The Comparative Performance of Micro- and Nano-topographically Complex Endosseous Implant Surfaces in Normoglycemic and Hyperglycemic Subjects

by

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

Institute of Biomaterials and Biomedical Engineering
University of Toronto

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2013

Abstract

Endosseous implants have notably high success rates, yet a small percentage of implants still fail for unidentified reasons. Recent literature points to hyperglycemia, resulting from untreated or undiagnosed diabetes, as a possible contraindication in an otherwise apparently healthy population. To investigate the effect of surface design on peri-implant healing in the presence of hyperglycemia, STZ-treated rats were implanted with custom rectangular implants of two surface topographies: grit blasted (GB) and grit-blast with a calcium phosphate nanotopography (GB-DCD). Tensile testing was conducted at 5, 7, and 9 days post-operative. Results demonstrated hyperglycemia to delay early stages of the peri-implant healing. Contact osteogenesis was increased along the GB-DCD surface, even in an environment of uncontrolled hyperglycemia, and the GB-DCD surface outperformed the GB surface in both healthy and hyperglycemic animals, showing peri-implant bone matured more rapidly on nanotopographically complex surfaces, even in the presence of uncontrolled hyperglycemia.
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<th>Description</th>
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<tbody>
<tr>
<td>BIC</td>
<td>bone-implant contact</td>
</tr>
<tr>
<td>S, value</td>
<td>surface roughness value</td>
</tr>
<tr>
<td>RMT test</td>
<td>removal torque test</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>TGF-b</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>SLA surface</td>
<td>sand blasted acid-etched surface</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor alpha</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>PC</td>
<td>perivascular cells</td>
</tr>
<tr>
<td>MSC</td>
<td>mesenchymal stem cell</td>
</tr>
<tr>
<td>OP</td>
<td>osteopontin</td>
</tr>
<tr>
<td>BSP</td>
<td>bone sialoprotein</td>
</tr>
<tr>
<td>CaP</td>
<td>calcium phosphate</td>
</tr>
<tr>
<td>TCA cycle</td>
<td>tricarboxylic acid (citric acid) cycle</td>
</tr>
<tr>
<td>T1DM</td>
<td>type 1 diabetes mellitus</td>
</tr>
<tr>
<td>T2DM</td>
<td>type 2 diabetes mellitus</td>
</tr>
<tr>
<td>GDM</td>
<td>gestational diabetes mellitus</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>IGT</td>
<td>impaired glucose tolerance</td>
</tr>
<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>OC</td>
<td>osteocalcin</td>
</tr>
<tr>
<td>ucOC</td>
<td>undercarboxylated osteocalcin</td>
</tr>
<tr>
<td>RunX2</td>
<td>runt-related transcription factor 2</td>
</tr>
<tr>
<td>BMD</td>
<td>bone mineral density</td>
</tr>
<tr>
<td>DXA</td>
<td>dual-energy X-ray absorptiometry</td>
</tr>
<tr>
<td>QUS</td>
<td>quantitative ultrasound</td>
</tr>
<tr>
<td>MAR</td>
<td>mineral apposition rate</td>
</tr>
<tr>
<td>MMP-13</td>
<td>matrix metalloproteinase 13</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>AGEs</td>
<td>advanced glycosylation end products</td>
</tr>
<tr>
<td>HbA1c</td>
<td>glycosylated haemoglobin</td>
</tr>
<tr>
<td>STZ</td>
<td>streptozotocin</td>
</tr>
<tr>
<td>GLUT-2 transporter</td>
<td>glucose transporter 2 protein</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>BSE</td>
<td>backscattered electron imaging</td>
</tr>
<tr>
<td>cpTi</td>
<td>commercially pure titanium</td>
</tr>
<tr>
<td>DCD treatment</td>
<td>discrete crystalline deposition of CaP nanoparticles</td>
</tr>
<tr>
<td>GB</td>
<td>grit blasted</td>
</tr>
<tr>
<td>GB-DCD</td>
<td>grit-blasted with DCD treatment</td>
</tr>
<tr>
<td>Gy</td>
<td>Gray (SI unit of absorbed radiation)</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>IFFT</td>
<td>inverse fast Fourier transform</td>
</tr>
<tr>
<td>FE-SEM</td>
<td>field emission scanning electron microscopy</td>
</tr>
<tr>
<td>DCM</td>
<td>Division of Comparative Medicine (University of Toronto)</td>
</tr>
<tr>
<td>C</td>
<td>control</td>
</tr>
<tr>
<td>HG</td>
<td>hyperglycemic</td>
</tr>
<tr>
<td>IV</td>
<td>intravenous</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>Tukey’s HSD</td>
<td>Tukey’s honestly significant difference test</td>
</tr>
<tr>
<td>ICC</td>
<td>intra-class correlation</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>BSEI</td>
<td>backscattered electron imaging</td>
</tr>
<tr>
<td>qBSE</td>
<td>quantitative backscattered electron imaging</td>
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</table>
Chapter 1.0 Introduction

The past 15 years have seen considerable progress towards understanding the role of implant surface design in promoting osteoconduction, defined as “the recruitment and migration of osteogenic cells through a 3-dimensional, transient, soft biological matrix.”

Previous research conducted in the Davies Lab has proven that surface topography is instrumental in establishing a bone-bonding interface during implant healing. This work, combined with significant recent advances in material science, has allowed for the creation of consistent, nanotopographically complex surface treatments that have resulted in revolutionary endosseous implants shown to accelerate the healing response in healthy subjects.

In general, endosseous implants are extremely effective with a clinical success rate of 95%. There are numerous factors that contribute to the failure of the remaining 5% of implants, of which recent dental literature suggests undiagnosed systemic diseases such as diabetes mellitus, and more specifically the associated hyperglycemia, to be a possible contributor.

In 2011, 18.8 million Americans were diagnosed with diabetes and 79 million were diagnosed with pre-diabetes, while an estimated 7 million additional people suffered from the disease yet remained undiagnosed. Demographics predict an increasing trend of diagnoses, suggesting a significant contraindication for the success of dental implants and other medical procedures in addition to the obvious impact on the entire healthcare field. While numerous strategies have been reported for maintaining a consistent blood glucose level in diabetic patients prior to surgical implantation, these guidelines are ineffective for patients who remain undiagnosed and therefore untreated. As such, developing endosseous implants capable of overcoming the delayed bone healing associated with hyperglycemia may be of paramount importance for improving clinical success rates.

This thesis addresses the performance of nanotopographically complex implant surfaces in an environment of uncontrolled hyperglycemia. To provide the relevant background a brief review of endosseous implants and peri-implant healing, including the impact of surface design, is provided below; followed by an overview of diabetes mellitus and its effects on the physical properties and healing abilities of bone, and relevant animal models to investigate such issues.
1.1 Endosseous Implants and Bone Healing

1.1.A The Importance of Surface Design

There has been significant progress over recent years in understanding the role of implant surface design in promoting osteoconduction.\(^1\) Similarly many theories have been presented to explain the phenomenon of bone bonding, including chemical\(^{13,14}\) and biochemical approaches,\(^{15}\) but these theories lack objective evidence\(^3\) and fail to explain the biological mechanisms occurring at the surface of a biomaterial. Indeed, the concept of “osseointegration” has failed to explain the increase in bone-implant contact (BIC) and increasing removal forces seen with implants of increasing surface topography but similar chemical characteristics.\(^{16}\)

Over the past two decades, it is surface topography that has emerged as the driving force behind so-called bone-bonding surfaces.\(^2,17-19\) Evidence shows the bone-bonding mechanism to be one of micro-mechanical interdigitation of bone tissue with the implant surface.\(^2,3,17,20\) Results from both the Davies lab,\(^21\) and elsewhere,\(^{16,22}\) show a lack of bone-bonding with smooth implant surfaces, yet when those same surfaces are treated to create topographical complexity of varying degrees with no change to the chemical composition of the surface, the result is a bone-bonding surface. Therefore, a biomaterial surface with topographical features in the same scale-range as that seen in normal bone remodeling sites—a microtopography with undercuts in the nano-scale range— is essential to successfully render a biomaterial bone-bonding.

