CTSL dc levels were log-transformed as was done for the previous analysis. A moderately-strong negative correlation was found for both CTSL sc and CTSL dc protein levels vs. pepsin digestibility of collagen (Figure 4-25: A; \( r = -0.51 \), \( p = 0.0005 \) and \( C_v = 25.6\% \) for CTSL sc level vs. pepsin digestibility. B; \( r = -0.46 \), \( p = 0.0019 \) and \( C_v = 20.8\% \) for CTSL dc level vs. pepsin digestibility). No major changes were found when analysing the samples separated by treatment (data not shown).

Figure 4-25: Linear regression analysis for CTSL protein level vs. pepsin digestibility of collagen
Correlation analyses for CTSL sc (A) and dc (B) protein level vs. the pepsin digestibility of collagen are shown, with regression lines (solid) and 95% confidence intervals (dashed). The correlation coefficients (\( r \)) and \( p \)-values, indicating whether the slopes of the regression lines are significantly different from 0, are displayed in the top right-hand corners of each graph. A value of \( p < 0.05 \) was considered significant.
Chapter 4: Diabetes-induced changes in the renal ECM and the effect of TETA

Additional analysis was performed to determine whether the collagen content in kidney cortices correlated with changes in CTSL protein levels in these tissues. Measures for CTSL sc and CTSL dc protein level were log-transformed as for the previous analyses. No significant correlation was found for the collagen content of kidney cortices vs. CTSL sc- or CTSL dc level (Figure 4-26: A; \( r = -0.16 \) and \( p = 0.30 \) for collagen content vs. CTSL sc level. B; \( r = -0.29 \) and \( p = 0.062 \) for collagen content vs. CTSL dc level). Again, no major changes were found when analysing the samples separated by treatment (data not shown).

![Figure 4-26: Linear regression analysis of collagen content vs. CTSL protein level](image)

The protease MMP2 is a major extracellular collagen-degrading proteinase (Creemers et al. 1998; Kerkvliet et al. 1999). MMP2 activity levels were not altered in diabetic rats or by treatment with TETA (see section 4.2.2.2) and consistent with this observation, no correlation with collagen levels was observed when all four groups were analysed together (Figure 4-27: A) or separated by treatment (data not shown). Activity levels of pro-MMP2 were shown to be increased in diabetic animals irrespective of treatment in section 4.2.2.2. No correlation was detected for pro-MMP2 running at 72 kDa when analysing all four groups together (Figure 4-27: B; \( r = 0.061 \) and \( p = 0.69 \)). An inverse correlation between pro-MMP2 activity and collagen content was detected when analysing the placebo-treated Sham and diabetic groups separately from the respective TETA-treated groups (Figure 4-27: C; \( r = 0.54 \),...
Chapter 4: Diabetes-induced changes in the renal ECM and the effect of TETA

Three regions of activity could be detected on the gelatinase gels. Two bands of activity were detected at 60 kDa (MMP2) as well as at 72 kDa (pro-MMP2), while one broad area of activity was detected, ranging from 130 kDa up to a molecular mass of 250 kDa and higher. No major changes in MMP2 activity levels were detected so that no correlation analysis was carried out for MMP2 activity. It was observed that activity levels of pro-MMP2 were generally

\[ r = -0.092 \]
\[ p = 0.55 \]

\[ r = -0.061 \]
\[ p = 0.69 \]

\[ r = -0.54 \]
\[ p = 0.010 \]

\[ r = 0.26 \]
\[ p = 0.25 \]

Figure 4-27: Linear regression analysis for collagen content vs. gelatinase activity at 60 kDa (MMP2) and 72 kDa (pro-MMP2)
Displayed is the regression analysis of the collagen content of rat kidney cortices vs. relative gelatinase activity for all four groups at 60 kDa (MMP2, A) and 72 kDa (pro-MMP2, B). Separate analyses of the placebo- (C) and TETA-treated (D) groups are also shown for the activity at 72 kDa. Shown are the regression lines (solid) and 95% confidence intervals (dashed). Correlation coefficients \((r)\) and \(p\)-values, indicating whether the slope of the regression line is significantly different from 0, are displayed in the top left-hand corner of each graph. A value of \(p < 0.05\) was considered significant.

\( p = 0.010 \) and \( Cv = 29\% \). This correlation was absent when analysing the TETA-treated Sham- and diabetic groups separately (Figure 4-27: D; \( r = 0.26 \) and \( p = 0.25 \)).
higher in both, the placebo- and TETA-treated diabetic groups, compared to respective Sham groups whereas activity in the broader higher molecular mass area was generally lower. It was thus of interest to determine whether these changes were correlated within animals. No correlation was observed when analysing all four groups together (Figure 4-28: A; \( r=-0.19 \) and \( p=0.21 \)). When analysing the groups separated by treatment, the correlation was significant for the placebo-treated Sham- and diabetic groups (Figure 4-28: B; \( r=-0.45 \), \( p=0.032 \) and \( C_v=20\% \)). No such correlation was observed for the TETA-treated Sham- and diabetic groups (Figure 4-28: C; \( r=0.048 \) and \( p=0.83 \)).

It was also of interest to determine whether the two post-translational modifications that were measured in collagen extracts from kidneys of the four groups would correlate with and thus
Chapter 4: Diabetes-induced changes in the renal ECM and the effect of TETA

potentially influence each other. As previously described, CML levels were log-transformed for analysis. Plotting collagen carbonyl levels vs. CML levels identified a weak but significant correlation (Figure 4-29: A; \( r = -0.29, p = 0.045 \) and \( \text{Cv} = 8.7\% \)). When the groups were analysed separated by treatment, the placebo-treated Sham- and diabetic groups displayed a strong inverse correlation (Figure 4-29: B; \( r = -0.52, p = 0.012 \) and \( \text{Cv} = 26.6\% \)). This was not the case for the TETA-treated Sham and diabetic groups, wherein no correlation was present (Figure 4-29: C; \( r = -0.092 \) and \( p = 0.67 \)).

![Figure 4-29: Linear regression analysis of collagen carbonyl level vs. CML level](image)

Correlation analysis of carbonyl levels from collagen extracts vs. CML levels from collagen extracts is displayed above. Shown are the regression lines (solid) and the 95 % confidence intervals (dashed). The correlation coefficients (\( r \)) and \( p \)-values, indicating whether the slope of each regression line is significantly different from 0, are also displayed in the bottom left-hand corner of each graph. A value of \( p < 0.05 \) was considered significant.

Protein levels of the collagen cross-linking enzyme, LOX may also influence the pepsin digestibility of collagen extracts from kidneys (Reiser et al. 1992). Thus the possible correlation between these two measures was investigated. LOX protein levels were acquired
Chapter 4: Diabetes-induced changes in the renal ECM and the effect of TETA

in the same way as CTSL sc and CTSL dc protein levels and were thus log-transformed for analysis as well. The $p$-value for the correlation analysis became significant and together with the obtained $r$-value would indicate a weak correlation (Figure 4-30: $r=0.30$, $p=0.045$ and $Cv=9.3\%$). However, one apparent outlier was present (Figure 4-30: Black circle) and excluding this data point resulted in a very weak correlation coefficient $r$, and a slope not significantly different from 0. Thus these values need to be interpreted with caution.

![Figure 4-30: Linear regression analysis of pepsin digestibility vs. Lox protein level](image)

Correlation analysis for collagen pepsin digestibility vs. Lox protein level is displayed above. Shown are the regression line (solid) and the 95% confidence interval (dashed). The correlation coefficient ($r$) is given in the bottom left hand corner of the graph. The $p$-value indicating whether the slope of the regression line is significantly different from 0 is also displayed in the bottom left hand corner of the graph. A value of $p<0.05$ was considered significant. One outlier (circled in black) strongly influenced the analysis casting doubt on the actual significance of the finding.
In analysing the gelatin zymography assay, the results of which are described in section 4.2.2.2, it was observed that in addition to the expected activity observed at the molecular mass for MMP2 and pro-MMP2, strong gelatinase activity occurred at a high molecular mass above 130 kDa. It was also observed that activity in this area, where the different isoforms of collagen run (starting at $M_{\text{Cola2(II)}}=130$ kDa up to values greater than 400 kDa for γ-band multimers of collagen) was decreased in placebo- and TETA-treated diabetic animals compared to placebo-treated Sham (see section 3.2.2.3 for analysis of pepsin solubilised collagen extracts). It was thus of interest to determine whether the gelatinase activity above 130 kDa correlated with the pepsin digestibility of collagen extracted from kidneys of the corresponding animals. A moderate but significant correlation was found between these two variables when all four groups of rats were analysed in parallel (Figure 4-31: $r=0.41$, $p=0.0043$ and $Cv=16.7\%$). No differences were observed between the treatment groups when they were analysed separately although the correlation coefficient was lower due to the decreased sample size (data not shown).

![Figure 4-31: Regression analysis of pepsin digestibility of collagen vs. gelatinase activity](image)

**Figure 4-31: Regression analysis of pepsin digestibility of collagen vs. gelatinase activity**

The correlation between the collagen pepsin digestibility and the gelatinase activity of kidney cortices of rats of the four groups measured between 130 kDa and >250 kDa is displayed. Shown are the regression line (solid) and the 95% confidence interval (dashed). The correlation coefficient ($r$) is given in the bottom right hand corner of the graph. The $p$-value indicating whether the slope of the regression line is significantly different from 0 is also displayed in the bottom right hand corner of the graph. A value of $p<0.05$ was considered significant.
4.3 Discussion

It is not clear whether enhanced AGE formation contributes to the development of diabetic complications in general, and diabetic nephropathy in particular (Goh et al. 2008). Doubt has been cast on one of the hypothesis, namely the AGE-RAGE interaction (Thornalley 2007). In support binding of CML modified proteins to RAGE has been shown to activate endothelial cells, smooth muscle cells and macrophages in vitro and the interaction has also been linked to the nuclear factor kappa B (NF-κB) (Morcos et al. 2002; Ramasamy et al. 2007). Critics of the hypothesis argue that concentrations of AGE modifications of endogenous proteins are not high enough to trigger RAGE in vivo, while food derived proteins may be modified to a higher extent if cooked at high temperatures are being degraded prior to internalisation and thus unable to trigger the receptor (Heizmann 2007). Another hypothesis is based on the modification of collagen and other long-lived proteins with AGEs, which are known to alter the proteolytic as well as mechanical properties of these proteins, possibly contributing to the fibrosis that occurs in diabetic nephropathy (Goh et al. 2008). In addition, elevated levels of the AGE CML are related to an increase in copper binding, which could in turn lead to an enhanced formation of AGEs in diabetes via metal-catalysed oxidative stress, possibly resulting in a vicious cycle (Saxena et al. 1999; Thorpe et al. 2002). The Cu(II) chelator TETA was previously shown to normalise proteinuria in kidneys STZ-diabetic rats, while its effect on AGE formation and other post-translational modifications with a potential relation to fibrosis in diabetic kidneys has not yet been investigated (Lu et al. 2010).

