The Enzymology of Fetuin: A Potential Link Between Periodontal Diseases and Calcifying Atheromas

by

Ryan Samuel Schure

A thesis submitted in conformity with the requirements for the degree of Master of Science

Discipline of Periodontology, Faculty of Dentistry
University of Toronto

© Copyright by Ryan Samuel Schure 2013
The Enzymology of Fetuin: A Potential Link Between Periodontal Diseases and Calcifying Atheromas

Ryan Samuel Schure

Master of Science

Discipline of Periodontology, Faculty of Dentistry
University of Toronto

2013

Abstract

Periodontal diseases may increase risk of vascular calcification in cardiovascular diseases but the potential mechanisms are not defined. Fetuin, a naturally-occurring serum glycoprotein in humans, protects against ectopic arterial calcification. We considered that patients with periodontitis could be at increased risk of developing calcifying atheromas because periodontal-disease associated enzymes may enter the circulation and subsequently degrade fetuin, thereby disrupting its ability to inhibit calcification. By in silico investigation, MMP -3 and -7 were predicted to cleave fetuin but only MMP-7 actually degraded human fetuin in vitro. MMP-7 degradation of fetuin was time- and concentration-dependent and was inhibited by an MMP Inhibitor. By mass spectrometry the presence of novel, MMP-7-mediated cleavage sites in fetuin were found. Fetuin bound tightly to MMP-7 (kd =2.96 x 10^{-9} M). The degradation of fetuin by MMP-7 could explain, at least in part, the apparent association between periodontal diseases and calcifying atheromas.
Acknowledgments

They say, “It takes a village...”. My village included the following people to whom I am forever indebted:

To my supervisors, Christopher McCulloch and Dr. Howard Tenenbaum: I want to start by thanking you for taking on an inexperienced student and being prepared for some of the challenges that came along with that. I can’t really express how much I appreciate all of the guidance and support you have provided to me. Your enthusiasm for your work is inspiring and has really opened up my eyes to the elation that can be gleaned in the field of research. I cannot imagine two kinder gentleman and model mentors to have been my supervisors and feel honoured and privileged to have had the pleasure of working with you both.

To Dr. Craig Simmons, my committee member: Thank you for supporting this project and providing novel insights and valuable feedback at our meetings.

To Carol Laschinger: Thank you for ‘showing me the ropes’ in the lab and teaching me the techniques necessary for this project. Your patience and instruction was outstanding. Carol completed many of the preliminary experiments that were necessary for my work to even begin and was involved in all of the assays discussed in this thesis. This work would not have been completed without her time and efforts.

To Wilson Lee and our summer student, Kerry D’Costa: Your work on the mineralization assays contributed greatly to the overall completion of this thesis. Thank you for being there to help out whenever needed.

To Dr. Reyhaneh Rezaei and Dr. Corneliu Sima: You have been great friends and co-residents and I know you will both be exceptional periodontists. Reyhaneh also contributed significantly to this project with her work evaluating the ability of fetuin to inhibit mineralization in vitro.

To my wife-to-be (Hila), my parents (Judy and Hugh), and my brother (Ali): Thank you for your unwavering support during this endeavour. It wasn’t always easy, but I appreciate your listening ears, your ability to keep me grounded, and you for being able to see the bigger picture. I would not be here without your encouragement. The good news- you probably now know more about fetuin than 99.9% of the population!
# Table of Contents

Acknowledgments ................................................................................................................. iii
Table of Contents .................................................................................................................. iv
Publication Arising from Thesis ............................................................................................. vi
List of Tables ........................................................................................................................... vii
List of Figures ........................................................................................................................ viii
List of Abbreviations ............................................................................................................. ix

**LITERATURE REVIEW** ..................................................................................................... 1

1  Association Between Periodontal Diseases and Cardiovascular Diseases ....................... 1
   1.1  Periodontal Disease ........................................................................................................ 1
       1.1.1  Classification of Diseases ....................................................................................... 1
       1.1.2  Epidemiology ........................................................................................................ 1
       1.1.3  Etiology ................................................................................................................ 2
       1.1.4  Pathogenesis .......................................................................................................... 2
       1.1.5  Enzymes that are Upregulated in Periodontitis ................................................... 4
       1.1.6  Matrix Metalloproteinases ....................................................................................... 6
   1.2  Cardiovascular Diseases ............................................................................................... 11
       1.2.1  Background and Epidemiology .............................................................................. 11
       1.2.2  Etiology and Pathogenesis of Atherosclerosis .................................................... 11
   1.3  Evidence of Associations Between Periodontal Diseases and Cardiovascular Diseases ......................................................................................................................... 12
       1.3.1  Periodontal Pathogens in Atherosclerotic Plaques ............................................ 12
       1.3.2  Periodontitis Associated with Carotid Artery Calcifications and Atherosclerosis .............................................................................................................................................. 13
       1.3.3  Improvement in CVD Biomarkers After Periodontal Therapy ......................... 14

2  Vascular Calcification ......................................................................................................... 16
   2.1  Vascular Calcification in CVDs .................................................................................... 16
       2.1.1  Regulation of Vascular Calcification .................................................................... 17

3  Fetuin .................................................................................................................................. 18
   3.1  Background ................................................................................................................ 18
   3.2  Structure .................................................................................................................... 19
   3.3  Biological Activity ....................................................................................................... 19
       3.3.1  Inhibition of Calcification .................................................................................... 19
       3.3.2  Inhibition of HMGB1 Release ............................................................................. 21
   3.4  Enzymatic Degradation of Fetuin ............................................................................. 21
STATEMENT OF THE PROBLEM ................................................................. 23
OBJECTIVES .............................................................................................. 23
HYPOTHESIS .............................................................................................. 23

4 ABSTRACT ............................................................................................... 24
5 INTRODUCTION ....................................................................................... 25
6 MATERIALS & METHODS ................................................................. 27
   6.1.1 Reagents ....................................................................................... 27
   6.1.2 MMP Assay ................................................................................... 27
   6.1.3 Fetuin Cleavage by MMPs ............................................................. 27
   6.1.4 Fetuin Binding to MMPs ............................................................... 28
7 RESULTS .................................................................................................. 29
   7.1.1 In Silico Analysis ......................................................................... 29
   7.1.2 Enzyme Activation ....................................................................... 29
   7.1.3 In Vitro Degradation Assays ......................................................... 30
   7.1.4 Mass Spectrometry ....................................................................... 31
   7.1.5 Binding Assays ............................................................................. 31
8 DISCUSSION ............................................................................................ 33
   Future Studies ......................................................................................... 36
   Conclusions ............................................................................................ 37
TABLES AND FIGURES ............................................................................ 38
Bibliography ............................................................................................... 49
Publication Arising from Thesis

List of Tables

*TABLE 1:* Definitions of Predictors for Periodontal Diseases..........................38
List of Figures

FIGURE 1: Structure of MMPs ................................................................. 39

FIGURE 2: Amino acid sequence of human fetuin ........................................ 40

FIGURE 3: Enzyme activity levels of non-activated, manufacturer-activated, and APMA-activated MMP-7 .......................................................... 41

FIGURE 4A: Time-course degradation of fetuin by MMP-3 and MMP-7 .......... 42

FIGURE 4B: Inhibition of degradation by MMP-Inhibitor II .......................... 43

FIGURE 5A: Peptide analysis by mass spectrometry .................................. 44

FIGURE 5B: Fetuin amino acid sequence analysis .................................... 45

FIGURE 6A: Assessment of fetuin binding to heparin agarose beads ............ 46

FIGURE 6B: Free versus bound fetuin at varying fetuin concentration .......... 47

FIGURE 6C: Catalytic activity of fetuin bound to heparin agarose beads ........ 48
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAP</td>
<td>American Academy of Periodontology</td>
</tr>
<tr>
<td>AHSG</td>
<td>Alpha-2-Heremans Schmid glycoprotein</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>APMA</td>
<td>4-Amino phenylmercuric acetate</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAL</td>
<td>Clinical attachment loss</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DMC</td>
<td>Dimethylaminocoumarin</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ESRD</td>
<td>End-stage renal disease</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GCF</td>
<td>Gingival crevicular fluid</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High mobility group box 1 protein</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>LJP</td>
<td>Localized juvenile periodontitis</td>
</tr>
<tr>
<td>MGP</td>
<td>Matrix Gla protein</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MT-MMP</td>
<td>Membrane-type matrix metalloproteinase</td>
</tr>
<tr>
<td>NBD</td>
<td>Nitro-2-1,3-benzoxadiazol-4-yl</td>
</tr>
<tr>
<td>NUG</td>
<td>Necrotizing ulcerative gingivitis</td>
</tr>
<tr>
<td>NUP</td>
<td>Necrotizing ulcerative periodontitis</td>
</tr>
<tr>
<td>OPN</td>
<td>Osteopontin</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear leukocytes</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative fluorescence unit</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinases</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
</tr>
</tbody>
</table>
LITERATURE REVIEW

1 Association Between Periodontal Diseases and Cardiovascular Diseases

1.1 Periodontal Disease

1.1.1 Classification of Diseases

Practicing dentists use a classification system for periodontal diseases that is based on clinical manifestations and observations for diagnostic purposes although little reliance is made on laboratory tests. The most recent, internationally recognized classification system for periodontal diseases (1999 International Workshop for the Classification of Periodontal Diseases organized by the American Academy of Periodontology) focuses on the major categories of gingival and periodontal diseases. From this classification, several general aspects of periodontal diseases are generally recognized. Gingival diseases do not typically affect the underlying alveolar bone and can be induced by bacterial biofilms, viruses, fungi, foreign bodies or trauma. In some instances, gingival diseases are manifestations of genetic disorders or other health conditions. Gingival inflammation can, in a small proportion of patients, progress to periodontitis, in which the supporting structures of the teeth are degraded. Periodontitis can manifest as chronic or aggressive sub-types, and can also be a manifestation of at least 16 different systemic diseases. Further, periodontitis can be associated with endodontic lesions in various combinations. Necrotizing periodontal diseases include necrotizing ulcerative gingivitis (NUG) and necrotizing ulcerative periodontitis (NUP). Abscesses of the periodontium are classified as gingival, periodontal or peri-coronal. Determining the correct periodontal diagnosis requires an assimilation and understanding of information derived from the medical and dental histories that are then followed by clinical examination.

1.1.2 Epidemiology

Periodontal diseases were once considered to be almost universally prevalent in middle-aged humans; it was thought that all individuals in the population were equally susceptible. This notion was challenged in the 1980s, most notably by Löe and co-
workers, who examined untreated male Sri Lankan tea workers in a longitudinal study of 15 years duration\textsuperscript{4}. The longitudinal experimental design allowed assessment of the natural history of periodontal diseases. The data showed that, even under similar environmental conditions, individuals were not equally affected by destructive periodontal diseases. Notably, a large number of clinical and epidemiological studies have estimated the prevalence of severe, generalized periodontitis. In general, these data indicate that \(~\text{5\%\textendash}15\%\) of the human population is affected by these disorders\textsuperscript{5\textendash}8. Specifically, according to the Canadian Health Measures Survey 2007-2009, 16\% of Canadian adults have moderate periodontal disease (pocket depth of 4 or 5 mm) and 4\% of Canadians have severe periodontal disease (pocket depth greater than or equal to 6 mm)\textsuperscript{9}. One of the reasons for the wide range of prevalence estimates is the lack of consistent definitions of periodontal diseases that have been used in different epidemiological surveys.