An undercut is a recessed feature on an implant surface creating a retention volume, into which bone tissue can be deposited. Bone ingrowth occurs between features in the micro- to macro-scale range, and provides resistance to the removal of bone in three orthogonal planes.

Independently, bonding of bone to an implant surface, which was first described during experimentation with bioactive glass,\(^{23}\) is achieved in two stages. First, proteins are deposited into submicron undercuts on the implant surface. Second, the cement line proteins mineralize. The undercuts therefore provide resistance to the removal of mineralized cement line matrix in three orthogonal planes, resulting in bone bonding. This can occur on surfaces absent of microtopography. Bone-bonding is demonstrated by some form of mechanical disruption assay, where failure occurs within either the implant or bone, but the interface remains intact.\(^{24}\)

Qualification of surface topography has long formed the backbone of literature surrounding implant surface design, with specific interest in the categorization of surface roughness with parameters such as \(S_a\) values.\(^{25-27}\) Although such numerical characteristics are useful in material science to determine
consistency of machined surfaces, their biological relevance has yet to be shown. While such work emphasizes the importance of surface topography, it is the complexity of the surface, and more specifically the undercuts, rather than the range of surface roughness, that allows for increased osteoconduction at the implant surface. Various reports describe an increased removal torque using screw implants of progressively greater $S_a$ values during removal torque (RMT) tests.\textsuperscript{16,22,28} Considering the use of increasingly complex physical surface designs on implants of the same material – that is to say, the same chemical characteristics – it stands to reason that, as increased surface complexity will accelerate osteoconduction, while increased $S_a$ values result in deeper pits into which bone is deposited via contact osteogenesis, an increase in shear strength would result as there is more bone resisting the removal torque. Indeed, it was shown that, while the fracture plane of machined surfaces occurred at the immediate bone-implant interface, surfaces of increasing topographical complexity resulted in a fracture plane in regions further from the interface,\textsuperscript{16} thus providing support for this conclusion. While these observations underline the importance of surface topography and emphasize the formation of bone in the peri-implant region, RMT tests are not representative of bone-bonding ability, but rather demonstrate the increase of bone formation in an area. In addition, such tests are difficult to compare, due to differences in implant design, specifically with respect to pitch and depth of threads.\textsuperscript{27}

Topographically complex implant surfaces increase the surface area available for fibrin attachment during early stages of bone healing, allowing for a stronger attachment of the clot to the implant surface. Of particular importance are undercuts present on the surface, which provide solid anchoring points for fibrin allowing for resistance against retractive forces during clot retraction.\textsuperscript{1} Retraction of the clot is a direct result of cell migration through the matrix, and, therefore, retention of fibrin onto the surface allows migratory cells required for osteoconduction and osteogenic cells to reach the implant surface, where they can undertake de novo bone formation.\textsuperscript{29}

Implant surface topography has also been shown to increase platelet activation,\textsuperscript{30} leading to the upregulation of neutrophils – the first leukocyte to arrive into the healing compartment.\textsuperscript{29} A number of growth factors released at this stage play a pivotal role in the bone healing cascade, including platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-b), both of which serve as chemotactic factors for osteogenic cells.\textsuperscript{31} Thus, surface topographical complexity also allows for stronger chemotactic pathways through the increased activation of platelets and subsequent release of their constituents, leading to increased osteoconduction. Furthermore, as witnessed by the reduced expression of mature dendrocytes – a bone marrow-derived dendritic cell residing in the dermis, which can perform the function of macrophages or antigen-presenting cells, and can participate in the homeostasis of macromolecules of the dermis\textsuperscript{32} – in response to modified sand blasted acid-etched (SLA) surfaces,\textsuperscript{33} a
decreased inflammatory response could result from the presence of topographically complex implant surfaces in the healing compartment, leading to faster healing and recovery.

1.1.B Primary Stages of Bone Healing: Hemostasis and Angiogenesis

Bone healing in healthy subjects, generally as a result of fracture or trauma, is a complex, yet well-ordered occurrence leading to the formation of a fracture callus, which is remodeled over time to form new bone. The first result of trauma to the bone and surrounding vascular network is bleeding, caused in experimental models by creation, for example, of a surgical drill-hole defect; this results in a hematoma at the site of injury.\(^{34}\) In response to contact with damaged vasculature, platelets adhere to the exposed collagen and begin to aggregate, becoming activated in the process. Within 10-60 minutes of activation, platelets release upwards of 300 different proteins,\(^{35}\) resulting in recruitment and activation of additional platelets, while also initiating coagulation of blood to form a fibrin clot.\(^{36}\)

The mass release of chemokines and growth factors stimulates healing through a well-orchestrated chain of events involving many different cell types, including neutrophils, followed by monocytes (which activate at the site of injury, becoming macrophages to engulf necrotic tissue and debris), fibroblasts, and endothelial cells.\(^{34}\) As the hematoma continues to develop, additional growth factors are secreted which regulate cellular proliferation and differentiation at the site of injury.\(^{34,37}\) The secretion of tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)), interleukins-1 (IL-1), IL-6, IL-11, and IL-18, among others, in the hematoma increases recruitment and differentiation of inflammatory and regenerative cells to the region, while also promoting angiogenesis.\(^{36,37}\)

The complex fibrin network forming the backbone of the clot, in addition to preventing blood loss, performs a critical role in allowing for the migration of cells involved with immune response and tissue regeneration. Within 3 days, cells expressing Type I collagen mRNA are found in the defect, while cells with a more osteoblastic mRNA profile are found around 7 days.\(^{38}\) The coagulum in the hematoma is gradually degraded by a front of macrophages, leading to the formation of a vascularized granulous tissue, indicating progression of bone healing and the invasion of cells to the site of injury.\(^{39}\) The metabolically active macrophages migrate through the clot to the site of trauma, creating a diminishing oxygen concentration gradient that acts as a chemotactic pathway for endothelial and mesenchymal cells to initiate angiogenesis.\(^{40}\)

Angiogenesis occupies a fundamental role in bone healing, and tissue regeneration in general, supplying factors to regulate osteoblast and osteoclast activity.\(^{41}\) Through the re-establishment of a complex microvascular network within the regenerating bone tissue, angiogenesis supplies nutrients and oxygen to resident osteogenic cells responsible for laying down new bone matrix,\(^{42}\) and also acts as a pathway to
transport circulating osteoclast and osteoblast precursors.\textsuperscript{43,44} In addition to the hypoxic environment caused by macrophage activity – which is accompanied by a decrease in pH due to the release of protons through a vacuolar-type \( \text{H}^+ \)-\text{ATPase} pump\textsuperscript{40} similar to that seen in the osteoclast – the release of vascular endothelial growth factor (VEGF) and endothelin from activated platelets aids in the recruitment of endothelial cells to the area.\textsuperscript{45} Newly formed endothelial sprouts depend on the presence of VEGF to branch and grow, until the arrival of pericytes – defined as cells that established a physical contact with the outer endothelial cell membrane via gap junctions, express at least one pericyte marker, and do not express any endothelial markers\textsuperscript{46} – which continue to secrete VEGF once platelet activation ceases\textsuperscript{47} and stabilize newly formed endothelial tubes.\textsuperscript{48}

It is difficult to differentiate between pericytes and perivascular cells (PCs) based on our current understanding, and it is debatable whether these terms are interchangeable. Regardless, this cell population plays a vital role in the early stages of bone healing since PCs from all different organs and tissues have been proven capable of differentiating into three mesenchymal stem cell (MSC)-derived lineages (cartilage, bone and fat).\textsuperscript{49} This recent discovery likely explains the vast body of literature describing tissue-resident MSC populations due to the presence of vasculature in all tissues, suggesting a portion of the PC population to serve as the MSC source during wound healing.\textsuperscript{50} Abnormalities in the migratory ability and function of perivascular cells and their interaction with endothelial cells result in numerous different pathologies including diabetic retinopathy,\textsuperscript{48} indicating the importance of these cells for successful angiogenesis and subsequent healing.