Here, the enhanced formation of the AGE CML on collagen extracts from diabetic kidneys was shown while treatment with the Cu(II) chelator TETA, resulted in a trend to reverse the increased CML content with no effect on blood glucose levels, thus consistent with a role of Cu(II) in CML formation. A potential reason for the trend towards a decrease in CML levels rather than a full normalisation may be that the collagen turnover is rather slow for some collagen pools (Laurent 1987; Verzijl et al. 2000). Thus, even if new CML formation is inhibited completely upon TETA administration, increased CML-levels would persist on collagen that was present before the start of the treatment. Furthermore, TETA administration is thought to inhibit the oxidative part of the pathway for CML formation but not the initial glycation reaction or CML formation from reactive dicarbonyls (see page 18 ff. for details of CML formation). Increased blood glucose levels were still present in the TETA-treated STZ-diabetic rats. A potential future treatment would thus have to target blood glucose levels in parallel, resulting in the inhibition of the glycation reaction as well as the oxidative part of the AGE formation pathway.
While it was previously shown that CML-modified BSA leads to increased formation of protein carbonyls in vitro, considered a marker of metal-catalysed oxidative stress, kidney collagen carbonyl levels were decreased rather than increased in diabetic animals with elevated collagen CML levels (Requena et al. 1999; Requena et al. 2001). Moreover an inverse correlation between collagen-carbonyl levels and -CML levels was found here in placebo-treated diabetic and Sham rats. A preferential glycation of certain collagen lysine residues over others has been shown previously while some lysine residues do not form glycation adducts at all (Reiser et al. 1992). The formation of carbonyls also takes place on lysine residues in addition to arginine residues which is another major site of AGE formation (Requena et al. 1999). The finding of an inverse correlation between carbonyl and CML levels could thus be due to the fact that the sites of formation on collagen for these modifications are limited in vivo. Furthermore, some of the protein-bound carbonyls subsequently form stable products like AGEs (Liggins et al. 1997). In this case, lower carbonyl levels do not indicate an (unlikely) decrease in oxidative stress in diabetic kidneys, but rather point to the (gradual) saturation of these sites with AGEs or AGE precursors which do not comprise DNPH reactive carbonyl groups. In this regard, it is important to note that the carbonyl group present in the Amadori compound does not react with the DNPH-based carbonyl assay (Liggins et al. 1997; Stefek et al. 1999). Decreased or unaltered protein carbonyl levels have previously been reported in cytosolic liver- or kidney protein extracts from STZ-diabetic rats, respectively in support of the current finding (Portero-Otin et al. 1999). No correlation between collagen-carbonyl levels and -CML levels was present in TETA-treated groups, possibly due to the trend towards decrease in CML levels in TETA-treated diabetic rats. TETA treatment did not lead to a subsequent increase in protein carbonyl levels in diabetic animals, which can be explained by the finding that the formation of the AGE precursor, Amadori compound is not inhibited by anti-oxidative conditions and would be expected to be present at higher levels in these rats, due to the elevated blood glucose levels (Fu et al. 1994).

The application of mPAGE for the analysis of collagen extracts for glycation after pepsin solubilisation was found not to be suitable. The analysis is based on a stronger retention of glycated vs. non-glycated molecules of the same molecular weight. Due to the high molecular mass of the collagen molecules (between 100 kDa and above 400 kDa for γ-multimers), these changes in retention were negligible while glycated BSA displayed a strong retention over non-glycated BSA. However, mPAGE analysis altered the retardation pattern of collagen in the gel compared to SDS-PAGE, and thus enabled the identification of several
Chapter 4: Diabetes-induced changes in the renal ECM and the effect of TETA

isoforms of the basement membrane collagen type IV, which were not visible after SDS-PAGE.

The pepsin digestibility of collagen from STZ-diabetic rat kidneys was found to be decreased in this study and a connection with AGE formation has been demonstrated previously (Schnider et al. 1981; Brownlee et al. 1986; Candido et al. 2003). While CML levels were increased in diabetes alongside decreased pepsin digestibility, no correlation between these parameters was present. Collagen pepsin digestibility may be affected by collagen synthesis and degradation as well as the enzymatic cross-link formation by LOX in addition to AGE levels (Reiser et al. 1992). All of these factors mentioned above were altered here in diabetic rats, including increased LOX mRNA and protein levels, which may explain the lack of correlation between pepsin digestibility and CML levels of collagen. Transcriptional levels of the Lox-processing enzyme BMP1 were also increased in diabetic rats, possibly contributing to the decreased pepsin digestibility, whereas no changes were seen after TETA treatment. Interestingly, increased collagen protein levels were not found alongside the decreased pepsin digestibility. This surprising finding is discussed in the following sections.

In this study, a decrease in collagen protein levels determined by hyp measurement was found while treatment with TETA normalised collagen protein levels in STZ-diabetic rats. Collagen levels by hyp measurement were previously shown to be increased in the kidneys of STZ-diabetic rats (Gong et al. 2008). One major difference in the hyp measurement of this previous study was the hydrolysis of tissue in 2 M NaOH in an autoclave for 20 min. This method of hydrolysis was found to grossly underestimate the kidney collagen content in the current study so that extensive hydrolysis in 6 M HCl was employed instead. Another difference was the perfusion of kidneys with saline buffer prior to tissue collection. The altered hydrolysis procedure as well as the perfusion of the kidney may have both contributed to the discrepancies in hyp levels shown here.

The decreased levels of glomerular as well as tubulointerstitial collagen mRNA in diabetic rats could partially explain the finding of decreased collagen protein levels in the placebo-treated group whereas TETA treatment did not have an effect on mRNA levels. Collagen mRNA levels were previously shown to be increased in kidneys of STZ-diabetic rats with a normalising effect of TETA treatment (Gong et al. 2008). The major difference compared to the current study was the use of one normaliser for mRNA levels while three normalisers were used in the study reported here. Protease levels were analysed as they may contribute to the decreased collagen levels in STZ-diabetic rats. Altered protease levels may also explain the normalisation of collagen levels after TETA treatment without significant effects.
Chapter 4: Diabetes-induced changes in the renal ECM and the effect of TETA

on collagen mRNA levels. As discussed in section 1.2.3 the extracellular protease MMP2 in combination with the lysosomal protease CTSL and possibly CTSB are thought to be important for the degradation of soft tissue collagen including the kidney.

Activity levels of free MMP2, determined by gelatinase assay were very low in kidney cortices and unaltered in diabetic rats, while pro-MMP2 activity levels were increased in diabetic rats in line with the trend towards an increase in Mmp2 mRNA levels. A possible reason for low MMP2 activity may be the association of the free enzyme with inhibitors or substrates (Aimes et al. 1995). Thus levels of unbound, active MMP2 may provide an inadequate representation of the total MMP2-dependent collag enolytic potential in vivo. The increased pro-MMP2 activity in the cortex of diabetic kidneys can be interpreted as a response to the decreased digestibility of collagen. Previous evidence for a compensatory increase of protease activity in STZ-diabetic rats was reported for glomerular basement membrane extracts and respective collagen extracts from kidneys (Knecht et al. 1987; Leber et al. 1987). Here, absolute collagen levels correlated with levels of pro-MMP2 in placebo- but not TETA-treated Sham and diabetic rats. The correlation of collagen with pro-MMP2 in placebo-treated rats supports the role of this protease in the degradation of the extracellular matrix. The presence of such a correlation with the pro-MMP2 enzyme suggests that the activation of MMP2 is not the rate-limiting step in collagen degradation in placebo-treated rats. The absence of the correlation in TETA-treated animals could be due to an effect on the activity of the enzyme in vivo or on the activation of the enzyme. An increased activation of MMP2 through H₂O₂ has been shown via the increased expression of Mt1-MMP (Yoon et al. 2002). This was mediated by nuclear factor kappa B (NF-KB) while this factor is increased in diabetic nephropathy in humans (Schmid et al. 2006). The activity levels of Mmp2 displayed a tendency for increase which was not significant in placebo-treated nor in TETA-treated diabetic rats. A broad area of EDTA-sensitive gelatinase activity was seen at molecular weights between 130 kDa up to 250 kDa and higher. As opposed to the activity level of pro-MMP2, activity at these molecular weights was decreased rather than increased in diabetic rats. It can be hypothesized that activity in this area resembles substrate-bound (e.g. collagen) MMPs so that the decreased activity in diabetic rats is due to a decrease in soluble collagen entering the gel. In support of this, the activity in this area correlated inversely with pepsin digestibility of collagen.

Here, increased CTSL sc and CTSL dc protein levels were found alongside decreased pepsin digestibility in diabetic rats. CTSL activity levels displayed a tendency for increase in both placebo- and TETA-treated diabetic rats. However, the activity assay employed kidney cortex tissue lysates in a standardised environment and an artificial substrate, so that it may
be difficult to draw conclusions from such a setting. CTSL sc and CTSL dc protein levels were both shown to correlate inversely with the pepsin digestibility of ex vivo kidney collagen both in placebo- and TETA-treated healthy and diabetic rats, supporting a compensatory increase of protease levels in diabetes (Knecht et al. 1987; Leber et al. 1987). Both CTSL and pepsin digest the non-helical region of collagen which may make them susceptible to the same post-translational modifications (Worrall et al. 1966; Garnero et al. 1998). A strong correlation between collagen CML levels and CTSL sc and dc protein levels was present in placebo-treated healthy and diabetic animals. The correlation was absent in those animals treated with the Cu(II)-chelator TETA. One possible reason for this lack of correlation could be the partially decreased CML levels in the STZ/TETA-treated group. A particular sensitivity of CTSL for modifications of collagen was previously shown for the enzymatic modification by glycosaminoglycans (GAG) where the activity was reduced 10-fold in modified compared to unmodified collagen (Li et al. 2004). CTSL sc and dc protein levels did not correlate with collagen levels although the correlation between CTSL dc and collagen approached significance. This may be due to the intracellular action of cysteine cathepsins. It takes place after the extracellular degradation by MMP2 and the phagocytic uptake of collagens explaining a previously demonstrated weak link to absolute collagen levels (Everts et al. 1996; Creemers et al. 1998).

Increased copper binding to CML-proteins may have additional effects on CTSL. One of the placebo- as well as the TETA-treated healthy rats displayed high collagen CML levels despite normal blood glucose levels, for unknown reasons. Interestingly, in the placebo-treated, but not the TETA-treated rat, this was associated with high CTSL sc and dc protein levels (indicated by black arrows in Figure 4-24 on page 120) suggesting that TETA also influences the correlation, independent of effects on CML levels. It is known that Cu(II) inhibits the activity of the parasite L-type cathepsins cruzain and falcipain-2 (Lockwood 2006). In section 4.2.2.2, it is shown that CuCl2 decreased CTSL activity in kidney lysates from healthy and diabetic rats while addition of TETA normalised CTSL activity in vitro. Considering that CML binds copper, TETA may act by extracting this copper pool, thus exerting a normalising effect on CTSL activity (Saxena et al. 1999; Eaton et al. 2002). It is well known that kidney copper content is increased in diabetes (Failla et al. 1981; Uriu-Adams et al. 2005) and TETA treatment was shown to normalise kidney copper in STZ-diabetic rats (Gong et al. 2008).

Messenger RNA levels of the cysteine protease Ctsk were also measured, as it is a strong collagenase, albeit of very low abundance in kidneys; levels were here found not to be altered in STZ-diabetic kidneys, and were unaffected by TETA treatment. CTSB is a much
Chapter 4: Diabetes-induced changes in the renal ECM and the effect of TETA

weaker collagenase than CTSL while more recent reports suggest that CTSB does not degrade collagen at all (Li et al. 2004). In this chapter, we show that the decrease in mRNA levels of Ctsb in diabetic rats was consistent with that of the non-collagen-degrading cysteine protease Cts h rather than the increase in mRNA levels seen for Ctsl. Also, CTSB protein levels were unaltered in the placebo-treated diabetic group, suggesting that altered CTSB protein levels do not contribute to the decrease in collagen levels in the diabetic rats. The slight decrease in CTSB protein levels independent of changes in mRNA levels in the TETA-treated healthy and diabetic rats is an interesting phenomenon which cannot be explained currently. Some clues may be obtained from future immunohistochemistry experiments.

Several additional observations were made at the transcriptional level. As opposed to the frequently implied importance of growth factors in chronic kidney disease arising from diabetes or other diseases, no changes in mRNA levels of growth factors Tgfβ1, Ctgf and Vegfa were found in this study (see section 1.2.2.1 for a summary). Such discrepancies may be explained by the use of different models, different-time frames of diabetes, and the use of different methods or normalisers for the measurement of mRNA levels. One study reported increased Tgfβ mRNA levels in the STZ-diabetic rats after up to 2 weeks of diabetes while the current study ran for a total of 16 weeks, so that the time frame may be responsible for the different findings (Park et al. 1997). Another study reported increased levels of Tgfβ mRNA by northern blotting in glomeruli from STZ-diabetic rats after 24 weeks while whole cortex lysates from kidneys were used here (Nakamura et al. 1993). Further factors influencing different reports regarding levels of growth factors may be the presence of different isoforms of Tgfβ. The isoform Tfgb1 analysed here is believed to be the major contributor to fibrosis in diabetic nephropathy (Reeves et al. 2000).