1.1.3 Etiology

Multiple factors contribute to the development and progression of periodontal diseases. In addition to microbial biofilms, environmental and acquired risk factors affect the onset, rate of progression and severity of disease, as well as response to therapy\textsuperscript{3}. A risk factor can be defined as “an action or event that is related statistically in some way to an outcome and is truly causal” (Table 1)\textsuperscript{10}. Overwhelming evidence shows that bacterial biofilms initiate tissue destruction\textsuperscript{11\textendash}17, however, other data that relate to separate risk factors, including smoking\textsuperscript{3, 7, 18\textendash}23, diabetes status\textsuperscript{24\textendash}26, and genetic background of people with periodontitis\textsuperscript{3, 7, 27\textendash}31, indicate that the etiology of periodontitis is multi-factorial.

1.1.4 Pathogenesis

The histopathological features of periodontal tissues in gingivitis and periodontitis have been investigated intensively and are well-known\textsuperscript{32}. One widely considered hypothesis for the progression of periodontal diseases suggests the existence of “initial, early, and established lesions”, which are thought to represent sequential stages following from the establishment of gingivitis. The putative “advanced lesion” may manifest clinically as periodontitis. These disease stages likely comprise the large majority of inflammatory
gingival and periodontal lesions in humans. The specific vascular and cellular changes involved in disease development are described below.

1.1.4.1 Changes in Tissue Vasculature

An alteration in vascularization, which is the overall organization and structure of the blood vessel supply to a tissue, is a frequent consequence of chronic inflammation. In chronic inflammatory lesions of the periodontium, there are marked increases in the number of blood vessels in the periodontal pocket wall compared to minimally inflamed tissues\(^{33-37}\) and the mean diameter of vessels is expanded\(^{33-36}\). As a result of capillary vasodilation, as well as increased microvascular permeability, there is enhanced extravasation of tissue fluid from capillaries into the interstitium during inflammation\(^ {38}\). This leads to an increase in osmotic pressure in the surrounding tissues. This has also been demonstrated in experimentally-induced periodontitis, suggesting a positive association between vascular permeability and the relative degree of tissue vascularization\(^ {39}\). Increased vascular remodeling facilitates increased production of a serum-like fluid that normally emanates from the gingival crevice at a physiologic rate: gingival crevicular fluid (GCF). The fluid is essentially a manifestation of host defense, but it also provides a pathway for bacterial irritants and various proteins including hydrolytic enzymes to enter the surrounding tissues, bone, and periodontal ligament in addition to drainage through lymphatic system\(^ {34,40}\).

1.1.4.2 Changes in Cellular Composition

New approaches for periodontal diagnosis now focus on developing more objective measures of disease such as the analysis of various biomarkers in GCF\(^ {41}\). These measures extend the clinical information obtained from traditional approaches such as assessment of clinical measurements of probing depths, bleeding on probing, clinical attachment levels, plaque index and radiographic examination\(^ {42}\). GCF includes a mixture of molecules from blood, host tissues and plaque biofilms. In a healthy sulcus, the amount of gingival fluid is very small while at inflamed sites the amount of fluid may increase, as alluded to above, up to 30-fold\(^ {43}\). The composition of GCF resembles that of an inflammatory exudate as assessed by examination of electrolytes, proteins, cytokines, antibodies, bacterial antigens, and enzymes\(^ {38,41,44-50}\).
1.1.5 Enzymes that are Upregulated in Periodontitis

Enzymes in GCF have been considered as potential biomarkers of periodontal disease activity but it is important to establish whether enzymes are of host or bacterial origin. Some putative periodontal pathogens, such as *Porphyromonas gingivalis* or *Treponema denticola*, produce virulence factors including proteases, which may be detected in plaque biofilms and GCF samples derived from patients with periodontitis. As GCF contains bacterial enzymes that can bind to and regulate the activity of host cell-derived enzymes, it is also possible that bacterial proteases may activate host enzymes.

According to Loos, ~100 constituents of GCF have been evaluated for potential utility in the development of periodontal diagnostic tests. Some of the more promising GCF components have been reviewed. The major enzymes that are associated with the inflammatory host response to periodontitis are outlined below. Briefly, a description of some of the statistically meaningful definitions that are used to characterize diagnostic tests will be included. The sensitivity of a test is defined as the proportion of people with the disease who will have a positive result. The specificity of a test is the proportion of people without the disease who will have a negative result. The positive predictive value of a test is defined as the proportion of people with a positive test result who actually have the disease. The negative predictive value is the proportion of people with a negative test result who do not have the disease.

Alkaline phosphatase (AP) is a host cell membrane-bound glycoprotein. It is a phosphohydrolytic enzyme that plays an important role in the normal function of the periodontal ligament, root cementum formation and maintenance, and bone homeostasis, including an important role in the mineralization of bone and other calcified tissues. AP hydrolyzes monophosphate ester bonds at basic pH and plays a role in superoxide generation. In the GCF, AP may originate from neutrophils (PMNs), which are an important part of the first line of the innate host defense to inflammation, but AP may also be produced by bacteria in the gingival sulcus. Elevated levels of AP are found in the GCF of patients with naturally occurring and experimental gingivitis. In a longitudinal study of untreated adult patients with periodontitis, elevated levels of AP preceded loss of clinical attachment and the amount of AP in the GCF was significantly higher in sites.
exhibiting rapid destruction as compared to sites that were stable. The specific predictive value of AP for future attachment loss is dependent on the threshold values selected. For example, if 900 µIU/30 s is used, the positive and negative predictive values for subsequent loss of attachment are 62% and 68%, respectively. However, when a threshold of 1300 µIU/30 s was selected, the positive and negative predictive values were 77% and 76%, respectively. As AP is associated with bone formation, the finding that it is increased in destructive inflammatory lesions of the periodontium suggests that the AP measured in GCF may originate from bacteria or may reflect increased bone turnover in marginal periodontal lesions.

The lysosomal enzyme β-glucuronidase is released by activated PMNs and is involved in the degradation of proteoglycans and matrix proteins by hydrolysis of glycosyl bonds. Increased levels of β-glucuronidase in GCF are thought to be good predictors of clinical loss of attachment and are associated with high values of diagnostic sensitivity (89%) and specificity (89%). The relative risk of disease progression at sites with increased β-glucuronidase activity ranged from 6-14 times higher than controls. Further, β-glucuronidase levels are increased at active sites compared to inactive sites. Increased enzyme activity is associated with the presence of putative periodontal pathogens such as spirochetes, *Porphyromonas gingivalis*, and *Prevotella intermedia*. Reduced activity of β-glucuronidase is observed after periodontal therapy.

Aspartate aminotransferase (AST) is a host cell cytoplasmic enzyme that is released after cell death by disease-related necrosis and may provide an indirect assessment of neutrophil migration and cell lysis in the gingiva. Initial studies that examined AST in a ligature-induced model of periodontitis in beagle dogs demonstrated the presence of increased levels of AST in GCF. Increased AST activity has also been associated with gingival inflammation in a population of patients treated for periodontitis and at sites where there is active destruction of periodontal tissues. Median levels of AST are significantly higher in sites that exhibit active clinical attachment loss (CAL) compared to stable sites. Similarly, levels of lactate dehydrogenase, which is also a marker of cell death, are associated positively with inflammation.
Neutrophil elastase accounts for the bulk of protease activity in GCF\textsuperscript{67}. Elastase cleaves a broad range of extracellular matrix proteins including elastin, collagen and fibronectin\textsuperscript{50, 53}. Levels of elastase in GCF increase after the induction of experimental gingivitis and decrease when plaque biofilms are removed\textsuperscript{68}. The levels of elastase correlate with periodontal probing depth\textsuperscript{69} and some evidence indicates that elevated levels of elastase activity may predict future attachment loss\textsuperscript{70}. Data have shown elastase activity to be correlated with both probing depth\textsuperscript{69} and inflammation\textsuperscript{71}.

Hyaluronidase activity increases in direct proportion to elevated levels of gingival inflammation\textsuperscript{72} and decreases after provision of periodontal therapy with concomitant reduction in periodontal inflammation\textsuperscript{72}. Hyaluronidase participates in periodontal destruction by cleaving the beta (1-4)-N-acetylglucosaminidine links in hyaluronic acid, chondroitin-4-sulphate, and chondroitin-6-sulphate, all of which are important structural components of the extracellular matrix\textsuperscript{50}. With regard to periodontitis though, a large proportion of hyaluronidase activity is of bacterial origin and therefore assessment of its levels and/or activity as a specific biomarker of host enzyme activity and disease might be questionable\textsuperscript{73}.

Cathepsin-like proteases are cysteine proteases that play a role in degradation of matrix molecules and collagen in particular. While evidence from longitudinal studies is limited, both cathepsin B\textsuperscript{49} and D\textsuperscript{50} appear to have potential as diagnostic markers of gingival inflammation and for the assessment of treatment outcomes.

### 1.1.6 Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are potentially the most important family of proteinases that participate in both the normal turnover (modeling and remodeling) of tissues comprising the periodontium, as well as destruction of the extracellular matrix (ECM) in periodontal diseases. The gene family of MMPs encodes a total of 24 homologous, zinc-dependent endopeptidases (MMPs 1-3 and 7-28)\textsuperscript{47}, which share structural domains but differ in substrate specificity, cellular sources and inducibility\textsuperscript{74}. Because of the potential for MMPs to degrade matrix proteins and to degrade and potentially activate other important regulators of biological and pathobiological processes in the periodontium such
as signaling molecules, the expression and activation of MMPs are regulated tightly.

In general, most MMPs are synthesized in a latent form (i.e. a ‘pro’-form) and require activation for full development of their proteolytic activity\(^{47,74}\). Activation may occur \textit{in vivo} by the action of other proteinases or superoxide ions; activation \textit{in vitro} can be mediated by the use of mercury compounds\(^{75}\). All MMPs are capable of degrading certain matrix components. They contain a Zn\(^{2+}\) at their active site, which is important for control of catalytic activity. MMPs require Ca\(^{2+}\) for stability and function optimally at neutral pH\(^{74}\). Specific inhibition of MMP proteolytic activity can be regulated by a family of proteins known as the tissue inhibitor(s) of metalloproteinases (TIMPs), which specifically and irreversibly inhibit the extracellular activity of certain MMPs.