1.1.C Key Processes for Peri-Implant Bone Formation: Osteoconduction, de novo Bone Formation, and Contact and Distance Osteogenesis

A critical aspect in bone healing, especially around implants, is the process of osteoconduction – defined as the recruitment and migration of osteogenic cells to the implant surface.\textsuperscript{1,24,29} Since there is no bone on the implant at the time of surgery, it is reasonable that, in order for bone to form directly on the surface, there must first be bone cells present to secrete the matrix. This concept is relevant to both normal bone remodeling and peri-implant bone formation, since new bone is deposited in a similar manner in both circumstances. In normal remodeling, osteoclasts act to resorb old bone, which leaves a topographically complex surface of exposed, demineralized collagen, with significant undercuts and complexity at both the micro- and nano-scale range.\textsuperscript{24} Thus, implant surfaces with effective bone-bonding properties mimic the biological topography of resorbed bone, leading to successful \textit{de novo} bone formation directly onto the implant surface, which forms in a predictable fashion. The process begins with the adsorption of non-collagenous bone proteins – namely osteopontin (OP) and bone sialoprotein (BSP) – to the topographically complex surface, creating a collagen-free organic matrix with nucleation sites for calcium
phosphate mineralization. Calcium phosphate nucleation is followed by crystal growth and the assembly of a collagenous matrix, which is interwoven into the collagen-free cement line. The final result is a collagen-free cement line, approximately 0.5 μm in thickness, separating the collagen compartment of new bone from either the resorbed surface of old bone or the topographically complex implant surface.\textsuperscript{1,29}

The process by which osteogenic cells are recruited to the implant surface (osteocoduction) resulting in \textit{de novo} bone deposition directly on the implant surface is known as \textit{contact osteogenesis}. This is contrary to \textit{distance osteogenesis} where bone is deposited in the area surrounding the implant, encroaching on and eventually surrounding the implant surface.\textsuperscript{1,24,29} Distance osteogenesis is expected for peri-implant cortical bone healing, as the vascular disruption of the cortex kills the surrounding cortical bone, and the remodeling capacity, based on osteoclast invasion from the medullary compartment, is very slow.\textsuperscript{51} Both processes will occur during bone healing and contribute to implant stability, but contact osteogenesis alone is responsible for the bone-bonding interface, making it a critical aspect of early bone healing for endosseous implants. It is important to understand as well that once differentiating osteogenic cells become osteoblasts and begin to secrete matrix, they stop migrating. Thus, bone ingrowth into 3-dimensionally complex surfaces is the result of continual recruitment and migration of osteogenic cells.\textsuperscript{20}

Calcium phosphate coatings on biomaterials allow for increased protein adsorption to the surface, of which OP and BSP play a pivotal role in the bone healing cascade. Ultimately, this allows not only for increased binding of fibrinogen, but also leads to increased platelet activation which further enhances fibrin binding and accelerates osteocoduction and early bone formation.\textsuperscript{29} The result of such activity is \textit{de novo} bone formation directly onto the surface of the implant, creating a micro-mechanical interdigitation of the cement line with the calcium phosphate layer at the implant surface, rendering the implant “bone bonding.”\textsuperscript{2,17,20,29}

1.1.D Nanotopographically complex surfaces accelerate early stages of healing

Nanotopographically complex implants have been shown to increase osteocoduction at the implant surface during early time points in a healthy population. This results in increased \textit{de novo} bone formation on the implant surface thereby improving implant stability and decreasing healing times.\textsuperscript{2} Mendes \textit{et al.} have shown such nanotopographically complex implants to be effective in increasing osteocoduction during the early stages of bone healing, while also proving it possible to render an implant “bone-bonding” by using these surface designs in healthy rats.\textsuperscript{2,3}

The topography of these implants closely mimics the resorption surface of old bone during normal bone remodeling (a result of osteoclast activity), which has a 3-dimensionally complex topography in the sub-micron range with undercuts due to the randomized orientation of collagen fibers.\textsuperscript{24} By imitating the bone
surface seen in the natural environment, the implant surface provides a highly topographically complex surface, both at a micro- and nano-scale, into which new bone matrix can be deposited and become interdigitated, as discussed above.\textsuperscript{124} Discrete crystalline deposition of CaP nanocrystals onto a microtopographically complex implant surface, which creates the nanotopographically complex surface used in this study, has been shown to increase the recruitment of osteogenic cells to the implant surface, resulting in significantly greater contact osteogenesis, better implant stability, and an increase of 150\% in the BIC in human clinical trials.\textsuperscript{52,53}

Nanotopography of biomaterials has recently been shown to have an effect at the cellular level as well, leading to altered cell and tissue responses that may aid the bone-healing cascade.\textsuperscript{54–56} Such surfaces result in improved adhesion of cell populations onto the implant surface, leading to increased proliferation efficiency of MSC populations\textsuperscript{57} and enhanced expression of osteoblast-specific gene expression.\textsuperscript{58} In osteogenic cell lines, nano-scale surface topography, independent of surface chemistry, leads to enhanced protein interactions and increased osteoblast adhesion to the surface of both nanophase alumina and titania,\textsuperscript{59} ultimately resulting in accelerated osteoconduction and increased contact osteogenesis.

1.2 Diabetes Mellitus

1.2.A The Disease

Diabetes mellitus is a systemic disease characterized by hyperglycemia together with disturbances in carbohydrate, fat and protein metabolism resulting from defects of insulin secretion (Type 1), insulin action (Type 2), or both.\textsuperscript{60–62} It is associated with reduced life expectancy, significant morbidity due to diabetes-related microvascular complications, increased risk of macrovascular complications (ischaemic heart disease, stroke, peripheral vascular disease, among others), as well as an overall diminished quality of life, particularly in later years.\textsuperscript{63} The disease centres around insulin, a peptide hormone produced mainly in the pancreatic β-cells and a primary moderator in whole body fuel homeostasis. In healthy individuals, activation of insulin receptors results in decreased blood glucose by encouraging glucose uptake in target tissues, while also promoting glycolysis, glycogen synthesis, and amino acids uptake for storage. At the same time, the presence of insulin inhibits lipolysis and ketogenesis, glycogenolysis, proteolysis, and gluconeogenesis, suppressing numerous processes that break down and release stored energy.\textsuperscript{64} Acting in skeletal muscle, adipose tissue, and the liver, insulin promotes the uptake of carbohydrate, fat, and amino acids for catabolism of storage molecules, while also stimulating glycolysis and tri-carboxylic acid (TCA) cycle activity in skeletal muscles, and glycerol synthesis and TCA cycle activity along with an antilipolytic effect in adipocytes.\textsuperscript{61}
1.2.A.1 Type 1 Diabetes

Type 1 diabetes mellitus (T1DM), also known as juvenile diabetes, is generally diagnosed in children or young adults, although older adults may present with a slower onset of the disease. It is caused by an auto-immune reaction, in which the insulin-producing β-cells in the Islets of Langerhans of the pancreas are attacked, thus halting production of insulin in the body. People suffering from Type 1 diabetes require daily insulin injections to survive. With appropriate treatment, it is possible for patients to lead a normal life, yet it does not generally prevent development of comorbidities over the patient’s lifetime.

1.2.A.2 Type 2 Diabetes

By far the most common form of diabetes is Type 2 (T2DM), accounting for over 90% of cases worldwide. Also known as non-insulin dependent diabetes and adult-onset diabetes, diagnosis of T2DM generally occurs after the age of 40, and can remain undetected for many years before diagnosis. At the outset, it is characterized by progressive insulin resistance, where insulin attaches normally to receptors on liver and muscle cells, but transportation of glucose into the cell is blocked. This is overcome in early stages by overproduction of insulin, known as hyperinsulinaemia. As the condition progresses and insulin resistance becomes more widespread, plasma glucose levels increase, while the pancreas is unable to maintain elevated insulin levels and drastically decreases its production, leading to hyperglycemia. Over time, the elevated blood glucose leads to a decrease in beta cell mass, thereby halting the production of insulin altogether and leading to frank diabetes and sustained hyperglycemic conditions. T2DM is strongly linked to obesity, poor diet, older age, low levels of physical activity, ethnic background and certain other genetic factors (Figure 1). Treatment begins with lifestyle changes, emphasizing a healthy diet and physical activity, and can be combined with insulin prescriptions and several oral medications on a patient-specific basis.