Regarding the enzymes involved in the defence against oxidative and glycoxidative stress, several interesting observations were made at the transcript level. Reduced glutathione is necessary as a co-factor for the hydrogen peroxide-detoxifying glutathione peroxidase (GPX). While mRNA levels of the extracellular-acting isoform Gpx3 could not be interpreted due to the large differences in relative mRNA levels within groups for unknown reasons, mRNA levels of glutathione synthetase reductase were decreased. However, this did not translate into altered GSR protein levels in the kidney in STZ-diabetic rats, consistent with previous reports of protein and activity levels (Wohaieb et al. 1987; Walter et al. 1991). Transcript levels of the superoxide dismutases (Sod1-3) as well as catalase (Cat) were unaltered in diabetic kidneys and no effect of TETA treatment was detected. Effects of diabetes or TETA treatment on activity levels cannot be excluded, however. A decrease of SOD1, SOD3 and CAT activity levels in STZ-diabetic rats was shown previously (Wohaieb et
The glyoxalase system, consisting of GLO1 and GLO2, is capable of the detoxification of AGE precursors like the α-dicarbonyl glyoxal and has thus been thought to ameliorate glycoxidative stress in the body, while altered levels in diabetes have been shown previously (see section 1.3.4.1). Here, messenger RNA levels of Glo2 were decreased in placebo-treated diabetic rats, while TETA treatment decreased Glo1 and Glo2 levels further. It seems unlikely that this deregulation of glyoxalase mRNA levels contributed to the enhanced formation of AGEs on collagen in diabetes, as levels of CML which may form via glyoxal, displayed a tendency for decrease after TETA treatment in diabetes, resulting in CML levels that were not significantly higher than those from placebo-treated rats. Previously, decreased activity levels of Glo2 have been reported in STZ-diabetic rats consistent with the decreased mRNA measurements obtained in the current study (Phillips et al. 1993).

The aldose reductases AKR1A1, AKR1B1 and AKR1C1 are also involved in the detoxification of α-dicarbonyls (Fujii et al. 1995; Suzuki et al. 1998); here, they did not display any changes on a transcript level in diabetes or after TETA treatment. The enzyme 2-oxoaldehyde dehydrogenase (ALDH1A1) can catalyse the enzymatic processing of 3-deoxyglucosone (Collard et al. 2007). The decreased levels found here in the diabetic rats may reflect the decreased body weight, as Aldh1a1 mRNA levels in visceral fat were found to positively correlate with BMI (Kiefer et al. 2012). While decreased mRNA levels of Aldh1a1 may contribute to the enhanced formation of other AGEs, it is unlikely that it influences CML levels as CML does not form via 3-deoxyglucosone (see page 18 ff. for CML formation).

It is known that CML-modified BSA, amongst other ligands, is capable of triggering the so called “receptor for AGEs” (RAGE) in vitro which can lead to a positive feedback loop, resulting in increased Rage transcription levels (Kislinger et al. 1999; Goldin et al. 2006). No changes of mRNA levels were found for Rage despite increased collagen CML levels. One possible reason could be that collagen CML adducts are not suitable for triggering Rage, as collagen is an anchored ECM protein which may not happen to co-localise with the receptor. In addition, the interaction between Rage and AGE levels present in vivo has been disputed in general in recent years (Heizmann 2007; Ramasamy et al. 2007; Thornalley 2007). The enzyme amine oxidase copper-containing 3 (AOC3), also known as semicarbazide sensitive amine oxidase (SSAO), is a potential contributor to the formation of AGEs while overexpression and inhibition studies implied a role in the deposition of the extracellular matrix (Langford et al. 2002; Gokturk et al. 2003; Obata 2006). Protein levels were previously
shown to be increased in kidneys of STZ-diabetic rats while TETA treatment normalised levels (Gong et al. 2008). Another study showed an initial increase of AOC3 activity due to an increase of protein level after 1-2 weeks of diabetes. A strong decrease in AOC3 activity was seen between 2 and 4 weeks of diabetes so that levels were the same as in healthy rats after 4 weeks of diabetes (Hayes et al. 1990). The decrease continued after 4 weeks of diabetes, and rats displayed a trend for decrease in AOC3 activity after 8 weeks of diabetes compared to healthy rats. Our current study shows a strong decrease of mRNA levels of Aoc3 in rats with 16 weeks of diabetes, and is thus consistent with the second study assuming that the trend towards decreasing AOC3 activity continues. TETA treatment did not have an effect on Aoc3 mRNA levels in STZ-diabetic rats, but might exert effects on AOC3 activity considering that copper is a co-factor of the enzyme.

In summary a lost equilibrium of collagen in diabetic kidneys was detected with evidence for a disturbed collagen synthesis paralleled by an altered collagen degradation pathway. TETA treatment restored this equilibrium through ameliorative effects on AGE formation while these effects were associated with changes on the degradation pathway. On the contrary TETA treatment did not exert any effects on an mRNA level. The finding of unaltered mRNA levels of Tgfβ1, Ctgf, Vegfa as well as Rage does not support an on-going inflammatory response in diabetic animals (Liu 2011). However NF-kappa B levels which were previously shown to be altered in diabetic kidneys and connected to a possible AGE-RAGE induced inflammatory response were not measured as this was not the target of the thesis (Morcos et al. 2002).
Chapter 5: Diabetes-induced changes in the ECM of cardiac LV and effect of TETA

Chapter 5 – Diabetes-induced changes in the ECM of the LV of the heart - the effect of TETA

5.1 Introduction

Fibrosis is a hallmark of both DN (as discussed in the previous chapter), and DCM, which is the focus of the current chapter. Molecular mechanisms leading to fibrosis in diabetic cardiomyopathy are still poorly understood. Proposed contributors to myocardial fibrosis include alterations in transcriptional regulation caused by growth factors, along with altered transcription levels of collagen isoforms and changes in the amounts and activity of collagen-degrading proteases (Asbun et al. 2006). AGEs have also frequently been considered to be implicated in the development of fibrosis in DCM, based in part on the finding that AGE formation is enhanced under high-glucose conditions, and that this in turn may lead to an alterations in collagen digestibility (for example see paragraph 1.3.3.1) (Bell 2003; Asbun et al. 2006). While this hypothesis is attractive, to date relatively little research has been focused on the effect of AGE-modified substrates on relevant proteases in vivo.

Here, the ECM of the LV of hearts from STZ-diabetic rats was analysed in order to further elucidate mechanisms that may contribute to the formation of myocardial fibrosis. The general approach employed was consistent with the investigations described in the previous chapter, wherein changes in the extracellular matrix of the kidney of diabetic rats were described. As for the kidney, the responses to treatment with the Cu(II) chelator, TETA, were also investigated. TETA treatment has been shown to improve the function of the diabetic heart in humans and rats (Cooper et al. 2004; Baynes et al. 2009; Cooper et al. 2009). On a molecular level it was previously shown that TETA treatment reverses the increases of mRNA levels corresponding to types I, -III and -IV collagen in STZ-diabetic rats (Gong et al. 2006). These observations coincided with increased mRNA levels of Tgfβ in diabetic rats while TETA treatment normalised Tgfβ mRNA levels. The current study was designed to confirm and extend the previous findings regarding the ECM in diabetes, while the focus was on the analysis on post-translational modifications and collagen homeostasis rather than growth factors. Changes in the ECM of hearts from STZ-diabetic rats were analysed at the transcript level as well as the protein level, with a major focus on the main protein constituent, collagen. Transcript and protein levels of collagen-degrading proteases were also determined. Equivalent to the approach described in the previous chapter for the kidneys, collagen extracts from the hearts of corresponding animals were analysed for the best characterised AGE, CML, as well as for levels of carbonyl. Pepsin digestibility of
collagen was also determined as a functional measure. All the parameters mentioned above were measured in the LV of placebo- and TETA-treated STZ-diabetic rats and compared to healthy counterparts that had also received placebo or TETA treatment.
5.2 Results

5.2.1 Transcriptional analysis of genes relevant to ECM metabolism and oxidative/glycoxidative stress in the heart

Consistent with the study conducted in the diabetic kidney, this analysis was initiated by measuring changes in the transcript levels of specific genes of interest, which in many cases were those also examined in the previous chapter. The results obtained were extended for identified genes of particular interest, by measurement of amounts and/or activity of certain proteins/enzymes. Genes of major interest were identified based on the experimental findings of the current study as well as information derived from the evolving literature. In addition, measurements of post-translational modifications in cardiac collagen extracts were performed.

Transcription levels of target genes were detected by RT-qPCR in the same way as it was performed for the kidney. RNA was extracted from LV tissue from the four groups of animals, transcribed into cDNA and subsequently analysed by RT-qPCR. The signal for each target gene was normalised for the level of reference genes which were measured in parallel for the same set of samples. As for the kidney, a set of three stably-expressed reference genes, in this case Ndc1, Rpl13a and Tbp, had previously been identified for the LV by Hogl in our group (Hogl 2009). Importantly, expression levels of these reference genes were unaffected by the disease state (Sham/STZ) as well as the treatments employed here (Plac/TETA). All target gene transcription levels were thus normalised to the geometric mean of the mRNA level of these three genes. In addition, relative mRNA levels were normalised to the average of the healthy placebo-treated (Sham/Plac) group. Details of primers used for individual target genes as well as the reference genes are given in the appendix on page 174.

Statistical analysis was carried out by two-way ANOVA followed by Tukey-Kramer post-hoc comparison, as described on page 64. Analogous to the measurements in the kidney, analysis was usually carried out for 9 animals, which generally yielded sufficient statistical power. For some target genes, 9 animals were insufficient however, so a second set of measurements was performed as required, using mRNA extracted from LV-tissue from a previous animal trial carried out in our group. This meant that statistical analysis could be carried out for twice the number of animals (n=18 per group), which resulted in an increased statistical power. As the amount of tissue available from the previous trial was limited, only a
Chapter 5: Diabetes-induced changes in the ECM of cardiac LV and effect of TETA

restricted set of genes could be analysed that way. Another limiting factor was the high cost of each RT-qPCR experiment.

5.2.1.1 Relative mRNA levels of collagen and genes involved in collagen processing and degradation, and growth factor responses

As for the kidney, the major collagen isoforms in the heart are types I, -III, -IV, -V and -VI (Bishop et al. 1995). Of those, collagen types I and -III are by far the most abundant types in the heart, respectively accounting for ~80% and ~11% of the total (de Souza 2002). Both types I- and -III collagens are of the fibrillar type, whereas type VI collagen is classified as a filamentous type (Bailey et al. 1998).

Relative mRNA levels corresponding to the different isoforms of type I-, III- and -VI collagens were measured in the LV of the 4 groups of rats. Levels of type I collagen isoforms, Col1a1 and Col1a2 did not differ significantly between the TETA- and placebo-treated diabetic groups compared to the placebo-treated Sham controls after Tukey-Kramer post-hoc comparison (Figure 5-1: A and B). Messenger RNA levels corresponding to the type III collagen isoform, Col3a1 were also unaltered in placebo- or TETA-treated diabetic rats compared to placebo-treated Sham rats (Figure 5-1: C). While none of the Tukey-Kramer post-hoc comparisons were significant for Col1a1, Col1a2 and Col3a1, for the comparison of placebo- and TETA-treated diabetic rats vs. placebo-treated Sham rats, the trend for a decrease in the diabetic group became significant when measured by the two-way ANOVA Disease term (Figure 5-1: A; \( p = 0.011 \), B; \( p = 0.0002 \) and C; \( p = 0.0011 \), all two-way ANOVA Disease terms for [Sham/Plac + Sham/TETA] vs. [STZ/Plac + STZ/TETA]).