1.1.6.1 Structure of MMPs

All MMPs share a common domain structure and catalytic mechanism (Figure 1). They contain an N-terminal signal peptide, which targets them for secretion from the cell to the extracellular environment. The signal peptide is followed by a pro-peptide that contains a conserved PRCGXPD sequence, and a catalytic domain, with a conserved HEXXHXXGXXH sequence\(^{76}\). The three histidine residues in the catalytic domain together bind a Zn\(^{2+}\) ion, which is critical for enabling the development of catalytic activity. Specific amino acids in the catalytic and hemopexin domains help to define substrate specificity by controlling interactions with substrates\(^{77}\). Substrate specificity is determined in part by sequences in exosites (e.g. substrate binding sites), which are located in domains that are separate from the catalytic domain\(^{76}\). These non-catalytic domains help to modulate and broaden the substrate specificity profile of MMPs by providing additional binding sites that lie outside of the catalytic domain\(^{47}\). The catalytic domain in most MMPs is followed by a proline-rich linker, which is known as the hinge region. This sequence is contiguous with the C-terminal hemopexin domain, which adopts a 3-dimensional structure resembling a four-bladed propeller and functions in substrate and inhibitor interactions. The exceptions to this general rule of MMP structure are matrilysin (MMP-7) and endometase (MMP-26), which lack the hemopexin domain, and MMP-23, which contains C-terminal cysteine- and proline-rich domains (cysteine array region) and an immunoglobulin-like domain instead of the hemopexin domain\(^{76,77}\).
1.1.6.2 Functions of MMPs

MMPs are generally classified into groups based on their functions. Collagenases (MMP-1, -8, -13, -18) cleave interstitial collagens I, II, and III at a specific site three-quarters of the length of the molecule from the N-terminus. This site is thought to be thermally unstable and enables collagenases to bind and degrade the collagen triple helix. The ¼- ¾ cleavage pattern of mammalian MMPs contrasts with bacterial collagenases, which cleave the collagen molecule at multiple sites leads to almost complete degradation. Consequently it is possible to differentiate between the actions of either bacterial or mammalian forms of collagenase, both of which would be present in an inflamed/infected periodontal site. In the periodontium, the rate of connective tissue destruction has been correlated to the amount of mammalian collagenolytic activity in GCF. Circulating MMP-8 (neutrophil collagenase) as well as MMP-9 (neutrophil gelatinase) levels are increased in patients with periodontitis and have been shown to decrease after therapy for this condition.

Gelatinase A (MMP-2) and B (MMP-9) are members of a second but equally important group of MMPs that can degrade collagen, gelatin, and various glycosaminoglycans in the intracellular ground substance. MMP-2 plays an important role in osteogenesis in humans since mutations of the MMP-2 gene can lead to an autosomal recessive form of multicentric osteolysis. Increases of MMP-9 activity have been associated with increases of CAL but correlate poorly with other traditional clinical parameters of periodontal health.

Stromelysin 1 (MMP-3) and 2 (MMP-10) have similar substrate specificities but MMP-3 catalyzes most substrates more rapidly than does MMP-10. In addition to digestion of ECM components, MMP-3 activates a number of pro-MMPs, including pro-MMP-1. MMP-11, also known as stromelysin 3, is usually grouped with "other MMPs" because the sequence and substrate specificity diverge from those of MMP-3.

From a structural point of view, the matrilysins represent a relatively simple type of MMPs and are characterized by the lack of a hemopexin domain. Matrilysins include MMP-7 (matrilysin 1) and MMP-26 (matrilysin 2, or endometase). Matrilysins digest a number of ECM components including fibronectin, laminin, type IV collagen, gelatin, elastin,
entactin, tenascin and proteoglycan core proteins. MMP-7 also processes some cell surface molecules such as pro-α-defensin, Fas-ligand, pro-tumor necrosis factor (TNF)-α, and E-cadherin.

There are six membrane-type MMPs (MT-MMPs): MMP-14, -15, -16, -17, -24, and -25. Some MT-MMPs are type I trans-membrane proteins that are anchored to the cell membrane by glycosylphosphatidylinositol (GPI). MT-MMPs can digest a number of ECM components and some exhibit collagenolytic activity. MMP-14-null mice demonstrate skeletal abnormalities, which is most likely due to the lack of collagenolytic activity. MMP-14 also plays an important role in angiogenesis.

Seven MMPs are not classified into the above categories. Metalloelastase (MMP-12) is expressed mainly in macrophages and is essential for macrophage-mediated digestion of ECM components. MMP-19 was identified as a T-cell-derived auto-antigen in patients with rheumatoid arthritis. Enamelysin (MMP-20) is located primarily within newly-formed tooth enamel; mutations at the cleavage sites of MMP-20 in the enamel protein amelogenin can lead to amelogenesis imperfecta. MMP-22 has been identified but its function is unknown. MMP-23 is also known as a cysteine array MMP and is expressed mainly in reproductive tissues. MMP-28, or epilysin, is found predominantly in keratinocytes, and may be involved in tissue hemostasis and wound repair.

### 1.1.6.3 Endogenous Inhibitors of MMPs

The functional activities of MMPs must be regulated in order to maintain homeostasis. The activity of MMPs is under the control of specific inhibitors, the most important of which are the TIMPs, as mentioned above. Four TIMPs (TIMP-1, -2, -3, and -4) have been identified in vertebrates. The TIMPs bind non-covalently to active MMPs in a 1:1 molar ratio. Inhibition is enabled by their ability to interact with the Zn$^{2+}$-binding site in the catalytic domain of MMPs. There is a certain degree of specificity among the TIMPs for MMP inhibition. TIMP-1 inhibits all MMPs except the MT-MMPs and MMP-2; TIMP-2 inhibits all MMPs except MMP-9; TIMP 3 can inhibit MMP-1, -2, -3, -9, and -13; TIMP-4 inhibits MMP-1, -3, -7, and -9. The tissue destruction seen in disease that has been...
attributed to MMPs may be caused by imbalances in the ratios between MMPs and TIMPs.\textsuperscript{90}

1.1.6.4 MMP-3 and MMP-7 in Periodontitis

In an attempt to understand the relationship between levels of biomarkers and severity/progression of disease, the levels of MMPs and TIMPs in patients with periodontitis have been assessed in several investigations. MMP-3 is potentially important from a clinical perspective as it is involved in the activation of latent pro-MMP-8, pro-MMP-9\textsuperscript{91} and the fibroblast collagenase MMP-1\textsuperscript{92}. Cross-sectional\textsuperscript{93} and longitudinal\textsuperscript{91} studies have shown that MMP-3 levels can be used to differentiate diseased from healthy sites in the periodontium and that sites with high levels of MMP-3 in the GCF are at significantly greater risk for progression of periodontitis as measured by loss of attachment or other parameters of tissue destruction.

MMP-7, which degrades numerous ECM and basement membrane components, is also involved in the activation of pro-MMP-8\textsuperscript{94} and is present in gingival tissues of patients with periodontitis\textsuperscript{95}. While MMP-8 and -13 were found to be the major collagenolytic enzymes in adult and localized juvenile periodontitis (LJP), MMP-7 was also found to be expressed at higher levels in patients with LJP as compared to healthy controls\textsuperscript{95}. While MMP-7 levels have not been shown to be elevated in the GCF of some patients with periodontitis compared to healthy controls\textsuperscript{94, 96}, one investigation demonstrated that the active form of the enzyme was detected in GCF derived from patients with adult periodontitis and localized juvenile periodontitis\textsuperscript{95}. Elevated MMP-7 levels have also been reported to be present in peri-implant sulcular fluid of failing implants\textsuperscript{97}.

In the context of this thesis I will discuss below the pertinent background information relating to cardiovascular diseases (CVDs) before examining potential functional and mechanistic relationships between periodontal diseases and CVDs. I will also address below the fact that there is currently a commercially available medication that has been approved for use in and can be used to modulate the activities of several MMPs (See Page 35).
1.2 Cardiovascular Diseases

1.2.1 Background and Epidemiology

CVDs are defined as diseases and/or injuries to the cardiovascular system, which include the heart, the blood vessels of the heart and the system of blood vessels (veins and arteries) throughout the body and within the brain\(^98\). CVDs are a leading cause of death in many countries, including Canada, where they account for \(\sim 30\%\) of all deaths\(^99\). The number of people suffering from CVDs is increasing: in 2005, approximately 1.29 million Canadians reported having heart disease, which represented an increase of 380,000 patients from 1994\(^98\). Important increases were also seen in the prevalence of major risk factors including hypertension, diabetes and obesity between 1994 and 2005\(^98\). Importantly, the majority of cases of CVDs and stroke are ultimately related to atherosclerosis.

1.2.2 Etiology and Pathogenesis of Atherosclerosis

Atherosclerosis involves the accumulation of lipids, lipid-laden immune cells, and apoptotic cells in the arterial wall, and results in the narrowing of arteries\(^100,101\). The cholesterol-rich plaques of atheromas may rupture, yielding thrombi that travel to occlude arteries and small vessels, resulting in a myocardial infarction or stroke downstream from the blockages\(^100\). Several factors increase the risk for atherosclerosis, including elevated cholesterol and triglyceride levels in blood, hypertension, diabetes, and cigarette smoking\(^102\).

Although atherogenesis has in the past been considered a constitutive process, more recent data suggest that inflammation plays a pivotal role in the genesis and pathogenesis of atherosclerosis, particularly in the context of CVDs\(^100,102\). Leukocytes have been shown to localize in early atherosclerotic lesions in humans\(^103\). While normal endothelium does not generally support the binding of white blood cells, patches of arterial endothelium with early lesions begin to express surface selective adhesion molecules, such as vascular cell adhesion molecule (VCAM)-1 that support binding. Impaired atheroprotective mechanisms occur at points in arteries where there is turbulent instead of laminar flow of blood over endothelial cells\(^104\). Ultimately, inflammatory mediators can inhibit collagen synthesis and promote the expression of collagenases by foam cells within the lesion. These alterations in
matrix metabolism can cause weakening of the outer fibrous cap of the lesion due to thinning of this structure. Accordingly, the fibrous cap becomes weaker and therefore more susceptible to rupture. In fact, most fatal myocardial infarctions are thought to occur due to a physical disruption or rupture of the atherosclerotic plaque as opposed to occlusion of an atherosclerotic vessel with a thrombus of some sort

1.3 Evidence of Associations Between Periodontal Diseases and Cardiovascular Diseases

As already alluded to above, there is a statistically significant association between the presence of periodontitis and the risk for CVD. One possible explanation might relate to the concept that both groups of conditions share common pathophysiological characteristics, and therefore would have similar risk factors. Indeed, increased risk for exhibiting either one of these diseases has been associated with aging, male sex, smoking, hypertension, lower educational status, reduced financial resources, and socially isolated and stressed people. Several reviews of epidemiological studies as well as meta-analyses have summarized the association between periodontal disease and CVDs. Assessment of the available evidence indicates that there is a moderate, positive and statistically significant association between the presence of these two diseases in humans, while the mechanisms underlying the relationship are still unclear, the similarity between their underlying pathophysiological mechanisms notwithstanding. Therefore, in addition to the concepts pointed out above, several other biologically plausible mechanisms that might link periodontitis and CVD in a causal way which have been postulated will be described hereunder.