![Figure 1: Risk factors for Type 2 diabetes mellitus. (Holt et al. 2010, pg 46)](image-url)
1.2.A.3 Gestational Diabetes

The third type of diabetes is called Gestational Diabetes Mellitus (GDM), and is far less common. GDM develops in 1 in 25 pregnant women worldwide, a large percentage of which occur in underdeveloped nations, and generally occurs during the later stages of pregnancy. Risk factors include being overweight prior to pregnancy, previous occurrences of gestational diabetes, and family history. In order to minimize risks to the developing fetus, blood glucose control throughout the pregnancy is critical. While it usually disappears after birth, it can lead to the development of permanent diabetes in the mother.

1.2.B Diabetic Pathologies and Diagnosis

While the underlying cause of each form of diabetes differs, all three are associated with a significant increase in plasma glucose levels, a condition known as hyperglycemia. It is this chronic hyperglycemia that leads to significant physiological complications, including disease of the cardiovascular system, kidney (nephropathy), eye (retinopathy), as well as nerve damage (specifically peripheral neuropathy). Clinical diagnosis is often driven by the presence of classic symptoms including thirst, polyuria, weight loss, and recurrent infections in the patient. A critical component in the final diagnosis is the level of venous plasma glucose. The World Health Organization (WHO) defines a plasma glucose level below 5.0mmol/L as healthy, 5.0-11.0mmol/L as uncertain, and anything greater than 11.1mmol/L as a definitive diagnosis (Figure 2). As clinical symptoms can take many years to develop, education and awareness in the general population is critical to diagnose and treat the disease at early stages, in an effort to effectively delay, or even curtail more serious manifestations of diabetes-related conditions.
Figure 2: 2006 WHO recommendations for criteria of diabetic and pre-diabetic diagnoses. (Holt et al. 2010, pg 48)

1.2.C Prevalence in Society and Future Indications: A Growing Concern!

Globally, diabetes is one of the most common non-communicable diseases and is a top-5 leading cause of death in high-income countries, responsible for approximately 4.6 million deaths in 2011. An estimated 366 million people around the world suffered from the disease in 2011, with another 280 million people diagnosed with pre-diabetes (7% and 6.4% of the global population, respectively), also known as impaired glucose tolerance (IGT) which leads to diabetes in over 70% of cases. Approximately 80% of all cases reside in low- and middle- income countries. Trends predict this number to rise to over 550 million diabetic cases and 398 million pre-diabetic cases globally (9.9% and 7.1% of the predicted global population, respectively) by 2030. As it can take multiple years for symptoms to manifest, diagnosis of the disease can be difficult, particularly in low-income countries where access to medical screening is limited. As such, an estimated 183 million people worldwide are unaware of their diabetic condition, which further complicates treatment and can lead to more rapid onset of co-morbidities as a result of uninhibited hyperglycemia.
In 2011, 18.8 million Americans were diagnosed with diabetes, 79 million were diagnosed with prediabetes, and an additional 7 million people were estimated to suffer from the disease yet remain undiagnosed and untreated.\(^8\) Diabetes is a considerable burden on healthcare systems worldwide, with almost US$218 billion spent on diabetes and related conditions in the US alone in 2007.\(^8\) Current trends predict 1 in 3 children born after 2000 to develop diabetes over their lifetime, leading to 36 million diabetic Americans by 2030 (Figure 3)\(^61\) and 48 million by 2050.\(^66\) Given the ratio of diagnosed to undiagnosed cases, this suggests over 18 million undiagnosed diabetics in the US population by 2050.

<table>
<thead>
<tr>
<th>Rank</th>
<th>Country</th>
<th>Persons (millions) 2030</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>India</td>
<td>87.0</td>
</tr>
<tr>
<td>2</td>
<td>China</td>
<td>62.6</td>
</tr>
<tr>
<td>3</td>
<td>United States</td>
<td>36.0</td>
</tr>
<tr>
<td>4</td>
<td>Pakistan</td>
<td>13.8</td>
</tr>
<tr>
<td>5</td>
<td>Brazil</td>
<td>12.7</td>
</tr>
<tr>
<td>6</td>
<td>Indonesia</td>
<td>12.0</td>
</tr>
<tr>
<td>7</td>
<td>Mexico</td>
<td>11.9</td>
</tr>
<tr>
<td>8</td>
<td>Bangladesh</td>
<td>10.4</td>
</tr>
<tr>
<td>9</td>
<td>Russian Federation</td>
<td>10.3</td>
</tr>
<tr>
<td>10</td>
<td>Egypt</td>
<td>8.6</td>
</tr>
</tbody>
</table>

Figure 3: Predicted global distribution of diabetes for 2030. (Adapted from: Holt et al. 2010, pg. 51)\(^61\)

1.3 Diabetes and Bone

Diabetes has far-reaching effects on many systems and tissues within the body, including the skeletal system. As the diabetic condition, namely chronic hyperglycemia, can exist for many years before a diagnosis is made, untreated diabetes has been suggested as a contraindication for many medical treatments, including implant-based dental therapies.\(^5\) While the impact of diabetes can be seen throughout the body and its physiological systems, its effect on bone is notable, yet not entirely understood. Literature reports vary significantly, depending on many factors including the type of diabetes, the time points of interest, and the animal models used for investigation.\(^67\)

1.3.A Effect on Physical Properties of Bone

The effects of diabetes on bone have been widely reported in the literature, yet conclusions are wide-ranging and often times contradictory.\(^67\) It is generally agreed that the two types of diabetes act in different manners,\(^68\) possibly due to the presence of hypoinsulinaemia (T1DM) or hyperinsulinaemia (T2DM).\(^69\) Regardless, diabetic bone, irrespective of the disease type, is generally weaker and more
It is likely that shared (due to hyperglycemic conditions) and different (due to insulin levels, other hormones, or age of onset) mechanisms jointly affect bone properties in T1DM and T2DM, of which many mechanisms are yet to be discovered and understood.72

1.3.A.1 Insulin Activity and Bone

Insulin, the hormone at the center of the disease, has been shown to play a crucial role in bone, with a significant anabolic effect.68,73 Osteoblasts express functional insulin receptors and respond to exogenous insulin by increasing production of anabolic markers, resulting in collagen synthesis,74 alkaline phosphatase (ALP) production,75 and glucose uptake.64,76 In healthy subjects, evidence now points to a bone-pancreas endocrine loop, potentially linked by osteocalcin (OC) – a factor produced strictly by osteoblasts, therefore providing a common measurable element of osteoblast activity.77–79 Insulin signaling in osteoblasts stimulates the production of OC, which is formed as pre-pro-osteocalcin and becomes pro-osteocalcin, also known as undercarboxylated osteocalcin (ucOC), following processing in the endoplasmic reticulum of osteoblasts. Before secretion, a vast majority of ucOC undergoes a vitamin-K dependent carboxylation, endowing the molecule with high affinity for bone matrix, yet there remains a small percentage of ucOC released into circulation which has been suggested to link bone to glucose homeostasis.80 The undercarboxylated form has been shown to enhance beta cell function81 and increase insulin secretion and sensitivity,82 likely through regulation of adiponectin.82,83 Emerging evidence suggests a loop in which insulin suppresses the Runx2 inhibitor Twist2 (an inhibitor of osteoblast development), thus promoting osteoblastic differentiation and the increased production of bone. In turn, the production and bioavailability of osteocalcin is increased, leading to increased osteoblast activity (OC), while the accompanying ucOC is released into circulation and acts as a hormone to regulate fat accumulation through adiponectin as well as insulin production in pancreatic beta cells and collective insulin sensitivity.64,77,81,82 As such, under hyper- or hypoinsulinaemic conditions, the production and maintenance of bone and regulation of adipose tissue would be negatively impacted.67