Transcript levels of the type VI collagen isoforms Col6a1, Col6a2 and Col6a3 were also analysed. No significant changes were detected for the comparison of placebo- or TETA-treated diabetic animals with placebo-treated healthy animals for isoforms Col6a1 and Col6a2 (Figure 5-1: D and E). Transcription levels of Col6a3 were significantly decreased in the LV of placebo- and TETA-treated STZ-diabetic rats compared to placebo-treated healthy animals (Figure 5-1: F; a, \( p = 0.0004 \) and b, \( p = 0.0007 \) for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). The comparison of TETA-treated diabetic rats with those receiving placebo was not significant for any of the collagen isoforms described above.
Chapter 5: Diabetes-induced changes in the ECM of cardiac LV and effect of TETA

Figure 5-1: Relative mRNA levels of collagen type I, III and VI in the LV of the four groups of rats

Relative mRNA levels of different isoforms of interstitial collagens. Displayed are levels of Col1a1 (A), Col1a2 (B), Col3a1 (C), Col6a1 (D), Col6a2 (E) and Col6a3 (F) for each of the four groups of animals (Sham/Plac, Sham/TETA, STZ/Plac and STZ/TETA). Shown are individual data points (n=9 per group) and mean ± SEM values as indicated. Groups significantly different from the Sham/Plac group at p<0.05 employing by Tukey-Kramer post-hoc comparison are indicated by the letter “a” for the STZ/Plac group or “b” for the STZ/TETA group.
Chapter 5: Diabetes-induced changes in the ECM of cardiac LV and effect of TETA

The transcription levels of three basement membrane-type collagen isoforms, Col4a1, Col4a4 and Col4a5 were also analysed in the LV of rats from the four treatment groups. Transcript levels for collagen type IV isoforms alpha 2 and 3 as well as the three different collagen type V isoforms alpha 1-3 were below the detection level in the LV, consistent with the findings in kidney cortex. Relative mRNA levels of type IV collagen isoform Col4a1 were significantly decreased in the LV of placebo- or TETA-treated diabetic animals compared to placebo-treated Sham animals (Figure 5-2: A; a, \( p=0.0034 \) and b, \( p<0.0001 \) for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). No changes were found for the collagen type IV isoforms Col4a4 and Col4a5 when comparing placebo- and TETA-treated diabetic rats with placebo-treated Sham animals, employing the Tukey-Kramer post-hoc comparison (Figure 5-2: B and C). As for the interstitial collagens, no changes after TETA treatment were found in the LV of diabetic rats compared to placebo-treated animals for the type IV collagens.

![Figure 5-2: Relative mRNA levels of collagen type IV in the cardiac LV of the four treatment groups](image)

Growth factors are another interesting target for analysis due to their potential influence on collagen synthesis. The sample size was increased for the analysis of the growth factors by including tissue from a previous animal trial, in order to increase statistical power. The trends
Chapter 5: Diabetes-induced changes in the ECM of cardiac LV and effect of TETA

were the same for the analyses of the two separate animal trials. The mRNA levels of *Ctgf* were significantly increased in placebo- and TETA-treated diabetic animals compared to placebo-treated healthy animals (Figure 5-3: A; *a*, *p*<0.0001 and *b*, *p*<0.0001 for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). The comparison of the TETA-treated healthy group with the placebo-treated group was also significant for *Ctgf* (Figure 5-3: A; *c*, *p*=0.046 for Sham/Plac vs. Sham/TETA). No differences between the four groups were found for *Tgfβ1* (Figure 5-3: B). As opposed to the mRNA levels of the growth factor *Ctgf*, levels of *Vegfa* were decreased in the placebo- and TETA-treated diabetic groups compared to the healthy placebo-treated group (Figure 5-3: C; *a*, *p*=0.0034 and *b*, *p*=0.0016 for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). TETA treatment did not have a significant effect on the diabetic groups compared to placebo treatment for any of the three growth factors that were analysed.

The pro-collagen processing enzymes ADAMTS2 and BMP1 were analysed for changes as well as the collagen cross-linking enzyme LOX (Kagan *et al.* 1984; Uzel *et al.* 2001; Wang *et al.* 2003). In addition, BMP1 serves as an activator of LOX and thus may influence collagen-crosslinking as well as extracellular collagen formation (Uzel *et al.* 2001). Neither transcript

**Figure 5-3: Relative mRNA levels of growth factors in the cardiac LV**

Shown are comparisons of relative mRNA levels of growth factor genes in the cardiac LV in the four treatment groups. Levels for *Ctgf* (A), *Tgfb1* (B) and *Vegfa* (C) are presented. Shown are the individual data points (*n*=18 per group) and the mean ± SEM values are as indicated. Groups significantly different from the Sham/Plac group at *p*<0.05 employing the Tukey-Kramer post-hoc comparison are indicated by the letter “a” for the STZ/Plac group or “b” for the STZ/TETA group. A significant difference between Sham/Plac and Sham/TETA is indicated by “c”.
levels of Adamts2 and Bmp1, nor Lox displayed changes in the LV of placebo- or TETA-treated diabetic rats compared with placebo-treated Sham rats (Figure 5-4: A, B and C). Furthermore, TETA treatment did not have an effect on mRNA levels in the LV of healthy animals compared to placebo-treated animals.

Considering that MMP2 is the major protease involved in the extracellular degradation of collagen (Creemers et al. 1998; Kerkvliet et al. 1999), relative mRNA levels for Mmp2 were determined for 18 animals in order to yield a greater statistical power. Relative mRNA levels of Mmp2 were significantly decreased in the LV of placebo- and TETA-treated diabetic rats compared to placebo-treated Sham rats (Figure 5-5: A, a, p=0.0072 and B, b, p=0.0048 for Sham/Plac vs STZ/Plac and STZ/TETA, respectively). TETA-treated diabetic rats did not display levels different from their placebo-treated counterparts. Transcript levels of the collagen-degrading lysosomal cysteine proteases Ctsl, b and k as well as the non-collagen-degrading lysosomal protease Ctsb were also measured (Soderstrom et al. 1999). The transcript levels of Ctsl and Ctsb were both higher in the placebo-treated diabetic group compared to the placebo-treated Sham group (Figure 5-5: B, a, p=0.0074 and C, a, p=0.012 for Sham/Plac vs. STZ/Plac of Ctsl (B) and Ctsb (C), respectively). Treatment of diabetic rats
with TETA significantly lowered transcription levels of \(Ctsl\) and \(Ctsb\) to levels equivalent to those in healthy placebo-treated rats (Figure 5-5: B; d, \(p=0.026\) and C; d, \(p=0.025\) for STZ/Plac vs. STZ/TETA of \(Ctsl\) (B) and \(Ctsb\) (C), respectively). Levels of the lysosomal collagen-degrading protease \(Ctsk\) were also significantly decreased in the TETA-treated diabetic group compared to the placebo-treated Sham controls (Figure 5-5: D; b, \(p=0.026\) for Sham/Plac vs. STZ/TETA). Although levels for \(Ctsk\) trended towards decrease in placebo-treated diabetic rats compared to placebo-treated Sham rats, this difference was not
significant. Messenger RNA levels for the lysosomal protease Ctsh, which does not act on collagen, displayed a pattern similar to those of Ctsl and Ctsb. Ctsh levels were significantly increased in placebo-treated diabetic rats compared to placebo-treated Sham animals (Figure 5-5: E; a, \( p=0.0041 \) for Sham/Plac vs. STZ/Plac). Ctsh levels trended non-significantly towards normal values in TETA-treated diabetic rats.

5.2.1.2 Relative mRNA levels in cardiac LV of genes relevant to oxidative and glycoxidative stress

In parallel to the analysis performed in the kidney, genes involved in protection against ROS and non-enzymatic glycation were analysed in the cardiac LV. Changes in function of (some of) these genes could contribute to enhancement of oxidative stress or AGE formation in the diabetic heart, or may alternatively represent adaptive responses to pathogenic increases of stressors such as ROS. GSR can regenerate glutathione and thus contribute to cellular antioxidant potential, while SOD1-3 and CAT detoxify the ROS superoxide and hydrogen peroxide, respectively (Meister 1988; Fattman et al. 2003; Ye et al. 2004). Relative mRNA levels of Gsr were increased significantly in both the placebo treated- and the TETA-treated diabetic groups compared to the placebo-treated Sham controls (Figure 5-6: A; a, \( p=0.018 \) and b, \( p=0.0007 \) for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). No significant changes were found for Sod1 between the placebo-treated Sham compared to the placebo- or TETA-treated diabetic groups, although levels trended towards increase in placebo-treated diabetic animals (Figure 5-6: B). TETA-treated diabetic animals group had significantly lower Sod1 mRNA levels than placebo treated-diabetic animals (Figure 5-6: B; d, \( p=0.014 \) for STZ/Plac vs. STZ/TETA). No changes in mRNA levels were found between the four groups for Sod2 and Sod3 (Figure 5-6: C and D). Messenger RNA levels of Cat were significantly increased in the placebo- and TETA-treated diabetic groups compared to the placebo-treated Sham controls (Figure 5-6: E; a, \( p<0.0001 \) and b, \( p<0.0001 \) for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). Levels of Cat were partially normalised by TETA treatment in the STZ-diabetic group (Figure 5-6: E; d, \( p=0.0146 \) for STZ/Plac vs. STZ/TETA).
Relative mRNA levels for Gsr (A), Sod1-3 (B-D) and Cat (E) are displayed for the four treatment groups. Shown are the individual data points (n=9 per group) and the mean ± SEM values are as indicated. Groups significantly different from the Sham/Plac group at $p<0.05$ employing Tukey-Kramer post-hoc comparison are indicated by the letter “a” for the STZ/Plac group or “b” for the STZ/TETA group. A difference between the STZ/Plac and the STZ/TETA group is indicated by the letter “d”.

Figure 5-6: Relative mRNA levels of genes implicated in the detoxification of ROS in the cardiac LV of rats
Relative mRNA levels of Glo1 and Glo2 were measured for 18 rats per group in the LV as was previously done for kidney cortices shown (see paragraph 4.2.1.2). These two enzymes comprise the glyoxalase system which can detoxify the reactive AGE precursors glyoxal, methylglyoxal and 3-deoxyglucosone to less reactive α-hydroxyacids (see paragraph 1.3.4.1). Levels of Glo1 were significantly decreased in the TETA- but not the placebo-treated diabetic group compared to the placebo-treated Sham controls (Figure 5-7: A; b, \( p=0.049 \) for Sham/Plac vs. STZ/TETA). No such effects of TETA treatment compared to placebo treatment were seen in the healthy group. Glo2 mRNA levels were decreased in both, placebo- and TETA-treated diabetic animals compared to healthy animals (Figure 5-7: B; a, \( p=0.010 \) and b, \( p<0.0001 \) for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). No effects of TETA treatment were present in the healthy or diabetic group when compared to the placebo-treated controls.

**Figure 5-7:** Relative mRNA levels of the two genes of the glyoxalase system in the cardiac LV of rats
Displayed are mRNA levels for Glo1 (A) and Glo2 (B) in the LV of the four treatment groups. Shown are the individual data points (\( n=18 \) per group) and mean ± SEM values as indicated. Groups significantly different from the Sham/Plac group at \( p<0.05 \) employing the Tukey-Kramer post-hoc comparison are indicated by the letter “a” for the STZ/Plac group or “b” for the STZ/TETA group.
Other enzymes including AKR1A1, AKR1B1, AKR1C1 and ALDH1A1 can also detoxify the AGE-precursors methylglyoxal and 3-deoxyglucosone (see section 1.3.4.2). Transcription levels for Akr1c1, which were previously measured in kidney cortices, could not be measured in the LV as they were below the detection limit. No differences in relative mRNA levels for the enzymes Akr1a1, Akr1b1 and Aldh1a1 were found between the four groups employing the Tukey-Kramer post-hoc comparison (Figure 5-8: A-C).