1.3.1 Periodontal Pathogens in Atherosclerotic Plaques

In view of the relationship between chronic infection and inflammation in the development of atherosclerosis, it has been hypothesized that oral microorganisms, including periodontal pathogens, might enter the bloodstream when transient bacteremias occur and bind to the vascular endothelium. Once bound to the endothelium it has been suggested that periodontal pathogens contribute to the progression of inflammatory atherosclerosis. Transient bacteremias are common after dental procedures regardless of periodontal status,
and occur frequently after mastication or oral hygiene measures\textsuperscript{111}. Some data have strongly suggested that the gingival sulcus is the main portal to the bloodstream for oral bacterial species detected in the blood\textsuperscript{105,112,113}. \textit{Porphyromonas gingivalis}, a common periodontal pathogen, can adhere to and invade various human vascular cells in culture\textsuperscript{114,115}. Infection of aortic endothelial cells by \textit{Porphyromonas gingivalis} can induce a pro-coagulant response, potentially contributing to a pathogenic mechanism\textsuperscript{105}.

Viable \textit{Porphyromonas gingivalis} and \textit{Actinomyces actinomycetemcomitans}, both major periodontal pathogens, have been recovered in cultures obtained from atheromatous plaques in a subject with periodontal disease\textsuperscript{116}. In a study where human specimens were obtained from carotid endarterectomies and then examined by polymerase chain reaction, 80\% of lesions were positive for at least one of the target periodontal pathogens (\textit{Actinomyces actinomycetemcomitans}, \textit{Bacteroides forsythus}, \textit{Porphyromonas gingivalis}, and \textit{Prevotella intermedia})\textsuperscript{117}. A review of the subject has suggested that periodontal pathogens could theoretically disseminate through the circulation and localize within atheromatous plaques where they participate in the development and progression of atherosclerosis leading to coronary vascular disease and other clinical sequelae\textsuperscript{105,111,117}.

1.3.2 Periodontitis Associated with Carotid Artery Calcifications and Atherosclerosis

Arterial stenosis due to the development of calcified plaques is frequently measured with duplex ultrasonography, although the less robust technique of dental pantomographic radiography has been used as well\textsuperscript{118}. In a retrospective study of digital pantomographic radiographs, subjects exhibiting unilateral and/or bilateral calcifications of their carotid arteries also had a mean percentage of alveolar bone loss of 24.2\% and 25.7\%, respectively, compared to those with no calcification (these subjects had approximately 10\% bone loss). The differences and indeed the relationship between carotid artery calcification were demonstrated to be statistically significant\textsuperscript{119}. In one study where periodontitis was defined to exist in patients with \( \geq 30\% \) of teeth with at least 4 mm of alveolar bone loss from the cementoenamel junction, the odds ratio for a positive relationship between arterial calcification scores and periodontitis was 38.4\textsuperscript{118}. A dose-response relationship between the extent of carotid calcification as shown in pantomographic radiographs and the severity of
periodontitis was also reported\textsuperscript{118}. A significant association has also been observed between tooth loss (which can in some patients, reflect current or past periodontal disease) and the prevalence of plaques in the carotid artery. Within this population, 46% patients with 0-9 missing teeth exhibited carotid arterial plaques, whereas amongst those with \( \geq 10 \) missing teeth, the prevalence of carotid arterial plaques was 60\%\textsuperscript{120}.

1.3.3 Improvement in CVD Biomarkers After Periodontal Therapy

A biomarker is a characteristic that can be measured objectively and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Table 1)\textsuperscript{41, 121}. Biomarkers have been used in five investigational fields: for screening, diagnosis, prognostication, prediction of disease recurrence, or therapeutic monitoring\textsuperscript{121}. Many biomarkers have been examined in an effort to define their relationship to CVDs\textsuperscript{122}. In this context, there is evidence to indicate that some of the biomarkers related to CVDs, which will be discussed below, are increased in patients with periodontitis and may improve following treatment for periodontal disease. These findings further support the existence of a true relationship between periodontal diseases and CVDs.

1.3.3.1 Markers of Systemic Inflammation

Systemic inflammation can be measured with several markers of inflammation and C-reactive protein (CRP) has been studied extensively and in the most detail. Many epidemiological studies of individuals with no prior history of severe cardiovascular events have demonstrated that a one-time measure of CRP is a predictor of future events, including myocardial infarction, stroke, peripheral artery disease, and sudden cardiac death\textsuperscript{105, 123}. Other inflammatory markers associated with increased cardiovascular disease or risk include phospholipase A2, myeloperoxidase, fibrinogen, and interleukin (IL)-6\textsuperscript{105}.

Similarly, periodontal inflammation can be characterized by increases in the levels of systemic markers of inflammation including CRP, TNF-\( \alpha \), IL-1, IL-6, and IL-8\textsuperscript{120-123}. CRP and IL-6 are also found to be increased in the sera of subjects with periodontitis as compared to those without periodontitis, even after adjustments for confounding variables\textsuperscript{124-131}. Levels of IL-6 and CRP in subjects with severe periodontitis, but who were
otherwise healthy, have been shown to be reduced significantly after scaling and root planing\textsuperscript{128} or after full-mouth tooth extraction to eliminate periodontal infection\textsuperscript{129}.

1.3.3.2 Blood Lipid Levels

The importance of cholesterol, particularly low-density lipoprotein (LDL) cholesterol, is well established as an important factor in the pathogenesis of atherosclerosis\textsuperscript{132-134}. Periodontal pockets\textsuperscript{134}, as well as other measures of periodontal health such as recession and clinical attachment levels, are associated positively with the levels of both total and LDL cholesterol\textsuperscript{135}. A case-control study showed that total cholesterol levels, LDL cholesterol, and triglycerides were significantly higher in patients with periodontal disease when compared to age and sex matched controls that did not have periodontal disease. Furthermore, successful treatment outcomes after periodontal therapy were more likely obtained in persons with lower lipid levels than higher levels\textsuperscript{136}.

1.3.3.3 Endothelial Function

The vascular endothelium is involved in the regulation of vasomotor tone, permeability and growth, interactions between the blood vessel wall and platelets, leukocytes, and monocytes, as well as prevention of thrombosis\textsuperscript{105}. Impaired endothelial function has been demonstrated in patients with atherosclerosis and is a risk factor for atherosclerosis; accordingly, endothelial dysfunction may be a common precursor of adverse atherosclerotic cardiac events\textsuperscript{133, 137, 138}, and may be an early manifestation of atherosclerotic vascular diseases\textsuperscript{105}. Similarly, subjects with advanced periodontal disease exhibit endothelial dysfunction\textsuperscript{133, 137}, which has been demonstrated to improve following non-surgical periodontal therapy\textsuperscript{137}. No significant difference was found between subjects with mild periodontitis and control subjects, which suggests a dose-response effect\textsuperscript{133}.

The mechanism by which periodontitis might affect endothelial function is undefined; however it has been suggested that periodontal pathogens may affect the endothelium directly\textsuperscript{137, 138}. Some support for this contention comes from a randomized-control trial which found that after scaling and root planing, acute, short-term endothelial dysfunction was caused by the bacteremia, while ultimate improvement in oral health after six months was associated with improved endothelial function\textsuperscript{138}. 

2 Vascular Calcification

2.1 Vascular Calcification in CVDs

Calcification is defined as the accumulation of calcium salts in tissues. This is a specific type of mineralization, which in contrast to calcification involves the deposition of any mineral\textsuperscript{139, 140}. Therefore, for the purpose of this discussion, the terms will be used interchangeably. Pathologic calcification of cardiovascular structures including that of vessels (i.e. vascular calcification) is associated with a number of diseases, such as end-stage renal disease (ESRD) and several cardiovascular diseases\textsuperscript{141, 142}. Pathological calcification involves the deposition of calcium phosphate and is highly correlated with serum calcium and phosphate levels\textsuperscript{142}. Vascular calcification can occur in the myocardium, cardiac valves and blood vessels, and can lead to serious medical problems including valve stenosis, increased stiffening and decreased vascular compliance, caliphylaxis, and increased risk of myocardial infarction\textsuperscript{142, 143}. While vascular stiffening and decreased compliance can potentially contribute to an increase in blood pressure, the morbid effects of vascular calcification alone explain only a small part of hypertension. Major arteries, for example the aorta (e.g. aortic arch in particular) are more likely to calcify than are smaller vessels and the increased stiffness of large vessels does not apparently contribute to hypertension. Instead, it is the smaller diameter arterioles that as a result of increased smooth muscle tone, endothelial and possibly fibroblast dysfunction, play important roles in the development of hypertension. Yet they rarely calcify. Notably, vascular calcifications may cause an increase in the surface roughness of the inner vessel wall thereby disrupting laminar blood flow. This alteration could disrupt laminar flow in the blood vessel and lead to increased thrombus formation, an important causative factor in ischaemic heart disease and stroke. Taken together, both of these putative mechanisms could contribute to the development of cardiovascular diseases but the latter proposed mechanism might actually be more impactful than the former.

Arterial calcifications do not regress spontaneously and the complications from calcifications are related to the location and quantity of calcifications. Deposits in the arterial wall may be detected as foreign bodies, thereby inducing granulomas and stimulating other inflammatory reactions including the development of foam cells and the
recruitment of lymphocytes and mast cells\textsuperscript{144}. Calcifications may also undergo osseous metaplasia causing the formation of osseous trabeculae (i.e. in these cases, not just ectopic deposition of mineral). Large calcifications may protrude into the vessel lumen and act as occluding polyploid masses. These lesions may be the starting points for thrombi to develop; they can also detach and lead to peripheral or distant embolism. Intimal calcification is observed in atherosclerotic lesions while medial calcification is more associated with aging, diabetes and ESRD\textsuperscript{141, 142}.

2.1.1 Regulation of Vascular Calcification

Evidently, vascular calcification is regulated actively and might be initiated and controlled by a number of different mechanisms. Importantly, vascular calcification is clearly associated with periodontitis\textsuperscript{118, 119}, despite the lack of a clear mechanism to explain this linkage. Therefore, further discussion relating to its pathogenesis is warranted here. Some of the ideas relating to the regulatory mechanisms of vascular calcification have been reviewed\textsuperscript{143} and include the following concepts: (1) loss of inhibition of calcification, (2) induction of metaplastic bone formation, (3) circulating nucleation complexes that induce calcification, and (4) cell death, such that dead cells may nucleate initial calcification. Importantly, several blood proteins are normally involved in suppression of vascular calcification and these will be discussed below.