1.3.A.2 Type 1 Diabetes and Bone

T1DM, which creates an hypoinsulinaemic and hyperglycemic environment, is linked to osteopenia,84–86 reduced bone mineral density (BMD) measured using dual-energy X-ray absorptiometry (DXA),67,87–90 reduced bone status of adolescents measured using quantitative ultrasounds (QUS),91 and increased fracture potential67,86,89,92–94 in human subjects. Bone resorption is increased, while bone formation is decreased or normal, suggesting a basis for reduced BMD.67 Further to this, serum OC levels are unchanged in asymptomatic T1DM patients, but are significantly decreased at the onset of microangiopathy, indicating a decreased bone turnover in the presence of diabetic pathologies.95 Due to the anabolic effect of insulin on bone, as discussed earlier, hypoinsulinaemia associated with T1DM
would contribute to the low bone mass and turnover rate. In addition, poor glycemic index in T1DM patients has been shown to exacerbate inflammatory cytokines, which results in decreased BMD while also accelerating peripheral neuropathy in both humans and animals.

The most drastic bone mineral loss occurs within the first 5 years after the onset of clinical symptoms. As might be expected, the age at onset of diabetes as well as the duration of the disease significantly impact the physical properties of bone, and increase the risk of fracture. However, there is little change reported in the structural properties of bone in otherwise healthy T1DM patients before the onset of diabetes-related clinical complications, suggesting a controlled glycemic index in T1DM could maintain healthy bone.

### 1.3.A.3 Type 2 Diabetes and Bone

Patients with T2DM have been shown to have decreased, unchanged, or elevated BMD. While comparisons between studies of BMD in T2DM are difficult due to the variance in anatomical locations of measurements, age of the patients, and varying levels of patient-specific medications in the sample population, most large-scale studies point to an unchanged or elevated BMD level. Despite the elevated bone density, there is also an increased risk of fracture, especially for those patients of longer duration. While this creates somewhat of a paradox, it suggests that the bone quality, instead of its density, may be a significant factor in the increased fracture risk—a component not easily measured using simple densitometric techniques. In addition, there is evidence confirming decreased bone formation in T2DM, as well as decreased bone resorption, leading to a slower bone turnover rate and therefore higher bone density than normal. This has been suggested as a contributor for increased fracture risk, as there is a decreased ability to remove accumulating micro-damage in the bone through the process of resorption. The reduction in bone formation and resorption may be due to decreased insulin sensitivity, which leads to the expression of Twist2, thus inhibiting osteoblastic differentiation and further upsetting the bone-pancreas endocrine loop through the reduced circulation of ucOC.

### 1.3.A.4 Diabetes and the Biomechanical Properties of Bone

Animal work in models of T1DM have demonstrated a significant decrease in biomechanical properties including bone stiffness, toughness, energy absorption, and torsional strength, in addition to a reduced BMD, and an increased modulus of elasticity. These changes in physical properties indicate an impact on the mineral content of diabetic bone. Delayed bone formation indicated by decreased mineral apposition rate (MAR), decreased osteoid surface, decreased growth of metaphyseal cartilage, and decreased bone mineralization at the metaphysis are also present in diabetic animals, suggesting a similarly reduced bone turnover in diabetic animal models. Evidence points
to an abnormal increase in cell death through apoptosis of osteoblasts\textsuperscript{96,107} and decreased levels of plasma osteocalcin,\textsuperscript{73,77,109,110} thus providing additional evidence for a decrease in bone production. Torsional strength, toughness and stiffness can be improved in certain instances with insulin treatment,\textsuperscript{71,97,112} although not always.\textsuperscript{113}

\textbf{1.3.A.5 The Influence of Hyperglycemia}

While the discussion on diabetes centres largely on insulin, it is hyperglycemia that plays the most significant role in the long-term health of the body. Hyperglycemia is arguably the most important systemic consequence of diabetes and has a significant impact on low bone turnover seen in the disease.\textsuperscript{67,102} Animal work in varying models of T1DM has shown hyperglycemia to influence osteoblast differentiation, impair bone formation, and inhibit bone mineralization,\textsuperscript{107,110,114} all of which has been confirmed in vitro.\textsuperscript{115–117} Exposure to chronic hyperglycemia, in addition to decreasing cell volume through hyperglycemia-associated hyperosmolality which also likely changes structural properties of the cell, modifies osteoblast gene expression, increasing ALP activity and expression while decreasing OC, MMP-13, VEGF, and GAPDH expression.\textsuperscript{116} These observations were also noted in short-term, acute hyperglycemia over a 48-hour period, suggesting a rapid change in osteoblast gene expression that is maintained under chronic conditions, potentially due to increased osmotic stress.\textsuperscript{118,119} However, while chronic hyperglycemia suppressed late stages of osteoblast differentiation and produced increased expression of adipocyte-phenotype markers, acute hyperglycemia only affected mature osteoblasts, suggesting two distinct responses to hyperglycemia in osteoblasts: an early acute response driven by hyperosmolality, and a late acute and chronic response predominantly driven by hyperglycemia.\textsuperscript{116} The suppression of osteoblast differentiation and function combined with increased adipocyte differentiation, provides support for accelerated bone loss and increased fatty deposits in diabetic bone under chronic hyperglycemic conditions.\textsuperscript{116,118–120} In addition, excess glucose can be involved with glycation (non-enzymatic glycosylation) of proteins, producing advanced glycation end products (AGEs), especially in hyperglycemic conditions. This has been shown to result in non-enzymatic cross-linking of collagen fibers in bone and overall reduced mechanical properties, as measured using a mechanical three-point bending test.\textsuperscript{121} Furthermore, increased glycation of collagen is related to compromised bone turnover,\textsuperscript{122} providing at least a partial explanation for the retarded mineralization of newly formed bone in hyperglycemic conditions.

\textbf{1.3.B Drill-Hole Model: A Model to Study Bone Healing at Early Time Points}

A femoral drill-hole model was implemented in this work, as it is easily reproducible and allows for the study of different stages of bone healing in a mechanically stable environment. Unlike fracture models, rats maintain full weight-bearing immediately after surgery. In addition, it avoids numerous complications
unique to endochondral ossification and the influence of diabetes during the chondrogenesis stage of fracture healing. The model allows for explicit observation of bone healing, specifically at early time points.2,3,50 While there is a significant body of literature surrounding the physical properties of bone and its formation, “the most important stages of [bone] healing precede bone formation”.29 The early stages of healing, from the immediate appearance of blood at the site of tissue damage to the mineralization of newly formed bone are the most important in understanding bone healing, and will determine the long-term properties and stability of the bone, especially that supporting an implant.

1.3.C Diabetes and Bone Healing

Diabetes is known to delay both hard and soft tissue wound healing.123 While the physical properties of bone in T1DM and T2DM can vary, as described earlier, both are associated with an increased risk of fracture.67,69,123 There is a noticeable delay associated with fracture healing in diabetic patients124,125 and animals,126,127 particularly in displaced and open fractures, with new osteon formation taking upwards of 2 to 8 times longer in diabetic bone.124 Biomechanical testing at the site of fracture in diabetic bone have shown significantly reduced tensile strength and stiffness two weeks post-fracture,128 and decreased failure torque, failure stress, structural stiffness, and material stiffness at 3 and 4 weeks post-fracture compared to healthy controls.126 There is also a significant delay in tendon-bone healing after rotator cuff repair, providing evidence that chronic hyperglycemia impairs the overall healing capacity of the subject, not just that of bone.129 This has significant clinical ramifications for the expected outcomes of all surgical treatments involving bone and soft tissue repair.