![Figure 5-8: Relative mRNA levels of additional enzymes involved in the detoxification of methylglyoxal and 3-deoxyglucosone](image)

The enzyme AOC3, also known as SSAO, may contribute to increased AGE levels via the formation of the AGE precursor methylglyoxal (Obata 2006). The trans-membrane receptor RAGE is capable of binding AGEs amongst other ligands and an AGE-RAGE interaction has been proposed to contribute to the formation of diabetic complications (see paragraph 1.3.3.1). Transcript levels of Aoc3 were unaltered when comparing the four groups by using Tukey-Kramer post-hoc comparison (Figure 5-9: A). No changes of mRNA levels between the placebo-treated diabetic and the placebo-treated healthy group were found for Rage employing the Tukey-Kramer post-hoc comparison. Messenger RNA levels of the TETA-treated diabetic group were significantly decreased compared with the placebo-treated healthy group (Figure 5-9: B; d, p=0.040 for Sham/Plac vs. STZ/TETA).
5.2.2 Protein levels of genes relevant to the ECM and anti-ROS defence in the healthy and diabetic rat heart, and the effect of TETA

Parallel to the approach from the kidney described in paragraph 4.2.1 the next step of analysis for the LV was to enhance the findings regarding the mRNA level for genes of particular interest. Collagen protein levels as well as protein levels of the collagen-degrading proteases CTSL and CTBS were analysed, in order to confirm whether the treatment effect seen at the mRNA level is also present at the protein level. Also, protein levels of ROS defence enzymes CAT and GSR were analysed, based on the changes seen at the mRNA level. Collagen protein levels were determined through the measurement of hyp content. Relative levels of all other proteins shown in the following sections were determined by Western Blotting. While beta-actin was used as a normaliser for the kidney, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) appeared to be a better normaliser for the LV despite the apparent decrease in GAPDH activity in diabetes (Brownlee 2001). For Western Blotting, band intensity was measured by densitometry and normalised for the respective GAPDH band intensity, followed by normalisation for the average value of the healthy placebo-treated group. Measurements were carried out for 11-12 animals per group. The possibility of increasing the sample size was not available for LV protein measurements as there were only small amounts of RNAlater-treated tissue suitable for mRNA analysis, and no other LV tissue was left from the previous animal study.

Figure 5-9: Relative mRNA levels of Aoc3 and Rage in the LV of rats
Relative mRNA levels of the four treatment groups are given for Aoc3 (A) and Rage (B). Shown are the individual data points (n=9 per group) and the mean ± SEM values are as indicated. Groups significantly different from the Sham/Plac group at p<0.05 employing the Tukey-Kramer post-hoc comparison are indicated by the letter “a” for the STZ/Plac group or “b” for the STZ/TETA group. A difference between the STZ/Plac and the STZ/TETA group is indicated by the letter “d”.

Chapter 5: Diabetes-induced changes in the ECM of cardiac LV and effect of TETA
Chapter 5: Diabetes-induced changes in the ECM of cardiac LV and effect of TETA

5.2.2.1 Collagen protein level in the cardiac LV of four groups of rats

Collagen protein levels in the cardiac LV were determined by measuring the hyp levels in acid hydrolysates of freeze-dried LV tissue using a colorimetric assay. No changes in hyp levels between the groups were detected using the Tukey-Kramer post-hoc comparison (Figure 5-10).

![Collagen content in the cardiac LV of rats from the four treatment groups](image)

**Figure 5-10: Collagen content in the cardiac LV of rats from the four treatment groups**
Collagen content in the four groups was determined by measuring the hyp level. Collagen levels were calculated assuming that 12.5% of collagen consists of hyp and the collagen content is given in μg of collagen per mg of dry tissue. Individual data points (n=11 per group) are shown and mean ± SEM values are as indicated. No significant between-group differences were detected using the Tukey-Kramer post-hoc method.

5.2.2.2 GAPDH as a normaliser for Western blot measurements in the cardiac LV of rats

It was initially thought that beta-actin would be a suitable normaliser for protein-level analysis of the LV in the four treatment groups. However, when equal amounts of protein were analysed by Western blotting and beta-actin was used to normalise values, it gave apparently inconsistent results. Thus Gapdh was run in parallel to beta-actin on a gel (n=3/4 samples per group) in order to test which of the two displayed the more stable pattern. Bands for beta-actin and Gapdh are shown in Figure 5-11. Bands displayed a more homogeneous appearance for Gapdh than for beta-actin. The initial impression was confirmed when the band intensity was measured and the SEM values were calculated, and shown to be smaller for Gapdh than for beta-actin (Figure 5-11: B and C with SEM=0.057 and SEM=0.043 for beta actin and Gapdh, respectively).
5.2.2.3 Protein level of collagen-degrading proteases in the LV of rats

The two lysosomal collagen-degrading proteases, CTSL and CTSB displayed increased transcript levels in diabetic LV, whereas TETA treatment reversed these increases. It was thus of interest to determine the corresponding protein levels in the four treatment groups. Unfortunately, MMP2 activity could not be measured in the LV due to the limited amount of available tissue which was less than in the corresponding kidneys.

Levels of CTSL dc trended towards increase in the placebo-treated diabetic group compared to the placebo-treated Sham group (Figure 5-12: A). The TETA-treated diabetic group...
Chapter 5: Diabetes-induced changes in the ECM of cardiac LV and effect of TETA

Displayed significantly increased CTSL dc protein levels compared to the placebo-treated Sham group (Figure 5-12: A; b, p=0.043 for Sham/Plac vs. STZ/TETA). The notion that diabetes had a significant effect on CTSL dc levels is supported by the fact that the disease term in the two-way ANOVA comparison was significant, while the treatment term was not (Figure 5-12: A; two-way ANOVA Disease term, p=0.0016 for [Sham/Plac + Sham/TETA] vs. [STZ/Plac + STZ/TETA]). No changes were seen for CTSL sc between the four groups of rats (Figure 5-12: B).

Figure 5-12: Relative protein levels of CTSL in the cardiac LV of rats
Shown are the protein levels of CTSL dc (A) and CTSL sc (B) in the four treatment groups. Individual data points (n=11 per group) are shown and the mean ± SEM values are as indicated. Groups significantly different from the Sham/Plac group at p<0.05 employing Tukey-Kramer post-hoc comparison are indicated by the letter “b” for the STZ/TETA group. A representative blot for CTSL sc and CTSL dc as well as the normaliser GAPDH can be seen below the two graphs (C).
Levels of CTSB dc were decreased in the LV of placebo-treated and TETA-treated diabetic rats compared to healthy placebo-treated rats (Figure 5-13: A; a, $p<0.0001$ and b, $p=0.0002$ for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). The same effect was observed for CTSB sc (Figure 5-13: B; a, $p=0.0091$ and b, $p=0.026$ for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). No effects of TETA were observed for CTSB dc or CTSB sc.

![Figure 5-13: Relative protein levels of CTSB in the cardiac LV of rats](image)

**Figure 5-13: Relative protein levels of CTSB in the cardiac LV of rats**
Protein levels of CTSB dc (A) and CTSB sc (B) in the LV of the four treatment groups are shown above. Individual data points ($n=11$ per group) and the mean ± SEM are as indicated. Groups significantly different from the Sham/Plac group at $p<0.05$ employing Tukey-Kramer post-hoc comparison are indicated by the letter “b” for the STZ/TETA group. A representative blot for CTSB sc and CTSB dc as well as the normaliser GAPDH are shown below the two graphs (C).

**5.2.2.4 Protein level of enzymes involved in the detoxification of ROS**

Transcript levels of the hydrogen peroxide-detoxifying enzyme Cat were shown to be increased in the LV of diabetic hearts in the first part of this (see paragraph 5.2.1.2). Increased transcript levels were also found in diabetes for Gsr, an enzyme that reduces glutathione, which is needed for the glutathione peroxidase-dependent detoxification of hydrogen peroxide. Thus, these two enzymes were also analysed on the protein level.
Chapter 5: Diabetes-induced changes in the ECM of cardiac LV and effect of TETA

Protein levels of CAT were increased in the LV of placebo- and TETA-treated diabetic rats compared to placebo-treated Sham controls (Figure 5-14; A; a, $p<0.0001$ and b, $p<0.0001$ for Sham/Plac vs. STZ/Plac and STZ/TETA, respectively). TETA treatment did not modify values in healthy or diabetic rats compared to those in placebo-treated animals.

GSR protein levels were increased in TETA-treated diabetic animals compared with their healthy, placebo-treated counterparts (Figure 5-15; A, b, $p=0.037$ for Sham/Plac vs. STZ/TETA, respectively). GSR levels also trended towards increase in placebo-treated diabetic rats compared to their placebo-treated counterparts but this did not become significant. However, this trend was supported by the observation that the disease term of the two-way ANOVA was significant (Figure 5-15: A; two-way ANOVA Disease term, $p=0.0005$ for [Sham/Plac + Sham/TETA] vs. [STZ/Plac + STZ/TETA]). Treatment with TETA in healthy-control or diabetic rats did not have an effect compared to their placebo-treated counterparts.
5.2.3 Biochemical analysis of collagen isolated from the LV of healthy and diabetic rats and the effect of TETA

Next, changes in post-translational modifications of ex vivo collagen between the four groups were determined, in parallel with the analysis that was performed in kidneys in the previous chapter. Levels of the AGE CML as well as carbonyl levels were measured in collagen extracts from the LV. Also, pepsin digestibility was determined as a functional measure of collagen quality.

5.2.3.1 Changes in pepsin digestibility of collagen extracts

Pepsin digestibility of collagen is a frequently-cited measure associated with post-translational modifications, and changes in pepsin digestibility have been shown in parallel with altered AGE levels of collagen glycated in vitro or collagen extracts from diabetic patients (see paragraph 1.3.3.1). Collagen extracts were incubated with pepsin in acetic acid...
for 24 h at 4 °C. Soluble collagen was separated from insoluble collagen by centrifugation, and the supernatant as well as the pellet were analysed for collagen content employing the hyp assay. The percentage of collagen that was solubilised with pepsin was calculated subsequently. As opposed to the collagen extracts from kidneys, no changes in pepsin digestibility could be observed between the four groups employing the Tukey-Kramer post-hoc comparison (Figure 5-16). This was confirmed by the two-way ANOVA analysis where neither the disease term nor the treatment term was significant.

5.2.3.2 Collagen CML levels in the LV of rats

CML levels were to be measured in collagen extracts from the LV of the heart as well. However, CML levels of heart collagen extracts were close to or below the detection level for a big proportion of the samples. This meant that CML levels could not be quantified and compared between the groups in collagen extracts from the LV of the heart in a reliable way.

5.2.3.3 Measurement of collagen carbonyl levels in the four groups of rats

Carbonyl levels of collagen extracts were measured as a marker of oxidative stress independent of glycation. Collagen was solubilised by digestion with a collagenase preparation. Carbonyl levels of solubilised collagen were detected using an ELISA kit, based on the derivatisation of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) to form
dinitrophenylhydrazone (DNP). The derivatised DNP-carbonyl-protein conjugates are applied to an ELISA plate followed by the detection of DNP via a primary antibody and a HRP-coupled secondary antibody. A substrate for HRP was added and the conversion can be followed via absorbance measurement. Absolute amounts of carbonyl were calculated from an internal standard curve which was run in parallel. Levels of carbonyls on the collagen extracts were unaltered between the four groups of animals when analysed by the post-hoc Tukey-Kramer comparison (Figure 5-17).
Chapter 5: Diabetes-induced changes in the ECM of cardiac LV and effect of TETA

5.3 Discussion

Collagen mRNA levels were decreased for the interstitial collagen type VI alpha 3, as well as collagen type IV alpha 1 in the LV of the heart of diabetic rats, with no effect from TETA treatment. As for the kidney, the current findings do not agree with the previously reported results of increased collagen mRNA levels in STZ-diabetic rat hearts, and the reported ameliorative effect of TETA (Gong et al. 2006). Again, this discrepancy may be due to the application of a different normalisation procedure. While mRNA levels of a single normaliser (18 S ribosomal RNA) was used for the previous analysis, three different normalisers (Ribosomal protein L13a (Rpl13a), Tata box binding protein (Tbp) and Nucleoporin 1 (Ndc1)) were used for the current study. These were shown to be stably-expressed in the LV of the four groups of rats in a previous set of experiments (Hogl 2009). No changes in collagen levels determined by hyp measurement were detected in diabetic rats while TETA treatment also had no effect. This is not surprising considering that mRNA levels of the major collagen isoforms in the LV, namely type I and type III collagens, were not significantly altered in diabetic rats in the study presented in this chapter (de Souza 2002). The current finding of unaltered collagen levels is in agreement with most previous reports of STZ-diabetic rats after 16 to 32 weeks of diabetes while one publication reports increased type 1 and 3 collagen protein levels after 16 weeks as determined by immunofluorescence (Modrak 1980; Norton et al. 1996; Candido et al. 2003; Cooper et al. 2004). Interestingly, there are several reports of increased collagen levels in STZ-diabetic rats after 4, 6 and 8 weeks of diabetes determined by hyp measurement and histochemistry (Modrak 1980; Reddi 1988; Miric et al. 2001; Van Linthout et al. 2008). Considering the unaltered levels of collagen in STZ-diabetic rats after 16 to 32 weeks of diabetes, it is possible that such a transient increase in collagen levels is based on a different mechanism (possibly via increased TGF-β levels discussed in the paragraphs below) than the increased collagen content found in the hearts of long-term diabetic monkeys and dogs (18 months and 12 months of diabetes, respectively) or diabetic humans (Haider et al. 1978; Regan et al. 1981).