2.1.1.1 Loss of Inhibition of Calcification

Osteopontin (OPN) is a regulator of calcification; it is abundant at sites of calcification in human atherosclerotic plaques and calcified valves but is not found in healthy arteries\textsuperscript{141, 143}. OPN acts as an inhibitor of apatite crystal growth, most likely through direct aggregation and adhesion to apatite crystals through specific amino acids\textsuperscript{141, 145}. Additionally, OPN stimulates bone resorption by promoting osteoclast function though binding to the $\alpha_v\beta_3$ integrin\textsuperscript{141}. OPN-null mice demonstrate accelerated and enhanced vascular and valve calcification compared to wild-type controls\textsuperscript{143}.

Phosphate levels were long thought to affect mineralization directly, however recent evidence shows that phosphate regulates and coordinates cell signaling and gene expression that is involved in calcification\textsuperscript{145}; its effects are more indirect but still important.
Heterogeneous, non-clonal populations of vascular smooth muscle cells (VSMCs) do not mineralize in culture spontaneously, but can be induced to mineralize by increasing phosphate levels in the culture medium\textsuperscript{146}. Elevated phosphate levels also induce phenotypic transition in VSMCs. Under normal conditions, VSMCs express smooth muscle lineage markers representative of a contractile phenotype, including alpha smooth muscle actin. After treatment with supra-physiological levels of phosphate, there is a dramatic loss of markers for the smooth muscle cell lineage and a simultaneous increase in markers of ‘osteochondrogenic’ cells including such proteins as OPN, AP, Cbfa-1/Runx2 and osteocalcin\textsuperscript{141, 142, 147}.

One of the first proteins discovered that was involved in the inhibition of calcification was matrix Gla protein (MGP). It is a relatively small molecule that exhibits unique post-translational modifications of its glutamic acid residues by gamma-carboxylation. This modification appears to be important in its function\textsuperscript{145}. Mice with a null mutation in MGP exhibit extensive arterial calcification leading to arterial rupture and heart failure\textsuperscript{148}. MGP inhibits calcium phosphate deposition by chelation of calcium\textsuperscript{149} and also inhibits bone morphogenic protein (BMP)-2 activity via matrix association\textsuperscript{150}. In addition, MGP binds elastin and may normally mask mineral nucleation sites\textsuperscript{143}. The effect of MGP on BMP-2 depends on the degree of MGP gamma-carboxylation as well as the concentrations of the two molecules. Lack of function (as a result of insufficient gamma-carboxylation), as opposed to the relative amount of MGP, seems to be related to increased risk for calcification. Indeed, MGP isolated from calcified atherosclerotic plaque from rats has incomplete gamma-carboxylation\textsuperscript{145, 151}. The gamma-carboxylated, but not the non-gamma-carboxylated form of MGP is carried in plasma by fetuin, another circulating inhibitor of calcification that will be discussed in more detail below\textsuperscript{152}.

3 Fetuin

3.1 Background

Alpha-2-Heremans Schmid glycoprotein (AHSG in humans), or fetuin (bovine), is a circulating glycoprotein that was first isolated from fetal bovine serum. It has been found since in many other species including sheep, pig, cow, and human\textsuperscript{153}. Fetuin is a member of
the cystatin superfamily of cysteine protease inhibitors.\textsuperscript{154} In humans, it is synthesized by hepatocytes and occurs in all extracellular fluids, where its concentration ranges from 0.3-1.5 g/L\textsuperscript{155,156}; normal values are \textasciitilde 0.7 g/L\textsuperscript{157}. Fetuin accumulates preferentially in the skeleton during mineralization due to its high affinity for hydroxyapatite\textsuperscript{158}.

3.2 Structure

The amino acid sequence of human fetuin is shown in Figure 2. Human plasma fetuin is unique; it is a two-chain protein whereas all other known mammalian fetuins, such as bovine fetuin, are made up of only a single chain\textsuperscript{153,156,159}. In all species, fetuin contains an 18-amino acid signal sequence at the N-terminus. The one-chain form of fetuin consists of 349 amino acid residues whereas the two-chain form contains one A chain and one B-chain; residues 1-282 make up the heavy A-chain, the connecting peptide consists of residues 283-321, and the light B-chain comprises residues 323-349\textsuperscript{156}. A disulphide bridge between cysteine-14 and cysteine-340 joins the A-chain and the B-chain while the other ten cysteine residues are consecutively linked within the heavy chain and form five loops, spanning 4-19 amino acids\textsuperscript{160}.

3.3 Biological Activity

3.3.1 Inhibition of Calcification

Initially, it was suggested that fetuin could influence an array of biological processes, including opsonization, lipid transport, cell proliferation, tyrosine kinase inhibition of the insulin receptor, protease inhibition, hematopoietic cell homing, transforming growth factor-\textbeta and bone morphogenetic protein cytokine binding, and hepatocyte growth factor binding\textsuperscript{161-163}. In 1976, Triffet et al.\textsuperscript{164} showed that fetuin was a major component of the non-collagenous proteins in bone and was also shown to accumulate in teeth. Accordingly, it has been suggested that fetuin may play an important role in bone metabolism and turnover. By targeted deletion of the fetuin gene in mice, an inhibitory role for fetuin in the prevention of calcification was noted\textsuperscript{161}. When fetuin-null mice were fed a mineral-rich diet they developed widespread calcification (ectopic mineralization) in the lungs, heart and kidneys. Thus fetuin was regarded as a potent inhibitor of systemic calcification. However, the gross morphological features of bone in newborn, adult heterozygous and homozygous
fetuin null mutant mice appeared normal suggesting that, under normal physiological conditions, fetuin might not play a major role in bone formation\textsuperscript{161}, possibly suggesting other redundant mechanisms.

The mechanisms by which fetuin inhibits calcification have been investigated in some detail. Initially, fetuin was noted to bind calcium in the serum and efficiently prevent \textit{de novo} formation of apatitic mineral\textsuperscript{165}. Heiss et al. \textsuperscript{166} showed that fetuin prevented precipitation of calcium phosphate by the transient formation of soluble, colloidal spheres, which contained fetuin, calcium and phosphate. These spheres were referred to as “calciprotein particles”. From these data it was considered that fetuin acts as a direct crystal inhibitor (“poison”) for calcium phosphate induction of mineralization\textsuperscript{166-168}. “Calciprotein particles” are thought to be \textasciitilde 30-150 nm in diameter, are initially amorphous and soluble, but over time become progressively more crystalline. This identification of “calciprotein particles” provided a novel conceptual framework to explain how insoluble calcium precipitates could be transported and removed from the body\textsuperscript{166}.

A second potential mechanism by which fetuin might inhibit mineralization involves the inhibition of differentiation of cells that regulate the process of mineralization (i.e. osteoblasts)\textsuperscript{162,169-171}. This mechanism may involve the interaction of fetuin with transforming growth factor (TGF)-\(\beta\) or BMPs and interference with their promotion of osteogenic differentiation\textsuperscript{169}. Notably, fetuin in human serum has a similar affinity for TGF-\(\beta\) (and BMP) (2 x 10\textsuperscript{-6} M)\textsuperscript{162} as do authentic TGF-\(\beta\) receptors, which is consistent with a role for fetuin in regulating TGF-\(\beta\) activity \textit{in vivo}\textsuperscript{169}. Indeed, fetuin has been demonstrated to contain a TGF-\(\beta\) receptor-like region\textsuperscript{162}. Hence, fetuin could act as a ‘decoy receptor’ for osteoinductive cytokines, thereby mopping up available proteins that would otherwise stimulate osteodifferentiation.

Based on the known and hypothetical physiological functions of fetuin, it should not be surprising that increases in coronary artery calcification and the development of calcifying atheromas are observed when serum levels of fetuin are reduced\textsuperscript{172,173}. Patients with stable angina, or recent myocardial infarctions with angiographic evidence of coronary artery disease, exhibit lower levels of serum fetuin compared with healthy cardiovascular control
Patients with chronic kidney disease who are on hemodialysis exhibit reduced levels of fetuin and a depressed capacity to inhibit precipitation of calcium phosphate. In these patients, low levels of fetuin could be a consequence of the chronic inflammatory state that characterizes ESRD, as fetuin is a negative acute-phase reactant. Levels of fetuin are correlated highly with the inflammatory states and nutritional conditions of these patients. Progressive calcification is also seen in the hereditary disorder pseudoxanthoma elasticum, which is characterized by calcification of elastic fibres of the matrix in skin, retina and the cardiovascular system. Serum fetuin concentrations in patients with pseudoxanthoma elasticum were shown to be significantly lower (0.55 g/L) than levels measured in first-degree relatives (0.7 g/L) and unrelated controls (0.8 g/L).

3.3.2 Inhibition of HMGB1 Release

Fetuin may inhibit the production of the high mobility group box 1 protein (HMGB1). This protein is a mediator of inflammation that is secreted by activated macrophages and monocytes. HMGB1 may also be released passively from necrotic or damaged cells, which can exacerbate the inflammatory response. The kinetics of HMGB1 accumulation in vivo have been studied in murine models of endotoxemia and sepsis. Serum HMBG1 levels appear to increase and then plateau around 16-32 hours after induction and remain elevated for at least 72 hours. The late appearance of HMGB1 distinguishes it from TNF-α and other pro-inflammatory mediators that are typically expressed relatively early on in inflammatory lesions. HMGB1 could also contribute to the progression of atherosclerosis and other cardiovascular diseases. Accordingly, inhibition of the production of HMGB1 by fetuin may inhibit the inflammatory response; hence, any molecule that degrades fetuin could enhance inflammation and the progression of atherosclerosis and formation of calcified atheromas.

3.4 Enzymatic Degradation of Fetuin

Interactions between fetuin and blood-borne enzymes may be important to examine in the context of prevention of calcification in pathophysiological situations. After reporting an interaction between MMP-2 or MMP-7 with bovine fetuin, Ochieng and Green examined
the interaction of these two enzymes with human fetuin\textsuperscript{184}. They showed that when incubated at a 10-fold molar excess of either MMP-2 or MMP-7, complete digestion of fetuin took place after 12 hours. However, at equimolar ratios, neither of the enzymes could degrade fetuin. Notably, MMPs can degrade and bind fetuin, which could potentially reduce its naturally occurring functions\textsuperscript{184, 185}. However, at the time Ochieng and Green’s study was published, the fundamental physiological role of fetuin was not defined and so the potential significance of their findings was not appreciated fully. Therefore, the physiological relevance of fetuin binding to MMPs and loss of fetuin function were not considered. A more recent study by Kubler et al.\textsuperscript{186} showed that both MMP-3 and MMP-7 cleave bovine fetuin at specific sites but no further study of the human protein has been performed.
STATEMENT OF THE PROBLEM

Vascular calcification contributes to the development of cardiovascular diseases. Untreated periodontal disease may be a risk indicator for the development of cardiovascular diseases. Currently the underlying biological mechanisms relating untreated periodontitis and cardiovascular diseases are not defined.