In fracture repair, there is a delay during the transition from cartilage to bone in diabetic subjects. While the initial cartilaginous matrix of the fracture callus is composed of Type I and III collagen, followed by Type II collagen from the first chondrocytes in the fracture callus, Type X collagen is produced once chondrocytes begin to hypertrophy during endochondral ossification.127 With decreased Type X collagen, an overall reduced level of collagen production 4-11 days post-fracture,128 a reduced proliferation of chondroprogenitor cells and chondrocytes in the early stages of healing,130 and an overall decrease in chondrogenesis and osteogenesis after 7 days,131 there is evidence of a physically smaller and more fragile fracture callus in comparison to healthy controls indicating deficient cartilage formation in fracture healing.130–133

In the later stages of fracture healing, there is a noticeable reduction in bone formation, thought to be the effect of hyperglycemia on the formation, function, and bone deposition activity of osteoblasts.123 As discussed earlier, diabetes is associated with decreased levels of OC and ALP, signifying a decrease in osteoblast activity.67,95,100,108 Animal models have shown increased bone marrow adiposity and decreased
OC mRNA levels in the tibia, confirming the decreased bone formation and increased fatty deposits within diabetic bones. In vitro work shows a decrease in proliferation and size of osteoblasts from diabetic rats, which suggests a significant impact on the formation and function of osteoblasts. In addition, there are reports of increased cartilage resorption resulting from elevated osteoclast numbers, a higher expression of genes promoting osteoclastogenesis, a sustained and more severe inflammatory response, and diminished primary bone formation. This would prematurely reduce the amount of cartilage available for endochondral ossification, leading to a physically decreased fracture callus during fracture healing and structurally reduced secondary bone in the diabetic population. Overall, results show a significant decrease in the anabolic aspects of fracture healing in the presence of diabetes.

Insulin treatment has been shown as an effective means to overcome the impaired physical properties as a result of the fracture-healing process, with potential to reverse the mechanical defects in diabetic patients. Diabetic rats treated with insulin were shown to have comparable levels of blood glucose, which resulted in unchanged size and formation of the fracture callus, as well as normalized osteoclastogenesis, chondrogenesis, chondrocyte apoptosis, cellular proliferation, and overall bone formation in comparison to healthy controls. Additionally, sustained release of insulin was shown to restore the overall fracture healing rate to control levels. However, insulin is not always effective and, in some situations, there is no measurable change.

1.3.C.1 Hyperglycemia Causes Delay in Early Stages of Healing

While the physical characteristics and long-term effects of diabetes on the physical properties and healing capacity of bone have been investigated, there are comparatively few studies elucidating its effect on early stages of healing, specifically the impact on osteoconduction and *de novo* bone formation which are of critical importance for early implant stability and bone-bonding. Recent evidence from the Davies lab has shown diabetes, more specifically uncontrolled hyperglycemia, to affect bone healing at early stages, causing a delayed response concurrent with the osteoconduction phase, and a decrease in trabecular number and thickness. As well, there was evidence of decreased mineralization at early stages, matching previous reports describing a mineralization disorder characterized by a reduced or retarded mineralization process, including decreased mineralization, apposition, formation, and timing of mineralization, in animals of uncontrolled hyperglycemia. These findings seem to correlate with other work that has shown diabetes to negatively impact the mineral apposition rate and decrease the bone-implant contact. Furthermore, an elevated and prolonged expression of pro-inflammatory cytokines and macrophage numbers has been described in diabetic bone tissue, which adversely affects the differentiation of MSCs. Thus, hyperglycemia resulting from undiagnosed diabetes could negatively affect the early phases of bone healing as well as the quality of bone surrounding the implant.
Events occurring at early time points set the foundation for a successful bone healing cascade. Propagation of early bone healing events in a timely manner becomes critically important in clinical situations, particularly those involving biomaterials. Successful healing around implantable biomaterials depends highly on the recruitment and migration of osteogenic cells to the peri-implant site, which leads to the contact osteogenesis necessary for bone-bonding and early implant stability, as well as for long-term success. The outcome of contact osteogenesis is generally measured through BIC, which is reportedly decreased in the presence of hyperglycemia. A delay in the early stages of bone formation, likely due to reduced osteoconduction, would lead to a decrease in de novo bone on the implant surface in clinical applications, thus leading to a reduced BIC and decreased clinical performance. Adverse effects at early stages of healing in the presence of hyperglycemia would negatively impact long-term endosseous implant stability, suggesting hyperglycemia could indeed be a significant contributor to implant failures in an otherwise apparently healthy population — a matter that has not been adequately explored in the literature.

1.3.D The Effect of Diabetes on Endosseous Implant Healing

Diabetes has been shown to have negative implications in endosseous implant healing, as well as other aspects of the oral cavity including increased risk of periodontitis, potentially as a result of increased risk of infection for those with uncontrolled or poorly controlled glycaemia. The metabolic status of the patient during initial stages of peri-implant bone formation has been suggested as an important factor in the long-term success of the implant, as early bone formation is paramount to long-term success rates. Yet, as discussed above, there is a discrepancy in the literature as to the overall impact of diabetes on success rates. Longer healing periods and impaired implant integration leading to reduced early implant stability has been observed in patients with altered glycosylated haemoglobin (HbA1c), as measured by resonance frequency analysis. Certain studies claim the disease to negatively impact success rates, others claim a high success rate provided the disease is “under control”, and others note no contraindication. An increase in failure rates after one year, corresponding with the uncovering of implants and the initiation of loading, suggests failure could be related to early stages of implant loading. As there is a delay in the early stages of peri-implant bone formation seen in diabetic patients, this could suggest a compromised bone-implant interface which would be exacerbated by the onset of loading due to forces and micromotion along the interface. Overall, there is a wide variance in how glycaemia levels are reported, which could explain the discrepancy in success rates seen in the literature. As well, there are a small number of evidence-based clinical trials from which to base conclusions. Despite incongruity, it is generally agreed that diabetes has a negative influence on the long term success of endosseous implants, although to what degree remains unknown.
results and conclusions in the literature emphasize the need for further examination and definitive characterization of glycemic control in such studies.

In animal models of diabetes, there is evidence of reduced BIC\textsuperscript{[143,151,152]} as well as decreased bone density, bone formation, and mineralization in the peri-implant region.\textsuperscript{[143,151]} One report shows a greater bone volume surrounding implants in diabetic rats at 14 days, which was corrected with insulin treatment.\textsuperscript{153} As hyperglycemia has been shown to cause delays in early bone formation, which leads to a delay in bone resorption of the fracture callus around healing bone,\textsuperscript{50} the delay in bone formation would lead to increased bone volume at 14 days compared to the control group, as the diabetic rats would not have initiated bone resorption at that time — a result that would also explain the decreased bone maturity observed around implants in this study. Insulin control has been shown to regulate, at least in part, the process of peri-implant bone formation,\textsuperscript{[141,153]} further emphasizing the need for proper glycemic control pre-implantation.

1.4 Animal Models of Diabetes Mellitus
Since von Mering and Minkowski’s famous discovery in the 1880’s of the link between the pancreas and diabetes, through to the ground-breaking discovery of insulin by Banting and Best in the 1920’s with their dog Marjorie, much work has been done to develop an accurate and sustainable animal model to study the disease. While no single model completely encompasses all aspects of diabetes, each illustrates various etiological and pathogenic mechanisms that could also operate in humans.\textsuperscript{154} It is important to understand the shortcomings of each in order to appropriately address relevant research questions. Much work in modern diabetes research centres around the use of rodents, which are cheaper and more manageable than larger animals, yet justification is occasionally sought for the use of cats, dogs, pigs, or primates.\textsuperscript{155} The Davies lab has used rats, which are more physiologically similar to humans than mice and much more cost-effective than larger animals.\textsuperscript{155} Rats provide an appropriately sized skeletal system in which to assess the bone healing around biomaterials in weight-bearing situations, specifically through placement of materials in the distal femur. Such implant-related investigations are more difficult in mice due to the diminutive bony structure. As such, all models involving mice and large animals have been omitted from this discussion. In addition, spontaneous and genetically altered diabetic animal models were not ideal for this project due to variance in the onset and severity of symptoms. A discussion of these models has been provided in Appendix A.