As opposed to findings obtained from kidney samples, mRNA levels of the extracellular collagenase Mmp2 were decreased in the LV of diabetic rats while TETA treatment had no effect. Decreased mRNA levels of Mmp2 were previously reported in hearts of STZ-diabetic rats after 4-6 weeks of diabetes and were associated with collagen accumulation in those animals, while no increase in collagen levels was found here (Li et al. 2007; Van Linthout et al. 2008). In contrast to levels of the extracellularly acting MMP2, mRNA levels of the lysosomal collagen-degrading cysteine proteases Ctsl and Ctsb were increased in diabetic
Chapter 5: Diabetes-induced changes in the ECM of cardiac LV and effect of TETA

rats, while TETA treatment normalised mRNA levels of both proteases. No changes were present at the protein level for CTSL sc while CTSL dc protein levels were higher in diabetic rats and TETA treatment had no effect on the protein level. A great discrepancy between mRNA and protein levels was observed for CTSB, where both CTSB sc and -dc protein levels were decreased in diabetic rats with no TETA effect. CTSL and CTSB are the major cysteine proteases expressed in the heart (Soderstrom et al. 1999). Up-regulation of CTSL found here, is thought to be a protective mechanism against the formation of fibrosis in hearts, as evidenced by fibrosis found in the heart of CTSL-deficient mice (Stypmann et al. 2002; Spira et al. 2007). The finding of an inverse correlation of Ctsl mRNA levels with the ejection fraction in cardiomyopathy was also interpreted in this context (Müller et al. 2012).

Interestingly, the concurrent decrease of Mmp2 mRNA levels in diabetic rats suggests a discrepancy in the response, regarding the extracellular and intracellular degradation pathway. An inverse correlation with the ejection fraction was also present in a previous study for CTSB mRNA and protein levels (Müller et al. 2012). Considering this, the finding of decreased CTSB protein levels with increased Ctsb mRNA levels in diabetic hearts are difficult to interpret. It may be due to a shorter half-life of CTSB protein in diabetic hearts, possibly due to the faster inactivation of cysteine proteases in the diabetic state (Zeng et al. 2006). This would suggest a distinct susceptibility of CTSB for these modifications, as CTSL protein levels were increased rather than decreased in diabetic hearts. Messenger RNA levels of the collagen-degrading cysteine protease Ctsk and the non-collagen degrading protease Ctsk were decreased and increased respectively, in the LV of diabetic rats with no effect from TETA treatment (Drake et al. 1996; Garnero et al. 1998). Levels for both of these proteases were previously found to be increased in dilated cardiomyopathy and hypertrophic cardiomyopathy in humans (Cheng et al. 2012). Overall, the findings for the different cysteine proteases suggest a disturbed protein catabolism in the heart of diabetic rats. The differential regulation of mRNA and protein levels in the diabetic state may represent an adaptation in response to alterations of their specific substrates of the different cysteine cathepsins. Roles independent of lysosomal protein degradation are also becoming increasingly obvious and may explain the distinct regulation of each cathepsin. CTSL for example, was shown to protect from cardiac hypertrophy via the interference with detrimental signalling pathways (Tang et al. 2009).

No changes of Tgfβ1 were seen at a transcript level in the LV of diabetic rats and no treatment effect of TETA was detected. Previously unaltered or increased mRNA levels of Tgfβ were reported in diabetic hearts (Way et al. 2002; Roestenberg et al. 2006; Westermann et al. 2007). The mRNA levels of Ctgf were strongly increased in the LV of diabetic rats while levels of Vegfa were decreased, both of which has been reported
Chapter 5: Diabetes-induced changes in the ECM of cardiac LV and effect of TETA

independently in previous publications (Candido et al. 2003; Daniels et al. 2009; Messaoudi et al. 2009). TETA treatment did not have any effect on the mRNA levels of the two growth factors. Both CTGF and VEGFA can be regulated by TGFβ but this seems unlikely, considering that Tgfβ1 mRNA levels were unaltered and that the two targets were dysregulated in opposing directions (Daniels et al. 2009). The two enzymes CAT and GSR both of which are involved in the detoxification of H₂O₂ were up-regulated at the mRNA and protein levels in diabetic rats in the current study, in agreement with previous findings (Wohaieb et al. 1987; Wohaieb et al. 1987). While TETA treatment partially decreased Cat mRNA levels, this did not translate to an effect on CAT protein levels, and no effects of TETA treatment were detected on GSR mRNA- or protein levels. Increased levels of CAT in the heart of diabetic rats may reflect a protective response of the heart in diabetes (Wohaieb et al. 1987). This may be due to the altered energy metabolism in the diabetic heart, considering that an increase in mitochondrial CAT levels has been shown in mice on a high fat diet recently (Rindler et al. 2013). There is an increased uptake and degradation of free fatty acids as opposed to glucose, and a parallel increase in the number of peroxisomes involved in fatty acid metabolism in diabetic hearts, possibly leading to increased amounts of intracellular ROS (Engels et al. 1999; Sakamoto et al. 2000; Glyn-Jones et al. 2007). CAT is present in peroxisomes and mitochondria in the heart and CAT levels may increase in both compartments, in order to protect hearts from such an increased intracellular oxidative stress (Yokota et al. 1992; Brownlee 2005). The overexpression of CAT in diabetic mice has been shown to protect cardiomyocytes, supporting the idea of a protective response of the heart (Ye et al. 2004). There is some evidence that TETA treatment improves the mitochondrial function and fatty acid metabolism in STZ-diabetic rat hearts (Glyn-Jones 2007). Thus the decrease in Cat mRNA levels with unaltered CAT protein levels may indicate an increased stability of the protein. A decrease in lipid deposits by Nile red staining was shown after TETA treatment alongside increased levels of enzymes involved in the metabolism of fatty acids in these animals (Glyn-Jones 2007). However, this also suggests that TETA treatment does not reverse the energy metabolism in the heart from fatty acid utilisation to glucose utilisation.

It has previously been shown that H₂O₂ can induce CTGF expression independent of TGFβ1 (Park et al. 2001). Thus it seems possible that increased CTGF levels are a consequence of the increased oxidative stress in the present study. Decreased Vegfa mRNA levels may again be a consequence of increased CTGF expression. Such an inverse regulation has been proposed recently, while it was also shown that the ratio of CTGF/VEGFA is the strongest predictor of fibrosis in DR (Kuiper et al. 2008). CTGF can further inhibit VEGFA activity by direct protein-protein interaction (Inoki et al. 2002). It may seem surprising that
Chapter 5: Diabetes-induced changes in the ECM of cardiac LV and effect of TETA

there is a lack of ECM accumulation as was evidenced by unaltered collagen levels by hyp measurement, while expression levels of collagen also tended to be decreased rather than increased, despite increased levels of CTGF. However, looking at the literature it seems that fibrosis is associated with increased TGFβ expression and protein levels rather than CTGF levels (Roberts et al. 1992). While Ctgf mRNA expression was constantly increased in STZ-diabetic mice after 2-9 weeks of diabetes, Col4a1 mRNA levels and fibronectin mRNA levels only increased significantly when Tgfβ mRNA levels increased strongly after 9 weeks of diabetes in the kidneys (Roestenberg et al. 2006). Another study in 6 week STZ-diabetic rats showed increased staining for type I and IV collagen in parallel with an increase of TGF-β and CTGF protein and mRNA levels (Aragno et al. 2008). Such a possibly transient increase of TGF-β expression may also explain the transient increase in collagen levels of the heart of STZ-diabetic rats mentioned in the beginning of this discussion, as I am unaware of reports of increased TGFβ protein levels in STZ-diabetic rat hearts with diabetes of 16 weeks or longer, although one report of increased Tgfβ1 mRNA levels exists (Gong et al. 2006).

As opposed to the changes described for CAT and GSR, protein levels of SOD1-3, which are involved in the detoxification of superoxide anion, were not altered in the LV of diabetic rats. Previous reports regarding levels of the copper-/zinc-containing SOD1 and SOD3 enzymes in hearts of STZ-diabetic rats have provided contradictory results (Wohaieb et al. 1987; Uriu-Adams et al. 2005), in that one group has reported increased activity levels (after 10 weeks of diabetes), while another group reported unaltered activity levels (after 12 weeks of diabetes), consistent with our current mRNA results. Levels of the manganese-containing enzyme SOD2 were not measured in the study by Wohaieb et al. and were reported to be unaltered in the study by Uriu-Adams et al.

The glyoxalase system, consisting of glyoxalase 1 (GLO1) and glyoxalase 2 (GLO2) catalyses the conversion of the AGE precursors methylglyoxal and glyoxal to less reactive α-hydroxyacids (Thornalley 2003). There are currently no reports of altered glyoxalase levels in the diabetic heart whereas decreased Glo2 mRNA levels were found in the LV of diabetic rats in the current study, with no effect of TETA treatment. Here, no changes for Glo1 mRNA levels were found in placebo treated diabetic rats while TETA treatment decreased Glo1 levels in diabetic but not healthy rats, compared to placebo-treated healthy rats. It was thought originally that GLO2 is the rate-limiting enzyme in the glyoxalase system (Thornalley 1990). However, it was recently shown that overexpression of GLO1 alone reduces hyperglycaemia-induced AGE levels which suggests that this is the rate-limiting enzyme in vivo, so that it is uncertain whether decreased Glo2 mRNA levels could exert an effect on in
vivo AGE formation (Brouwers et al. 2011). The decreased mRNA levels of Glo1 in TETA-treated diabetic rats compared to the placebo-treated controls appears to be a combined effect of the disease and the treatment, with no effect of the disease or treatment on Glo1 mRNA levels alone.

The enzymes AKR1A1, AKR1b1, AKR1C1 and ALDH1A1 are also implicated in the defence against reactive α-dicarbonyls (see section 1.3.4.2), in addition to the glyoxalase system. However, no changes on mRNA levels were present in the LV of diabetic rats for Akr1a1, Akr1b1 and Aldh1a1, while levels of Akr1c1 were below the detection limit. Treatment with TETA also had no effect on these enzymes. There is no previous data available regarding the regulation of these enzymes in diabetic hearts. Also, these enzymes have relatively broad substrate specificities which may explain the absence of changes in diabetic rat hearts (Bohren et al. 1989; Xiao et al. 2009).

The copper-containing enzyme AOC3, also known as SSAO was found to be strongly decreased at the mRNA level in the kidney of diabetic rats, in the current study. As opposed to the kidney, there were no detectable changes in the mRNA levels of Aoc3 in the hearts of diabetic rats nor were there effects of TETA treatment, consistent with the tissue-specific regulation of this enzyme. There are no previous available reports regarding AOC3 expression in diabetic hearts. This enzyme was included in the analysis, due to its potential involvement in ECM synthesis and the formation of AGE precursors (Obata 2006). It has also been reported previously that TETA treatment normalises AOC3 activity in the retina of diabetic rats (Hamada et al. 2005). However, considering that the expression levels in the heart are very low, the enzyme may not be directly involved in the development of diabetic complications of the heart (Ochiai et al. 2005). It could however exert effects via the aorta where it is strongly expressed (Ochiai et al. 2005). In support of this hypothesis, the inhibition of AOC3 leads to the disruption of collagen and elastin architecture of the blood vessel wall (Langford et al. 2002). Also, mice overexpressing AOC3 displayed a similar pathologic phenotype in the elastic lamina of the aorta to that in non-transgenic diabetic rats, further corroborating the importance of AOC3 for the aorta and a role in diabetes (Gokturk et al. 2003; Jüllig et al. 2010).