Fetuin is an inhibitor of vascular calcification and may be degraded by enzymes that are increased in periodontally-diseased tissues and in the serum of individuals with untreated periodontitis. Investigation of the mechanisms of the degradation of fetuin, possibly related to proteases released into the bloodstream in patients with periodontitis could explain, at least in part, the association between untreated periodontal diseases and cardiovascular diseases in general, and vascular calcification in particular.

OBJECTIVES

1. Employ *in silico* methods to identify enzymes that degrade fetuin.
2. Examine whether enzymes upregulated in periodontal diseases cleave fetuin.
3. Identify fetuin cleavage fragments by mass spectrometry.
4. Quantify MMP-fetuin binding interactions.

HYPOTHESIS

Fetuin is cleaved *in vivo* by enzymes that are associated with periodontal diseases as demonstrated by *in vitro* studies.
4 ABSTRACT

Background and Objective: Human subjects affected by inflammatory diseases such as periodontitis may be at increased risk for the development of cardiovascular diseases and calcification of atheromas, but the potential mechanisms are not defined. Alpha-2-Heremans Schmid glycoprotein (fetuin) is an abundant ~49 kDa serum glycoprotein that inhibits ectopic arterial calcification. We examined whether matrix metalloproteinases (MMPs), which are increased in inflammatory diseases including periodontitis, bind and degrade fetuin and alter its ability to inhibit calcification in vitro.

Materials and Methods: Binding and cleavage of fetuin by MMPs were assessed by SDS-PAGE, in silico analyses and mass spectrometry.

Results: From in silico analyses and literature review, we found that only matrix metalloproteinases 3 (MMP-3; stromelysin) and 7 (MMP-7; matrilysin) were predicted to cleave human fetuin at levels that were physiologically relevant. In vitro assays showed that MMP-7 and to a lesser extent MMP-3, degraded human fetuin in a time- and dose-dependent fashion. Fetuin peptides generated by MMP-7 cleavage were identified and sequenced by mass spectrometry; novel cleavage sites were found.

Conclusion: MMP-7 cleaves fetuin in vitro. Therefore, the MMPs increased in inflammatory diseases such as periodontitis could affect fetuin’s ability to regulate mineralization and potentially enhance the risk of calcified atheroma formation.
5 INTRODUCTION

Calcification in the intima of blood vessels is associated with several cardiovascular diseases. Vascular calcification within atheromas requires the deposition of hydroxyapatite; the amount of calcification is a marker of the atherosclerotic burden and may mediate loss of arterial elasticity. Several cohort and cross-sectional studies have found positive associations between periodontitis and increased risk of cardiovascular diseases and vascular calcification but the underlying mechanisms that explain if and how periodontitis and other inflammatory diseases enhance the formation of calcifying atheromas are not defined. While there is no definitive evidence for a causal association between cardiovascular diseases and periodontitis, it is nevertheless important to identify potential pathophysiological mechanisms that could contribute to this link.

Vascular calcification may be initiated by several mechanisms that include loss of inhibition of calcification, induction of bone formation and cell death, which can nucleate initial calcification processes. Notably, several proteins may be involved in suppressing calcification in atheromas including osteopontin, matrix Gla protein, osteocalcin and the alpha-2-Heremans Schmid glycoprotein (human homologue of fetuin A). Fetuin is an abundant serum sialoprotein (Mr ~49,000) that is a member of the cystatin superfamily and is synthesized in the liver. Fetuin, which is incorporated into mineralized tissues and plays an important role in bone formation and resorption, inhibits calcification and prevents precipitation of calcium phosphate. Fetuin may act as a crystal poison but may also inhibit mineralization by interfering with the differentiation of cells with a mineralizing phenotype. Low levels of fetuin in the serum are associated with increased levels of vascular calcification. In view of the relationship between low levels of intact fetuin in the serum with increased risk for vascular calcification, we considered that MMPs upregulated in periodontitis could reduce levels of fetuin in serum as a consequence of their ability to degrade this protein. Several enzymes that are increased in gingival crevicular fluid of periodontitis patients, such as alkaline phosphatase, ß-glucuronidase, aspartate aminotransferase, lactate dehydrogenase, neutrophil elastase and
cathepsins B\(^49\) and D\(^50\), could potentially inhibit the function of fetuin, but none of these enzymes has been reported to degrade fetuin.

MMPs are an important family of zinc-dependent endopeptidases that are involved in the destruction of the extracellular matrix in periodontal diseases\(^91-93\). Notably, the concentrations of MMP-3\(^91-93\), MMP-7\(^94, 95\), and MMP-8\(^195\) are markedly increased in the gingival crevicular fluid of patients with periodontitis. While MMP-9 is increased in the blood of patients with periodontitis\(^194\), there are limited data on the relationship between concentrations in blood of other MMPs and periodontal status. As fetuin can interact with MMPs\(^183\), we considered that the binding of MMPs to fetuin may facilitate fetuin degradation\(^184, 185\).

Previous studies have shown that complete digestion of human fetuin occurs within 12 hours when it is incubated with a 10-fold molar excess of either MMP-2 or MMP-7\(^184\). In contrast, at equimolar ratios, neither of these enzymes can degrade fetuin, suggesting that MMP-2 and MMP-7 do not affect regulation of mineralization by fetuin \textit{in vivo}. While MMP-3 and MMP-7 can cleave bovine fetuin at specific sites in the C-terminus of the molecule\(^186\), the impact of these cleavages on the regulation of mineralization has not been examined. Accordingly, we assessed MMPs that are upregulated in periodontitis and how they interact with and degrade human fetuin.
6 MATERIALS & METHODS

6.1.1 Reagents

Heparin agarose beads and 4-amino phenylmercuric acetate (APMA) were obtained from Sigma-Aldrich (Oakville, ON). Human fetuin was purchased from MyBiosource (San Diego, CA) and antibody to human fetuin was bought from Alpha Diagnostics (San Antonio, TX). Human recombinant pro-MMP-3, human recombinant MMP-7, manufacturer-activated human recombinant MMP-7 (active), antibody to human MMP-7 and MMP Inhibitor II (N-Hydroxy-1,3-di-(4-methoxybenzenesulphonyl)-5,5-dimethyl-[1,3]-piperazine-2-carboxamide) were obtained from Calbiochem (San Diego, CA). 196

6.1.2 MMP Assay

A fluorimetric MMP assay kit (SensoLyte®) was obtained from Anaspec (Fremont, CA). This assay detects MMP-7 and MMP-3 activities in biological samples using a 7-dimethylaminocoumarin (DMC)-4-acetate/nitro-2,1,3-benzoxadiazol-4-yl (NBD) fluorescence resonance energy transfer peptide (FRET). When the FRET peptide is not cleaved, the fluorescence of DMC is quenched by NBD. After cleavage of the peptide by MMP-7 or MMP-3, the fluorescence of DMC is restored and is measured at excitation/emission wavelengths of 370 nm/460 nm in a fluorescence plate reader. MMP-7 and MMP-3 that were either unactivated, supplier pre-activated or that were activated in vitro with APMA in the laboratory were used (1 mM in TNCB for 1 hour at room temperature as described earlier 197) to determine their relative activity levels.

6.1.3 Fetuin Cleavage by MMPs

Fetuin was incubated in a buffer (50 mM Tris, pH 7.5, 10 mM CaCl₂, 150 mM NaCl, 0.05% BRIJ 35; designated TNCB buffer) with MMP-7 or MMP-3 for 0, 1, 2, 4, 8 or 24 hours at a fetuin:MMP ratio of 1:60 and at varying concentration ratios of fetuin:MMP as indicated in the Results. These assays were repeated in triplicate. Dithiothreitol was not included in the buffer as there was no need to reduce disulphide bridges in fetuin for
degradation assays\textsuperscript{198}. In some experiments, the reversible, binding site-targeting MMP Inhibitor II\textsuperscript{199} was co-incubated with fetuin and enzymes in digestion experiments. This inhibitor has an IC\textsubscript{50} 18.4 nM for MMP-3 and an IC\textsubscript{50} of 30 nM for MMP-7\textsuperscript{196, 199}.

Proteins from a fetuin tie-course digestion were separated by SDS-PAGE gels (12\%) and stained with Coomassie blue. Protein bands were excised individually and de-stained with ammonium bicarbonate. Gels were shrunk and alkylated, and the peptides were subjected to trypsin digestion before peptide extraction. Samples were evaporated to dryness and analyzed by tandem mass spectrometry (Protein Technology Service, Hospital for Sick Children, Toronto, ON). For prediction of cleavage sites, Scaffold 3 (Proteome Software, Portland, OR) was used with a web-based database for the amino acid sequence of human fetuin (Swissprot database: http://web.expasy.org) and applied to the analysis of the peptides produced from the digestions. Further, \textit{a priori} knowledge of MMP-7’s known preferred cleavage sites was applied\textsuperscript{192, 193}.

### 6.1.4 Fetuin Binding to MMPs

We assessed whether intact fetuin was bound by MMP-7 prior to potential fetuin digestion by MMP-7. Purified MMP-7 (or bovine serum albumin [BSA] as a control) was attached to heparin agarose beads and incubated with fetuin for 20 min. MMP-7 that had been bound to beads previously was incubated with fetuin at specific dilutions. Quantification of the bound whole fetuin versus free fetuin was estimated by dot blotting proteins onto nitrocellulose and then identified using immunodetection with antibody to fetuin. Appropriate dilutions of human fetuin standards were dot-blotted in parallel. Quantification of dot densities were used to estimate the k\textsubscript{d} and BMAX for MMP7-fetuin binding by Scatchard plots. This assay was repeated twice ensure consistent results. In separate experiments, the efficacy of MMP-7-mediated digestion of fetuin (with the MMP-7 bound to agarose) was assessed by separating fetuin degradation products by SDS-PAGE.
7 RESULTS

7.1.1 In Silico Analysis

We searched online databases (ExPASy PeptideCutter, http://expasy.org/tools/peptidecutter; MEROPS Peptidase Database, http://merops.sanger.ac.uk; CutDB, http://cutdb.burnham.org) for both predicted and reported cleavages of human fetuin by enzymes that are reported to be increased in periodontitis including as alkaline phosphatase, β-glucuronidase, aspartate aminotransferase, lactate dehydrogenase, neutrophil elastase and cathepsins B and D, MMP-3, MMP-7, and MMP-8. There was no predicted cleavage of human fetuin by most of these enzymes, however human fetuin was reported to be degraded after overnight incubation with MMP-7 at a very high enzyme:substrate ratio. Bovine fetuin was reported to be cleaved by both MMP-3 and MMP-7.