The simplest and most common method of inducing diabetes is through non-surgical means, specifically through injection of toxins such as streptozotocin (STZ) and alloxan, which destroy insulin-producing beta cells of the pancreas and create a model of T1DM. Such methods have been used successfully for
decades. STZ-induced diabetes is discussed below. Alloxan has an analogous structure to glucose and is transported into beta cells using the same low affinity GLUT2 glucose transporters. Redox reactions between the drug and its reduction product, dialuric acid, result in the production of oxygen free radicals, specifically hydrogen peroxide, which leads to necrosis of the beta cells and, ultimately, systemic conditions similar to that of T1DM or insulin-dependent diabetes.

The progressive development of diabetic animal models has led to a substantial increase in knowledge surrounding the disease, especially as it relates to the skeletal system. However, while the body of literature surrounding diabetes continues to grow, a conclusive mechanistic pathway through which diabetes affects bone healing is yet to be described. Much of the literature is focused on the long term impact on healing and the final outcome, often times neglecting the effect of the disease on early time points, which are critical for bone healing and the overall success of endosseous implants. In addition, many models of T2DM, while obviously useful, do not allow for independent assessment of individual components of the disease, specifically hyperinsulinaemia and hyperglycemia, and the impact of each on the healing capacity. While such models simulate the pathogenic mechanisms seen in humans, due to the complexity of the disease and its pathologies, it becomes difficult to determine etiology, which becomes especially important when designing biomaterials for implantation into the body. In addition, the nature of these models makes it difficult to control environmental factors and assess the onset of symptoms with any precision, which adds an element of uncertainty to the analysis. Thus, in the work presented herein, it was decided to isolate one major component of the disease – hyperglycemia – and elucidate the mechanisms through which it affects bone healing by examining early time points.

1.4.A Model Selection: Streptozotocin (STZ)-Induced Hyperglycemia in Rats
An animal model of streptozotocin (STZ)-induced diabetes was chosen for this investigation. The model was cost-effective and generated consistent and predictable onset of hyperglycemia, which allowed for a consistent surgical schedule, while limiting the variation seen in many other animal models. While genetic models spontaneously develop full-blown diabetes, making them an attractive option, the severity and chronology with which the disease develops is varied in these animals, which generates additional variables, complicates the surgical schedule, and obscures the number of animals required. The Davies lab has previous experience with the STZ rat model, during which it was shown to accurately represent diabetes-associated pathologies while successfully isolating a consistent and reproducible level of hyperglycemia from other factors and allowing for a consistent surgical schedule. The STZ-induced rat is a common model for investigating bone formation and osteopenia under diabetic conditions, as discussed earlier. As both types of diabetes result in sustained hyperglycemia, while hyperinsulinaemia is present only in the early stages of T2DM and only for a comparatively short period of time at that, the
decision was made to isolate hyperglycemia exclusively and investigate its effect on early stages of bone healing independent of other diabetic pathologies.

A repeatable and sustainable model was successfully created by injecting streptozotocin to induce rapid hyperglycemia, waiting one week for blood glucose to stabilize at a consistently high level, and then conducting experimental procedures. This approach allowed for the impact of uncontrolled hyperglycemia on bone healing to be accurately assessed in the absence of diabetic pathologies which had not yet developed, thus eliminating external variables. As such, the term “hyperglycemic” is used forthwith to describe the treated animal group, as there was no evidence to suggest the animals had become truly diabetic by the time of surgery.

As explained earlier, chronic hyperglycemia is known to have a significant impact on bone. Furthermore, as a model for hyperinsulinaemia (with insulin resistance) does not exist in the absence of hyperglycemia, this approach created a baseline for which to compare the more accurate T2DM models in future work, at which point the individual effects of hyperinsulinaemia can be elucidated through process of elimination. It is important to note that the majority of diabetic patients suffer from T2DM, and, since the selected model failed to address the hyperinsulinaemia likely present in the targeted human population, results cannot be directly applied to human diabetic patients.

1.4.B Drug Activity and Mechanism: How does the model work?

Streptozotocin is a nitrosourea derivative isolated from Streptomyces achromogenes which causes select necrosis of pancreatic beta cells resulting in a state of insulin-dependent diabetes. STZ is a toxic, hydrophilic glucose analogue which causes interference with glucose transport and glucokinase function, and induces strand breaks in DNA. The glucose moiety in its chemical structure enables STZ to enter beta cells via the low affinity GLUT-2 transporter in the plasma membrane. As such, beta cells with low expression of this transporter are unaffected by the presence of STZ.

The toxicity of streptozotocin is derived from the ability of its methylnitrosourea moiety to successfully alkylate strands of DNA, specifically at the sixth oxygen of guanine, which results in DNA fragmentation and the ultimate necrosis of pancreatic beta cells. Upon entering the cell, before consequences of alkylation are evident, there is an STZ-induced depletion of NAD+, which results in a decreased production and secretion of insulin. These symptoms eventually become permanent functional deficiencies. Interestingly, there is evidence of beta cells surviving the initial induction at low concentrations of STZ which are then able to maintain most of their metabolic functions, demonstrating the importance in selecting an appropriate dosage for the animal model to effectively eliminate beta cell function in treated animals.
In response to STZ administration, there is a predictable triphasic pattern of hyperglycemia. The first stage of hyperglycemia begins mere minutes after administration and lasting for approximately 2-4 hours, resulting from an inhibition of insulin secretion due to toxic effects of the drug. Approximately 4-8 hours after administration, the synchronized bursting of beta cell membranes results in a mass release of insulin, creating hypoglycemia in the animal. The final phase is permanent hyperglycemia due to degranulation and loss of beta cell integrity usually within 12-24 hours after administration. As a result, injection of STZ creates an environment similar to that of T1DM, or insulin-dependent diabetes.

### 1.4.C Additional Physiological Effects of the STZ Model

While hyperglycemia is a characteristic symptom of STZ induction, there are numerous other noteworthy effects of the drug throughout the body. The expression of GLUT-2 transporters in several organs means that hepatocytes and renal tubular cells, among others, are impacted, leading to varying degrees of damage to the kidneys and liver over longer periods of time. As such, this animal model is routinely used to investigate diabetic nephropathy as well as neuropathy, while there is also evidence of impaired systolic and diastolic function due to atrial and ventricular stiffening as well as development of cataracts.

### 1.5 Rationale for the Proposed Work

While endosseous implants have a markedly high clinical success rate, the delayed healing evident in hyperglycemic conditions suggests a contraindication for clinical success rates in diabetic patients, specifically those who remain undiagnosed. Combined with the mounting global diabetes epidemic, this suggests potential for increasingly compromised success rates of endosseous implants in the future. Despite the significant body of literature addressing the effect of diabetes on bone and implants, there has been little emphasis on the early stages of healing critical for long-term success of endosseous implants. From a clinical perspective, an implant surface capable of accelerating early stages of the bone healing cascade is desirable, as it could allow for successful implant-based treatments regardless of a patient’s glycaemia. For the future, such a surface may be paramount for sustaining and improving clinical success rates.

Recently engineered nanotopographically complex implants have been shown to increase osteoconduction during early time points in a healthy population, resulting in increased de novo bone formation on the implant surface thereby improving implant stability and decreasing healing times. The success of such surface designs has received attention in recent years, yet their modulating effect on bone healing has yet to be investigated in environments of compromised healing. The aim of this project is to investigate
nanotopographically complex implant surfaces and their modulating effect on bone healing in an animal model of hyperglycemia.

1.6 Hypothesis
It is hypothesized that, although hyperglycemia is expected to delay osteoconduction on both micro- and nanotopographically complex surfaces, osteoconduction on a nanotopographically complex surface in an environment of uncontrolled hyperglycemia will be greater than on a microtopographically complex surface in normoglycemic conditions.