No significant changes of Rage mRNA levels, the receptor for AGEs, were found in diabetic rats compared to healthy rats, while TETA treatment led to a small but significant decrease of Rage mRNA levels in diabetic- but not in healthy rats. As was mentioned in the previous chapter, CML-modified BSA, amongst other ligands, is capable of triggering RAGE in vitro which can lead to a positive feedback loop, resulting in increased Rage transcription levels.
Chapter 5: Diabetes-induced changes in the ECM of cardiac LV and effect of TETA

(Kislinger et al. 1999; Goldin et al. 2006). CML levels in heart collagen were below the detection level, so that such an effect seems unlikely. It is possible that effects on RAGE are exerted via serum AGEs or serum CML in the heart, which are increased in diabetes (see pages 24 ff.). In this case, it would be expected that Rage mRNA levels are increased in diabetic rats compared to healthy rats, which was not the case in our study. Considering that an interaction between RAGE and AGES in vivo has been disputed in recent years, it is possible that TETA treatment in diabetic rat hearts exerts effects on RAGE independent of AGES (Heizmann 2007; Ramasamy et al. 2007; Thornalley 2007). However, this finding was not investigated further due to the lack of changes in placebo treated diabetic rats. In addition, TETA treatment in healthy rats did not decrease Rage mRNA levels compared to placebo-treated healthy rats suggesting the possibility that a combined effect of the disease and the treatment led to the observed alteration in mRNA levels.

The findings regarding changes in post-translational modifications of collagen extracts obtained from the LV of diabetic rat hearts were very different from the findings for kidney collagen extracts. This was in spite of the fact that the types of collagen obtained after the extraction procedure (characterised in the studies described in chapter 3), were very similar. While pepsin digestibility and carbonyl levels of collagen extracts from kidneys were decreased in diabetic animals, no such changes were detectable in the collagen extracts from the LV. Furthermore, while CML levels in collagen extracts from kidneys could be detected, levels in LV collagen extracts were below the detection limit. At the same time, pepsin digestibility for LV collagen extracts appeared to be lower than for kidney collagen extracts. These observations need to be interpreted with caution, as no direct comparison for CML levels and pepsin digestibility between LV and kidney collagen extracts was carried out. In support of low CML levels found in cardiac collagen extracts in this study in relation to kidney collagen CML levels, a previous study has shown that glycated proteins in the heart overall are low compared to kidneys and are not increased in STZ-diabetic rat hearts as opposed to kidneys (Myint et al. 1995). This observation suggests an overall increased susceptibility for glycation in the proteins of the kidney as opposed to the heart.

Another possible explanation for low CML levels of collagen in the LV of the heart compared with the kidney may be the relatively short half-life of collagen in the heart, which according to some measurements has a turnover twice as fast as the collagen in the kidney (Gineyts et al. 2000). It has previously been reported that the half-life of collagen is an important determinant of AGE accumulation (Verzijl et al. 2000). Nevertheless, AGES do appear to be important in the development of DCM as a correlation between CML levels in small
myocardial vessels, and heart failure with reduced ejection fraction was shown (van Heerebeek et al. 2008).

The seemingly low pepsin digestibility of cardiac collagen as opposed to renal collagen may be due to a different quantity or quality of enzymatic cross-links mediated by LOX (Robins 2007). This is not necessarily in contradiction to a faster collagen turnover in the heart, as the turnover is determined by the expression and activity level of intracellular and extracellular collagen-degrading proteases, as well as rates of collagen synthesis in addition to the digestibility of collagen (see page 10 ff.). A fast turnover of heart collagen may also explain the lack of differences between pepsin digestibility of cardiac collagen from healthy and diabetic rats as less time remains for such differences to form. At the same time, this could explain the unaltered levels of collagen carbonyl levels in the LV of diabetic- compared to healthy rats, as these post-translational modifications accumulate over time under oxidative stress (see page 32). Again, the faster turnover in the heart could also explain relatively low collagen carbonyl levels in the LV of the heart compared to the kidney in the healthy rats. Collagen carbonyl levels in the diabetic rats were similar in hearts and kidneys, but this was due to the decrease in carbonyl levels in kidneys of diabetic animals compared to healthy rats (as discussed on page 129 ff.).
Chapter 6 – Summary and conclusion

The diabetic rat model was characterised and described in chapter 3. A method suitable for the extraction and solubilisation of kidney and heart tissue was established, which provided a basis for the experiments in chapters 4 and 5. The collagen extraction method employed was that published by Avery and Bailey almost two decades ago for the extraction of intramuscular collagen but has not been used frequently, judging by the observation that it has received only 10 citations (as of 9th of February 2013) according to Google scholar, none of which related to the field of diabetes (Avery et al. 1995). This is despite the massive interest that collagen research has attracted in relation to diabetes and AGEs. The authors mention that the method produces collagen of high purity with no inadvertent chemical modifications during its extraction from muscle. It was found in these studies that this method was well suited for the extraction of heart muscle collagen as well as kidney collagen. Therefore, the method was chosen as it was the best for fulfilling the requirements for collagen extraction required for this thesis work.

The major findings from the study described in chapter 4 are summarised in Figure 6-1 (below). First, evidence was provided for dysregulated collagen metabolism in the kidneys of diabetic rats: alterations included decreased collagen mRNA and protein levels, reduced collagen digestibility and altered levels of collagen-degrading proteases CTSL and MMP2. These alterations were partially ameliorated by treatment with the Cu(II) chelator TETA, which acted at the protein level without measurable effects at the transcriptional level. Secondly, data from this study lends support to the hypothesis that copper chelation lowers glycoxidative stress in vivo, as evidenced by a trend for decreased levels of the AGE, CML, in collagen in the kidneys of diabetic rats after TETA treatment (Wolff et al. 1987; Baynes 1991; Chace et al. 1991; Elgawish et al. 1996; Saxena et al. 1999). Thirdly, correlation analysis demonstrated a linkage between collagen CML levels and the lysosomal collagen-degrading protease CTSL in the kidneys of placebo-treated healthy and diabetic rats. The strong correlation between CTSL and CML levels was abrogated by TETA treatment, consistent with an involvement of copper in this linkage. One interesting observation pointing to a copper-dependent connection between CML and CTSL was that high CML levels in one healthy placebo-treated animal were associated with high CTSL protein levels, whereas high CML levels in a TETA-treated healthy animal did not show such an association. Finally, the copper sensitivity of CTSL from kidney lysates shown here in vitro, and the binding of redox-active copper to CML provide a possible explanation for the connection between CTSL and CML reported here (Qian et al. 1998; Saxena et al. 1999; Monnier 2001; Eaton et al. 2002).
Chapter 6: Summary and conclusions

Figure 6-1: Summary of essential results in the kidney
Shown is a summary of the most important findings regarding the effects of diabetes and TETA treatment in kidneys of STZ-diabetic rats. The connections are explained in detail in the accompanying text.

Advanced Glycation End-Products

Cu(II) chelation by TETA

ROS

Extracellular copper levels

Collagen CML levels are increased in vivo
Trend for normalisation of collagen CML levels after TETA treatment in vivo

Advanced Glycation End-Products

Kidney copper levels

TETA normalises copper induced decrease in CtsL activity in vitro

CtsL and Mmp2

Collagen pepsin digestibility decreased
Collagen mRNA- and protein levels are decreased
TETA treatment normalises collagen protein levels

Correlation between CtsL protein levels and collagen CML levels is absent after TETA treatment in vivo

Correlation between CtsL mRNA and protein levels is increased in vivo
Pro-Mmp2 levels are increased in vivo
CtsL mRNA and protein levels are increased in vivo

Negative correlation between Pro-Mmp2 activity and collagen protein levels in vivo

Correlation between Pro-Mmp2 activity and collagen protein levels in vivo

Indicates connections in the literature including references
----- Indicates possible new connections inferred from thesis results
New findings regarding diabetes
New findings regarding TETA treatment

References:

Chapter 6: Summary and conclusions

Both, the protease CTSL as well as CML have been associated with the degree of renal injury determined by albuminuria (Morcos et al. 2002; Bauer et al. 2011). Proximal tubular cells make up the largest portion of the kidney cortex and CTSL is most abundant in the lysosomes of these cells, so that dysregulation of CTSL in the cortex could well represent changes in proximal tubular cells (Pfaller 1982; Yokota et al. 1988). While it is believed that fibroblasts are the major cell type responsible for the synthesis and degradation of collagen, epithelial cells are also capable of phagocytosis and the intracellular degradation of collagen (Salonen et al. 1991; Everts et al. 1996). Considering the strong expression of CTSL in the proximal tubular cells, it is possible that these contribute to the basal degradation of tubular basement membrane or tubulointerstitial collagen in kidneys. Increased expression of CTSL may compensate for the decreased digestibility of collagen in combination with increased collagen CML levels in diabetes reported here, which is supported by the correlation between CTSL and CML as well as CTSL and collagen digestibility.

Alternatively, kidney collagen CML levels may be an indicator of the amount of CML being formed in serum over time, due to the slow turnover of collagen (Verzijl et al. 2000). The main purpose of proximal tubular cells is to retrieve and digest albumin and other serum proteins and peptides (as well as other nutrient molecules, salts and water), which pass through the filtration barrier of the glomerulus (Birn et al. 2006), but the importance of proximal tubular cells in the development of proteinuria has been underestimated until recently (Jefferson et al. 2008). Peptides and proteins are increasingly modified in diabetes with AGEs in general and CML in particular (Kilhovd et al. 1999; Morcos et al. 2002). Increasing amounts of serum protein-AGE adducts, peptide-bound AGES and non-protein bound AGES (so called AGE-free adducts) are taken up via the brush border located at the apical membrane of proximal tubular cells in diabetes, and are degraded in the lysosomes by CTSL amongst other proteases (Gugliucci et al. 1996; Miyata et al. 1998). In line with these observations, there is an accumulation of cytoplasmic CML in proximal tubular cells, and it has been concluded that this CML is likely to be of soluble origin rather than CML-protein adducts and possibly located alongside or inside the lysosomes (Alderson et al. 2004; Uchiki et al. 2012).

Elevated levels of copper alongside elevated CML levels are present in the serum of many diabetic humans (Mateo et al. 1978; Sjögren et al. 1986; Walter et al. 1991; Zargar et al. 1998; Kilhovd et al. 1999; Morcos et al. 2002). In addition, diabetic rats display increased kidney copper levels while such data for diabetic humans is currently missing (Failla et al. 1981; Gong et al. 2008; Cooper 2012). This abnormality could result in a vicious cycle of increased CML formation, through copper-catalysed ROS formation, in a process termed...
Chapter 6: Summary and conclusions

glycoxidative stress in diabetes (Saxena et al. 1999). The uptake of redox-active copper bound to CML-modified albumin or CML-peptides via proximal tubular cells may also contribute to the increased copper levels seen in the kidneys of diabetic rats. A potential pathogenic role of AGE-modified albumin in proximal tubular cells has previously been proposed in a publication entitled “Albuminuria and renal injury - beware of proteins bearing gifts” (Iglesias et al. 2001). The increased amount of redox-active copper bound to CML-modified albumin or CML-peptides as the unwelcomed “gift” was not mentioned in that context (Saxena et al. 1999; Eaton et al. 2002). Enhanced copper uptake is toxic to lysosomes resulting in lysosomal enlargement and lysosomal instability in vitro and in vivo (Romeo et al. 2000; Pourahmad et al. 2001). In this regard it is important to note that, based on its hydrophilic properties, TETA is thought to chelate only extracellular Cu(II), but TETA administration does influence intracellular copper levels in vitro (Logie et al. 2012). Thus TETA may ameliorate a toxic, lysosomal copper overload and glycoxidative stress, via the chelation of extracellular CML-bound copper.