7.1.2 Enzyme Activation

The enzyme activity of three different MMP-7 formulations (unactivated, manufacturer-activated and APMA-activated (1 mM APMA in TNCB for 1 hour at room temperature as described earlier)) was quantified. Enzyme activity was estimated with a fluorimetric MMP assay where increased fluorescence occurs after MMP-3 or MMP-7-induced cleavage of the MMP substrate. In time-course experiments that evaluated substrate degradation by the MMP-7 formulations, enzyme activity plateaued at about one hour (Figure 3), which was considered to be the optimal time used for APMA activation. While the APMA-activated MMP-7 exhibited the highest activity of the three formulations throughout the time course, the activity levels were not markedly different (<5% difference between groups at one hour). For all subsequent experiments, APMA-activated MMP-7 and APMA-activated MMP-3 were used as these preparations exhibited the highest consistent levels of catalytic activity.
7.1.3  In Vitro Degradation Assays

7.1.3.1 Time Course Assay

Time-course studies were conducted (at 37°C) using MMP-3 and MMP-7 incubated with fetuin at an enzyme to substrate ratio of 1:60. Analysis of fetuin degradation was assessed by SDS-PAGE, and a representative gel is included (Figure 4A). MMP-3 and MMP-7 were not detected in the PAGE gels because they were present at much more lower molar concentrations than fetuin. MMP-3 generated limited cleavage of human fetuin that was detectable only after 24 hours of incubation while marked degradation of fetuin was readily observed after 1 hour incubation with MMP-7. Using MMP-7, it was demonstrated that there was no intact fetuin left by 24 hours. Following 2 hours of treatment with MMP-7, the prominent fetuin degradation fragments were shown to have molecular weights of ~40 and ~18 kDa; after longer incubations the 18 kDa fragment predominated. Notably, based on densitometry of intact fetuin, MMP-7-mediated degradation of fetuin was inhibited by >90% with the MMP II inhibitor (1 µM; Figure 4B; IC$_{50}$ = 30 nM;\textsuperscript{196}), suggesting that the cleavage was attributable to the catalytic activity of MMP-7 and not due to non-specific degradation by undefined mechanisms in the assay.

7.1.3.2 Varying Enzyme-Substrate Ratio Assay

To complement the time-course experiments, digestions of human fetuin by MMP-3 and MMP-7 were conducted using various enzyme-substrate ratios for 4 hours and the relative amounts of full-length fetuin were evaluated by densitometry of one of the silver-stained stained gels. For MMP-7, 24% of fetuin was degraded at enzyme:substrate (E:S) = 1:60, 71% at E:S = 1:30 and 100% at E:S = 1:15. For MMP-3, even at E:S = 1:10, only 6% of fetuin was degraded (data not shown). As the relative amount of MMP-7 was increased relative to the amount of substrate (i.e. elevated enzyme:substrate ratio), MMP-7 caused more extensive fetuin degradation and the relative amount of intact fetuin was decreased even more. Therefore, MMP-7 but not MMP-3 appeared to be relevant for study of putative pathophysiological fetuin degradation.
7.1.4 Mass Spectrometry

Following MMP-7 degradation of fetuin for a period of 24 hours, fragments were cut out of PAGE gels at sites in the gels that were expected to contain fetuin and fetuin fragments (Figure 5A), and were analyzed by tandem mass spectrometry using Scaffold 3 analysis of the Swiss-Prot protein sequence database. From a series of analyses, continuous amino acid sequences (i.e. without interruption) of fetuin digestions by MMP-7 were compared to the reported cleavage sites of bovine fetuin by MMP-7. Based on these comparisons and the previous in silico identification of predicted sequences of all of the peptides obtained through the various analyses, a high probability (>95%) cleavage site in human fetuin was identified at R317-H318. Novel predicted cleavage sites (A167-L168; P192-L193), which were based on examination of the fetuin fragments of <20 kDa (Figure 5B) that included assembly and contiguity analyses of the sequences of the various tryptic peptides that were detected in all samples were also shown. In contrast to the initial in silico analyses, based on the online databases ExPASy PeptideCutter, MEROPS Peptidase Database, CutDB and Swissprot, MMP-7 was predicted to cleave fetuin at the sequence YDLR-HTFM (amino acid positions 312-313 at the cleavage site) but this did not occur.

7.1.5 Binding Assays

As MMP-7 evidently cleaved human fetuin (Figure 4A), the initial interaction of MMP-7 with fetuin by in vitro binding studies with MMP-7 bound to heparin agarose beads was examined. The amount of MMP-7 that bound to beads by dot blot analysis and compared this with known amounts of MMP-7 as standards (Figure 6A) was measured. For assessment of the binding of fetuin to MMP-7, fetuin was incubated with MMP-7 bound to heparin agarose beads at various concentrations (1.1-111 µM) and the relative amounts of bound and free fetuin after 20 min binding were quantified by immunodetection and densitometry of dot blots (Figure 6B). A 20 minute time period for binding was chosen as it was anticipated that there would be very limited degradation of fetuin by MMP-7 after this period of time. Measurements of the free versus bound amounts of fetuin allowed computation of BMAX (0.87±0.17) and $k_d$ (2.96±1.92 x $10^{-9}$ M; $r^2$=0.87), indicating high affinity binding of fetuin to MMP-7 (Figure 6B).
As MMP-7 binds tightly to heparin, we attached MMP-7 to heparin agarose in binding buffer and the catalytic efficacy of the bound enzyme was assessed. The binding of the MMP-7 to heparin agarose did not affect catalytic activity and bead-bound MMP-7 efficiently degraded fetuin (24 hours digestion; Figure 6C). Therefore the heparin that was used to attach MMP-7 for the binding assays (Figure 6B) did not evidently occlude functionally the catalytic site of MMP-7.
8 DISCUSSION

The cleavage of fetuin by two specific MMPs which are known to be increased in the crevicular fluid of patients with periodontitis, MMP-3\textsuperscript{91-93} and MMP-7\textsuperscript{94, 95}, was examined. The rationale for focusing on MMP-3 and MMP-7 was based on the initial \textit{in silico} analyses, which indicated that of those enzymes known to be increased systemically in patients affected by periodontitis, cleavage of fetuin was only predicted for these two MMPs. Despite the reported cleavage of bovine fetuin by MMP-3 and MMP-7\textsuperscript{186}, we found that only MMP-7 degraded human fetuin efficiently. Notably, the digestion of human fetuin by MMP-7 was strongly reduced by an MMP inhibitor (IC\textsubscript{50} = 30 nM), suggesting that the digestion was MMP-specific. Even when incubated at high enzyme-substrate ratios, human fetuin was not efficiently degraded by MMP-3. Based on these findings, subsequent studies were focused on the interaction between purified MMP-7 and fetuin. These experiments indicated very tight binding of these two proteins, consistent with our data showing that MMP-7-mediated degradation of fetuin increased proportionately when the enzyme-substrate ratio was increased.

The manufacturer-activated and unactivated MMP-7 had similar levels of activity in the fluorometric MMP assay. This observation may relate to the biological nature of MMP-7, which even when not activated, exhibits the ability to degrade certain substrates (i.e. the FRET peptide used here for catalytic assays) but not necessarily extracellular matrix proteins, which are its primary substrates. Since MMP-7 is a minimal-domain MMP, it presumably has less substrate specificity than an MMP with additional domains (e.g. hemopexin domains) and so it would be more likely to bind to and cleave a substrate even in its inactive form. This difference in domain structure and substrate specificity could also explain why MMP-7, but not MMP-3, degraded human fetuin in the \textit{in vitro} assays. Further, to ensure that the activities of the MMPs used for fetuin degradation were as high as possible, we decided to use the APMA-activated enzymes as they consistently produced the highest levels of catalytic activity.

Bovine fetuin and human fetuin exhibit \textasciitilde70\% amino acid homology\textsuperscript{153} and the similarity of the arrangement of cysteine residues and of disulfide loops have led to the proposal that
bovine and human fetuin are equivalent proteins. We found that MMP-7-mediated digestion fragments of human fetuin exhibited novel and unexpected cleavage sites that were different than the predicted cleavage sites for bovine fetuin\textsuperscript{186}. These variations between predicted and actual cleavages by MMP-7 indicate that small, species-dependent differences of fetuin structure could explain the discrepancies of the observed versus predicted cleavage sites.

In related work by Rezaei\textsuperscript{200}, it was found that the inhibition of mineralization by fetuin is reliant on intact fetuin structure. The inhibitory effect of fetuin on mineralization was reduced after digestion by MMP-7 but was not substantially altered by MMP-3 under the digestion conditions used here. Further, it appears that the ~18 kDa fetuin fragment is not as effective as the full-length molecule for the inhibition of mineralization. These data indicate that MMP-7 may be important for the control of biological mineralization \textit{in vivo}.

There is considerable interest in the potential association between inflammatory periodontal diseases and the development of cardiovascular diseases in general\textsuperscript{201} and in the formation of calcified atheromas in particular\textsuperscript{105}. However, apart from suggestions that periodontal pathogens may play a role in this association\textsuperscript{105}, little is known about other putative mechanisms, based on biochemical processes that could explain the statistical evidence for this relationship. Conceivably, linkages may exist between different inflammatory diseases and cardiovascular diseases, which could be mediated by reductions in the levels of intact fetuin in serum. In this context, Abilia found a 30\% reduction in the level of fetuin in patients with severe osteoarthritis\textsuperscript{202}. Although not investigated in patients with osteoarthritis, MMPs released into the bloodstream because of severe inflammatory joint disease could also contribute to degradation of fetuin.

MMP-7 has been implicated in the pathogenesis of atheroma formation\textsuperscript{203}. If MMP-7 that is produced in periodontitis is important for the regulation of calcification of atheromas, then there would need to be increased concentrations of MMP-7 in the blood. Notably, the concentration of MMP-9 is increased in the blood of patients with periodontitis\textsuperscript{194}; hence, it might be expected that the blood-levels of MMP-7 would also be increased in the bloodstream of patients with untreated chronic periodontitis, but this requires further study.
Based on the findings reported here, MMP-7 may be able to cleave serum fetuin, potentially increasing the risk of vascular calcification. If this notion is correct, the assessment of MMP-7 levels in the blood may be instructive for assessing risk of vascular calcification in inflammatory diseases such as periodontitis and possibly other inflammatory diseases such as osteoarthritis. The definition of such a mechanism, if it were demonstrated \textit{in vivo}, could explain in part the putative relationship between inflammatory periodontal diseases and calcification in cardiovascular diseases.

Recognizing this relationship between fetuin and MMP-7 could prove useful in developing clinical therapies for the treatment of inflammatory conditions in which the levels of MMP-7, or other MMPs, are increased. For example, a collagenase inhibitor (Periostat ®) is utilized in the treatment of certain types of periodontal diseases, and this is the only MMP inhibitor approved for use currently. While close to 60 MMP inhibitors have been pursued as clinical candidates for various conditions, most have been discontinued due to safety reasons\textsuperscript{204}. There remains tremendous interest in this drug class and clinical trials are underway in the development of new drugs in the MMP inhibitor class\textsuperscript{205}.