Altered proteolytic properties of collagen in vitro were amongst the first changes described for AGE-modified proteins while most subsequent studies have focused on collagen and other substrates rather than the possible effect of such modifications on proteases in vivo (Hamlin et al. 1975; Thomas et al. 2005). An inhibitory effect of AGE formation on the activity of the lysosomal proteases CTSB and CTSL has been shown in vitro while AGE-modified albumin was also degraded more slowly by these proteases than was un-modified albumin (Sebekova et al. 1998; Zeng et al. 2006). Further support comes from another study where altered processing of glycated albumin was shown for proximal tubular cells in vitro and it was proposed that such an altered digestibility may directly contribute to the formation of proteinuria (Ozdemir et al. 2008). Another group reported a decreased capacity of proteosomal processing in the kidneys of glyoxalase 1-deficient and STZ-diabetic mice (Queisser et al. 2010). Consequentially, a “glycation-altered proteolysis as a pathobiologic mechanism” has been proposed recently (Uchiki et al. 2012). Further support for this hypothesis is provided by this thesis. The study described in chapter 4 provides evidence for a previously unrecognised connection between the AGE, CML and the lysosomal protease CTSL in the kidneys of diabetic rats, while the partial amelioration of these effects by TETA treatment suggest the involvement of copper in this potentially pathogenic process. An involvement of copper is also supported by the in vitro experiments where CuCl₂ decreased CTSL activity and the addition of TETA normalised the activity.

Although controversy exists regarding the excretion of albumin in the urine, some workers have reported that >98 % of albumin is excreted in the form of peptides generated in
Chapter 6: Summary and conclusions

proximal tubular cells, which are not detected by standard albumin assays in healthy kidneys (Gudehithlu et al. 2004). Albuminuria has been linked to alterations in the lysosomal pathway in type 1 diabetic patients (Osicka et al. 2000) which agrees well with an altered digestion of glycated albumin in vitro (Ozdemir et al. 2008). Thus the findings reported here also provide a possible explanation for the amelioration of albuminuria and the normalisation of kidney copper levels in diabetic rats after TETA treatment, which was previously reported in several studies by our group (Gong et al. 2008; Lu et al. 2010).

In order to further strengthen the evidence for a connection between CML, CTSL and copper, an immunohistologic study for CTSL and CML could be carried out in healthy and diabetic rats treated with placebo or the Cu(II) chelator TETA. Furthermore, additional experiments regarding the interaction of AGEs with proximal tubular cells and lysosomal proteases could be performed. CML-modified peptides could be employed as opposed to the previous use of non-specifically glycated peptides or albumin, to determine the effects on lysosomal proteases of proximal tubular cells in vitro (Sebekova et al. 1998). While in vitro glycated albumin is still employed for research on AGEs and their role in diabetes-mediated tissue damage, it is increasingly becoming clear that it is not a suitable model compound to measure diabetes- or AGE-induced pathologic processes in vivo or in vitro, due to the extensive uncharacterised modifications of such substances (Heizmann 2007; Thornalley 2007). In that respect, the application of a specific CML-modified peptide alongside a corresponding, unmodified lysine-containing control peptide would enable one to draw more direct conclusions. Potential changes in protease activity in such a model could then be investigated further for copper sensitivity by the application of the Cu(II) chelator TETA. A recently discovered copper probe could also be employed in such studies (Wang et al. 2010).

Regarding the findings of the LV of the heart of diabetic rats described in chapter 5, no conclusive effects of TETA treatment were found, while changes seen in diabetic rats mostly agreed with previous reports. Some changes after TETA treatment were seen at a transcriptional level in diabetic rats but none of these findings could be corroborated at the protein level. This is in contrast to previous findings that TETA treatment improved physiological parameters in diabetic humans and STZ-diabetic rats (Cooper et al. 2004; Baynes et al. 2009; Cooper et al. 2009). The molecular basis for these pathologic changes in DCM are still not well understood although AGEs do appear to play an important role in this process, as a correlation between levels of the AGE CML in small myocardial vessels, and heart failure with reduced ejection fraction has previously been reported (van Heerebeek et al. 2008). TETA treatment may act by normalising AGE levels in the diabetic heart, but
collagen CML levels from the LV of heart collagen extracts were too low to be measured, possibly due to the faster turnover of collagen in the heart as compared to the kidney (Gineyts et al. 2000; Verzijl et al. 2000).

While the changes on an mRNA level in diabetes regarding collagen were similar in the heart and the kidney, other changes were very different in the two tissues. Proteases acting on collagen and non-collagen degrading proteases were dysregulated in diabetic rats in both tissues but often in opposite directions with CTSL, which was up-regulated or displayed a tendency to be up-regulated in both tissues in diabetes, being an exception. The current finding of unaltered collagen levels is in agreement with previous reports from STZ-diabetic rats after 16 to 32 weeks of diabetes (Modrak 1980; Norton et al. 1996; Candido et al. 2003). An absolute accumulation of collagen in the heart, based on hyp measurements has been reported in long-term diabetic dogs or monkeys after 18 months and 12 months of diabetes, respectively (Haider et al. 1978; Regan et al. 1981). Thus, it could be that the duration of diabetes is not long enough for the development of fibrosis in STZ-diabetic rats, considering that the longest studies ran approximately half as long as the studies in monkeys. The massive collagen accumulation reported in short to mid-term studies in STZ-diabetic rat hearts after 4-8 weeks appears to be transient with a complete normalisation after 18 weeks, casting doubt on its relevance in diabetic humans (Modrak 1980; Reddi 1988; Miric et al. 2001; Van Linthout et al. 2008).

The strong increase in CAT protein levels in the heart of STZ-diabetic rats were reported previously, while no such changes were present in the kidneys. These discrepancies may well be a reflection of the different nature of dysregulation in energy metabolism in diabetic hearts compared to diabetic kidneys. While the heart increasingly utilises fatty acids in the diabetic state, larger amounts of glucose are being taken up in diabetic kidneys, with an apparent decrease of fatty acid utilisation alongside unaltered or decreased levels of proteins associated with the fatty acid metabolism (Korner et al. 1994; Meyer et al. 1998; Engels et al. 1999; Sakamoto et al. 2000). Such a discrepancy is also present in mice fed with a diet rich in fatty acids where again the heart, but not the kidney displays a strong increase in CAT levels (De Craemer et al. 1994; Rindler et al. 2013). The finding that collagen carbonyl levels as an indicator of oxidative stress are unaltered in diabetic rat hearts suggests that oxidative stress in the extracellular matrix of the heart is unaltered (Berlett et al. 1997; Stadtman 2004). However, considering the finding of an inverse correlation of carbonyl levels with CML levels in the kidney, it may also reflect the fact that potential carbonyl formation sites are modified with other post-translational modifications in heart collagen.
Chapter 6: Summary and conclusions

In order to further elucidate the impact of TETA on CML levels in diabetes, analyses of the diabetic heart by immunohistochemistry for CML may be a useful tool. That way CML in small myocardial vessels in diabetes could be detected in continuation of the finding by van Heerebeek et al. and a potential treatment effect of TETA could thus be investigated (van Heerebeek et al. 2008). TETA has also been shown to restore the aortic relaxation in STZ-diabetic rats while aortic stiffness is a contributor to cardiomyopathy (Keegan et al. 1999; Austin et al. 2010). Considering that AGEs are increased in the aorta and that levels correlate with aortic stiffening in diabetes, TETA may also improve the diabetic heart via the reduction of AGE levels in the aorta (Sims et al. 1996; Meng et al. 1998; Xu et al. 2003). Thus, analysis of the aorta in healthy and diabetic rats treated with a placebo or TETA by immunohistochemistry in parallel to the LV may shed further light on the molecular basis of the beneficial physiologic effects seen after TETA treatment.

Future studies to investigate the treatment effects of TETA could be carried out using models like the Zucker diabetic fatty rat (Peterson et al. 1990) or one of the numerous other type 2 diabetic models (Yokoi et al. 2013). The reason we chose the STZ-diabetic rat model was that it represents a well-established hyperglycaemic model allowing for good comparability with previous studies (see p. 40 ff for detailed discussion). While the current setup of TETA treatment after 8 weeks of established diabetes was also used for reasons of easier comparability to previous studies and to simulate a more realistic treatment regime in humans it may be of interest to look at treatment beginning with the induction of diabetes. Such an earlier application of the chelator could yield more distinct information regarding the effects of Cu(II) as opposed to glucose regarding in vivo AGE formation. Other future studies could focus on the kinetics of AGE formation in vivo. Their formation in vitro has been shown to take place within days at a temperature of 37 °C and pH 7.4 while the formation in vivo is thought to take weeks or longer (Brownlee et al. 1988; Booth et al. 1997; Valencia et al. 2004). However, a recent in vivo study showed that levels of the AGE CML were already increased, by over ~2-fold after 3 weeks of diabetes which was the earliest time point measured, with no further increases after 12 weeks of diabetes suggesting that these products may form quicker than was originally thought (Duran-Jimenez et al. 2009).

In summary, this thesis presents evidence for a disturbed collagen metabolism in diabetic rat kidneys which is partially normalised by TETA treatment through effects on AGE formation and the collagen degradation pathway. Evidence for a disturbed collagen metabolism in the diabetic rat heart was less pronounced and TETA treatment did not show any effect.
### STZ certificate of analysis

**Certificate of Analysis**

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<td>Carbon (anhydrous)</td>
<td>Conforms</td>
</tr>
<tr>
<td>Nitrogen (anhydrous)</td>
<td>0.6 %</td>
</tr>
<tr>
<td>% Purity (HPLC)</td>
<td>36.6 %</td>
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<tr>
<td>Purity (HPLC)</td>
<td>15.7 %</td>
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<tr>
<td>≥75%</td>
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<tr>
<td>Alpha Anomer by HPLC</td>
<td>91 %</td>
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**LOT 119K1591 RESULTS**

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<td>Appearance (Color)</td>
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<td>Appearance (Form)</td>
<td>Powder</td>
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<td>Solubility (Turbidity)</td>
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<tr>
<td>¹³C NMR Spectrum</td>
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<td>Water (by Karl Fischer)</td>
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<tr>
<td>Carbon (anhydrous)</td>
<td>0.6 %</td>
</tr>
<tr>
<td>Nitrogen (anhydrous)</td>
<td>36.6 %</td>
</tr>
<tr>
<td>% Purity (HPLC)</td>
<td>15.7 %</td>
</tr>
<tr>
<td>Purity (HPLC)</td>
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<tr>
<td>Alpha Anomer by HPLC</td>
<td>91 %</td>
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**Recommended Retest Period:**

- Specification Date: NOV 2000
- Date of QC Release: NOV 2009
- Recommended Retest Date: NOV 2011
- Print Date: DEC 01 2009

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Rodney Burbach, Manager  
Quality Control  
St. Louis, Missouri USA

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**Appendix figure 1:** Certificate of analysis for the lot of STZ used
## Primer Sequences for RT-qPCR

### Appendix Table 1: Target Primer Sequences (continued on next page)

<table>
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<th>Gene Name</th>
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<tbody>
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<tr>
<td>Mmp2</td>
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<td>Sod2</td>
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<td>Vegfa</td>
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### Appendix table 2: Reference primer sequence

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<tr>
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<td><em>U2af</em></td>
<td>CCATTGCCCTTTGAACATT</td>
<td>CCTCCCGTACTTCTCTTCC</td>
</tr>
</tbody>
</table>
Appendix

NMR data from methacrylamido phenylboronic acid synthesis

Appendix figure 2: NMR of 3-methacrylamido phenylboronic ester
Shown are $^{13}$C (top) and $^1$H (bottom) NMR for the product of reaction number 3 given on page 68. Both NMRs confirm that the obtained product is the desired 3-methacrylamido phenylboronic ester.
Appendix figure 3: NMR of 3-methacrylamido phenyl trifluoroborate
Shown is the $^1$H NMR for the product of the de-protection reaction to yield 3-methacrylamido phenyl trifluoroborate for reaction number 4 described on page 69. The NMR confirms that the desired product was obtained.
The $^{13}$C (top) and $^1$H (bottom) NMR can be seen for the final product obtained from reaction number 5 described on page 70. Both NMRs confirm that the final product obtained is 3-methacrylamido phenylboronic acid.
References


References


References


182
References


References


References


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References


References


References


References


References


References


Thornalley, P. J. (2007). "Dietary AGEs and ALEs and risk to human health by their interaction with the receptor for advanced glycation endproducts (RAGE)--an introduction." Mol Nutr Food Res 51(9): 1107-1110.


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