It is also possible that the relationship between fetuin and MMPs \textit{in vivo} has ramifications beyond regulation of ectopic calcification. It is known that the increased presence of MMPs in the periodontium during periodontal diseases contributes to the breakdown of the extracellular matrix associated with these diseases. The data reported here showed that fetuin and MMP-7 bind tightly to one another, as indicated by the $k_d$ (2.96±1.92 x 10$^{-9}$ M). If fetuin, which circulates systemically, is present in periodontal tissues at significant concentrations, it may potentially interact with MMPs to modulate matrix protein degradation. The relationship between fetuin and MMPs needs to be examined further to understand better the potential impact of their interaction.
Future Studies

Arising from the work presented here, one potential next step would be to sequence the fetuin degradation products after MMP-7 digestion, synthesize these fetuin fragments, and examine their effects on calcification. As intact fetuin inhibits calcification, it would be useful to define whether the degradation products can inhibit calcification. While fetuin levels are lower in some chronic inflammatory conditions such as osteoarthritis and pseudoxanthoma elasticum, serum levels of fetuin in patients with chronic periodontitis have not been examined. These data could be used for determining the plausibility of the mechanistic link proposed here. Finally, it would be prudent to examine the effects of some of the other enzymes that are up-regulated in inflammation *in vitro*, and to determine what effects these enzymes exert on fetuin’s ability to regulate calcification. While MMP-3 was reported to cleave bovine fetuin, it did not degrade human fetuin. Further, based on our *in silico* search, MMP-7 was not predicted to cleave human fetuin but it did degrade fetuin *in vitro*. Accordingly, further *in vitro* studies that utilize other, inflammation-associated enzymes and their degradation of human fetuin may provide useful insights.
Conclusions

Based on an \textit{in silico} examination, both MMP-3 and MMP-7 were reported to cleave bovine fetuin. None of the other enzymes upregulated in periodontitis were predicted to cleave human fetuin. Human fetuin was degraded by MMP-7 \textit{in vitro}; the amount of degradation increased over time and when the enzyme-to-substrate ratio was increased. Very little, if any, digestion was mediated by MMP-3, even with longer incubation times and at high enzyme-to-substrate ratios. Novel cleavage sites in human fetuin were identified based on analysis of the MMP-7-mediated digestion products by mass spectrometry. The binding between fetuin and MMP-7 is very tight, as indicated by the $k_d$ (2.96±1.92 x 10^{-9} \text{ M}).
TABLE 1 - Definitions of Predictors for Periodontal Diseases\textsuperscript{9, 40, 117}

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Risk Marker</td>
<td>An attribute or event that is associated with increased probability of disease but is not necessarily a causal factor</td>
</tr>
<tr>
<td>Risk Indicator</td>
<td>An event that is associated with an outcome only in cross-sectional studies</td>
</tr>
<tr>
<td>Risk Factor</td>
<td>An action or event that is related statistically in some way to an outcome and is truly causal</td>
</tr>
<tr>
<td>Risk Determinant</td>
<td>An attribute or event that increases the probability of occurrence of the disease</td>
</tr>
<tr>
<td>Biomarker</td>
<td>A substance that is measured objectively and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention</td>
</tr>
</tbody>
</table>
**FIGURE 1 – Structure of MMPs.** The domain organization of MMPs is as indicated: S, signal peptide; Pro, propeptide; Cat, catalytic domain; Zn, active-site zinc; Hpx, hemopexin domain; Fn, fibronectin domain; V, vitronectin insert; I, type I transmembrane domain; II, type II transmembrane domain; G, GPI anchor; Cp, cytoplasmic domain; Ca, cysteine array region; and Ig, IgG-like domain. A furin cleavage site is depicted as a black band between propeptide and catalytic domain.

<table>
<thead>
<tr>
<th>MMP</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>7, 26</td>
<td><img src="image" alt="MMP Structure" /></td>
</tr>
<tr>
<td>1, 3, 8, 10, 12, 13, 18, 19, 20, 27</td>
<td><img src="image" alt="MMP Structure" /></td>
</tr>
<tr>
<td>2, 9</td>
<td><img src="image" alt="MMP Structure" /></td>
</tr>
<tr>
<td>21</td>
<td><img src="image" alt="MMP Structure" /></td>
</tr>
<tr>
<td>11, 28</td>
<td><img src="image" alt="MMP Structure" /></td>
</tr>
<tr>
<td>14, 15, 16, 24</td>
<td><img src="image" alt="MMP Structure" /></td>
</tr>
<tr>
<td>17, 25</td>
<td><img src="image" alt="MMP Structure" /></td>
</tr>
<tr>
<td>23</td>
<td><img src="image" alt="MMP Structure" /></td>
</tr>
</tbody>
</table>
FIGURE 2 – Amino acid sequence of human fetuin. The first 18 amino acids make up the signal sequence, and are represented by negative numbers. The heavy (A) chain contains amino acid residues, the connecting peptide regions consists of residues 283-321, and the light (B) chain comprises residues 323-349\(^{150}\). A disulphide bridge between cysteine-14 and cysteine-340 joins the A-chain and the B-chain\(^{153}\).

-18  -11  42
MKSLVLLLCL AQLWGCHS AP HGPGLIYRQP NCDDPETEEA ALVAIDYINQ NLPWGYKHTL
NQIDEVKVWP QQPSGELEFI EIDTLETTCH VLDPTPVARC SVRQLKEHAV EGDCDFQLLK
LDGKFVVAYA KCDSSPDSAECVRKVCQDCP LLAPLNDTRV VHAAKAALAA FNAQNNGSNF
QLEEISRAQL VPLPPSTYVE FTVSgtDCVA KEATAAKCN LLAEKQYGFC KATLSEKLGG
AEVAVTCTVF QTQPVTSQPQ PEGANEAVPT PVVDPDAPPSPPLGAPGLPP AGSPPDSHVL
LAAPPGHQLH RAHYDLRHTF MGVVSLGPS GEVSHPKTR TVVQPSVGAA AGPVVPPCPG
RIRHFKV
FIGURE 3 – Enzyme activity levels of non-activated, manufacturer-activated, and APMA-activated MMP-7. The catalytic activities of recombinant MMP-7 preparations were measured with a DMC/NBD FRET. The three different MMP-7 preparations evaluated were: APMA-activated, unactivated (control), and manufacturer-activated (active MMP-7). After cleavage by MMP-7, the fluorescence of DMC is restored. In this assay, the fluorescence was measured at excitation/emission wavelengths of 370 nm/460 nm in a fluorescence plate reader. Data are relative fluorescence units (RFU) and were measured after indicated incubation times (seconds).
FIGURE 4A – Time-course degradation of fetuin by MMP-3 and MMP-7. Fetuin was incubated with purified enzymes at a 1:60 enzyme:substrate ratio for the indicated times. The degradation fragments were separated by SDS-PAGE and stained with Coomassie blue.
FIGURE 4B – Inhibition of degradation by MMP-Inhibitor II. MMP-7 and human fetuin were co-incubated for 1 or 24 hours or with the addition of MMP Inhibitor II (C$_{21}$H$_{27}$N$_{3}$O$_{8}$S$_{2}$; 1 µM) for 1 or 24 hours as indicated.
**FIGURE 5A – Peptide analysis by mass spectrometry.** Typical SDS-PAGE gel of fetuin after no treatment (0 hr) or degradation by MMP-7 (1:60 enzyme:substrate ratio) for 24 hours that was used for mass spectrometry analysis of fetuin degradation. In this example, an ~18 kDa fragment (circled) was cut out of the gel, subjected to tryptic digestion, and analyzed by mass spectrometry. Data from a single sample are shown here. Four uninterrupted, matched (>95% probability) tryptic peptides from the cut-out fragment are shown in yellow highlighter (below) and are overlaid over the whole amino acid sequence for human fetuin. Green shading shows amino acids with post-translational modifications detected by mass spectrometry.
**FIGURE 5B – Fetuin amino acid sequence analysis.** Human fetuin amino acid sequence (see Fig. 2) with location of disulfide bond (Cys14-Cys340, red), predicted cleavage site by MMP-7 based on reported data with bovine fetuin (Arg299-His300, green), and predicted novel cleavage sites in human fetuin based on data from mass spectrometry (Arg149-Lys150 and Pro174-Lys175, blue).

```
MKSLVLLLCL AQLWGCHS AP HGPGLYRQP NCDPETEEA ALVAIDYINQ NLPWGYKHTL
NQIDEVKVWP QQPSGELFEI EIDTLETCH VLDPTPVARC SVRQLKEHAV EGDCDFQLLK
LDGKFSVYVA KCDSPDQCSQ DLRPLNDTRV VHAAKA A | LAA FNAQNNNGSNF
QLEEISRAQL VP | LPPSTYVE FTVSCTDCVA KEATEAACKN LLAEKGYFQ KATLSEKLG
AEVAVTCTVF QTPVTSQPQ PEGANEAVPT PVVDPDAPPS PPLGAPGLPP AGSPPDSHVL
LAAPPGHQLH RAHYDLR | HTF MGAVSLGSPS GEVSHPRKTR TVVQPSVGAA AGPVVPP CP
RIRHFKV
```
FIGURE 6A – Assessment of fetuin binding to heparin agarose beads. MMP-7 was eluted from a fixed volume of pelleted heparin agarose beads, dotted onto nitrocellulose membrane, and then stained with antibody to MMP-7. MMO-7 standards were included to estimate the amount of MMP-7 bound to the beads.
FIGURE 6B – Free versus bound fetuin at varying fetuin concentration. Data from dot blots were used to calculate free fetuin and bound fetuin at various concentrations. Computations of BMAX (0.87±0.17) and $k_d$ (2.96±1.92 x $10^{-9}$ M; $r^2$=0.87) were performed by Scatchard analysis.
FIGURE 6C – Catalytic activity of fetuin bound to heparin agarose beads. MMP-7 or BSA was bound to heparin agarose beads and incubated with human fetuin for 24 hours. Supernatants or materials eluted from the beads were separated by SDS-PAGE and stained with Coomassie blue. For MMP-7-bound beads, the bead eluent protein migrates at ~20 kDa, which is the approximate size for the fetuin digest products identified in Fig. 2. For beads bound with BSA, fetuin is only found in the supernate fraction and there is no fetuin or digestion products attached to beads.
Bibliography


94. Emingil G, Tervahartiala T, Mantyla P, Maatta M, Sorsa T, Atilla G. Gingival crevicular fluid matrix metalloproteinase (MMP)-7, extracellular MMP inducer, and


99. CANSIM Table 102-0529: Deaths, by cayse, Chapter IX: Diseases of the circulatory system (I00 to I99), age group and sex, canada, annual (number), 2000 to 2006. No. Statistics Canada, Released May 4, 2010. (Statistics Canada publication)


194. Soder PO, Meurman JH, Jogestrand T, Nowak J, Soder B. Matrix metalloproteinase-9 and tissue inhibitor of matrix metalloproteinase-1 in blood as