1.7 Objectives
There were four main objectives for the presented work:

1. To establish a reliable animal model of sustained hyperglycemia.
2. Conduct an in vivo experiment to compare a micro- and nanotopographically complex surface using an established bone healing model.
3. Re-design previous disruption test model to effectively isolate the peri-implant region and remove bias resulting from geometrical asymmetries along the bone arch.
4. Conduct mechanical testing to investigate the effects of hyperglycemia on early stages of endosseous healing.
Chapter 2.0 Materials and Methods

This study focussed on successfully and reproducibly inducing hyperglycemia into a rat population, and evaluating the performance of microtopographically and nanotopographically complex surface designs within the animal model of common systemic disease. Commercially pure titanium (grade IV) was used to manufacture custom implants, implant surfaces were treated to create micro- and nanotopographically complex surfaces, and those surfaces were then characterized. Healthy animals were injected with saline (healthy control), or streptozotocin (STZ) to create an environment of uncontrolled hyperglycemia. Implants were placed bilaterally in the distal femur of both healthy and hyperglycemic rats, and sacrificed on a timeline appropriate for investigating early healing. A new mechanical testing model was developed, based on a previous model, but with important modifications to more effectively isolate the bone in the peri-implant region. Finally, the mechanical integrity of the bone-implant interface was assessed, and block face staining and backscattered electron (BSE) imaging were conducted to assess the surrounding tissue.

2.1 Custom Implant Fabrication

Implants were manufactured from grade IV commercially pure titanium (cpTi), also known as medical grade titanium. This material has a proven history of biocompatibility and is widely used in biomedical applications. The implant design has been previously published in work from the Bone Interface Group at the University of Toronto and technical drawings can be found in Appendix C. The rectangular implant has dimensions 4mm x 2.5mm x 1.3mm (Length x Width x Height) with a hole drilled centrally down the long axis (diameter = 0.7mm) (Figure 4). All implants were custom manufactured by the Technology Development division of Biomet 3i (Palm Beach Gardens, FL, USA).
2.1.A Implant Surfaces

All implants underwent a standard grit blasting treatment to create a microtopographically complex surface. Half of the implants were then further treated with calcium phosphate nanoparticles to create a nanotopographically complex surface (DCD), as discussed below. These two implant surface designs were used as the control (grit blasted, GB) and treated (GB-DCD) groups, respectively. Grit blasting was chosen to create a consistent microtopographically complex surface which served as a baseline for both implant groups. The treated group then had a single additional variable with the superimposed nanotopography, which allowed for an independent assessment of the DCD treatment.

2.1.A.1 Grit Blasting Treatment (GB implants)

All implants were grit-blasted using a standard commercial grit blaster (Empire Abrasive Equipment, Longhorne, PA, USA). Before treating the implants, a flow rate analysis was conducted on the machine to ensure a consistent flow rate and blasting cycle for each implant. A custom apparatus was locked to the outer rim of the blast gun, with an air-tight clamp fitted with a custom sock impenetrable to the blast medium. The empty apparatus was measured before each test, placed over the blast nozzle to capture all of the media released during a single 15 second blast cycle, and then weighed again to calculate the amount of media released in a single cycle. This was repeated ten times and the average flow rate was calculated to be 1.345g/s.
Implants were fixed onto a conveyor belt using custom pressure-fitted blasting fixtures, which held the implants steady throughout the blasting process. The conveyor belt rotated the implants through a fully enclosed blasting chamber into the path of the blast nozzle. Each implant was grit-blasted uniformly with a blast time of 15 seconds per cycle using a CaP hydroxyapatite medium (particle size: 180-300 μm).

To remove embedded particles and debris from the grit blasting treatment, implants were cleaned in 18% nitric acid solution at 60°C using an ultrasonicator (Crest Ultrasonics, Trenton, NJ, USA) and then dried in an oven at 106°C for 2 hours. Visual inspection of the implants was conducted to assess for water marks resulting from improper drying, which can compromise the integrity of the implant surface.

2.1.A.2 Discrete Crystalline Deposition of Calcium Phosphate Nanocrystals (DCD Treatment) (GB-DCD implants)

For the treated group, a secondary treatment was applied following grit blasting to create a complex nanotopography on the implant surface through discrete crystalline deposition (DCD) of CaP nanoparticles. This is an additive process accomplished by suspending implants in a colloidal solution of 0.1 w/w% hydroxyapatite (calcium phosphate) nanocrystals. Following immersion in this solution, implants were immersed in three successive water baths, dried in an oven at 106°C for 2 hours, and stored in a clean room to prevent contamination.

2.1.B Packaging and Sterilization

Following surface treatment and subsequent inspection, all implants were packaged inside a clean room and sterilized using 60Co-gamma irradiation at a dosage range of 25-38 kGy, which effectively killed all surface contaminants and transmissible agents. All implants were considered sterile and appropriate for surgical implantation following this process.

2.1.C Surface Characterization

2.1.C.1 Grit Blasted Surface Analysis

A qualitative assessment was conducted for the grit-blasted implants to ensure any CaP particles and debris that may have become embedded in the implant surface during the grit blast procedure had effectively been removed during the cleaning process. For this analysis, 6 implants were randomly selected and visually analyzed using a JEOL JSM-6460LV scanning electron microscope (SEM; JEOL, Tokyo, Japan) operating at 15kV and a working distance of 10mm.

A quantitative analysis of the surface roughness was then conducted using a MicroXAM-100 3D surface profiler and optical interferometer (KLA-Tencor Corporation, Milpitas, CA, USA) to assess the 3-dimensional microtopography and surface roughness of the implants following grit-blasting. The 3D
surface profiler is a non-contact (and therefore, non-destructive) interferometric microscope used to measure variations in height along a surface with 10nm accuracy. Due to its limited resolution it is unable to assess the nanotopography applied by the DCD treatment, which requires a field emission SEM (FE-SEM). The surface profiler was operated at 312.5X magnification, which provided an analysis area of 262.2µm x 199.7µm. Using MapVue software (KLA-Tencor Corporation, Milpitas, CA, USA), a surface assessment was conducted and 3-dimensional surface maps were created for each respective surface (Figure 5). Raw data was filtered in MapVue using a Butterworth high pass filter, and then smoothed using an Inverse Fast Fourier transform (IFFT) (wavelength = 55218 nm) to remove the contribution of low frequency roughness values from the overall roughness calculations. The final surface roughness ($S_a$) value was calculated and recorded for a randomized selection of 15 implants from each of the GB and GB-DCD groups. Measurements were taken at 3 randomized locations on each side of the implant (6 measurements for each of the 30 total implants).

Figure 5: Three-dimensional surface maps were created to assess the microtopography on each implant surface. Raw data from these maps was exported and filtered to calculate the final $S_a$ values for each implant.
2.1.C.2 DCD-Treated Surface Analysis

To assess the nanotopography created by the DCD treatment, a JEOL JSM-7500F field emission scanning electron microscope (FE-SEM) (JEOL, Tokyo, Japan) was used, which can achieve an accurate resolution of 1.0nm, making it ideal for viewing the complex nanotopography of these implants. No preparation of the implants was necessary. From the DCD-treated group, 15 implants were chosen at random to assess and analyze the effectiveness of the treatment. As the nanotopography is sensitive to contamination and damage, all implants were handled with gloves and sterile tweezers. For the same reason, these implants could not be used and were discarded following analysis. All imaging was conducted using secondary electron (SE) imaging at 3kV and a working distance of 3.1mm.

Using the FE-SEM, the implant surfaces were first examined visually for voids, improper particle size and agglomerations, inconsistent distribution of nanoparticles, and environmental contaminants. Next, a quantitative analysis was conducted on 15 randomly selected implants to assess the overall coverage of CaP nanoparticles over the implant surface. One image was randomly generated at 30 000x magnification from each of the 15 implants. Each image was loaded into Scandium (Olympus Soft Imaging Solutions, Münster, Germany), which generated an automatic threshold to identify crystals from the underlying titanium. The surface coverage of the DCD crystals was then calculated as a percentage by dividing the total area of the crystals by the entire area of the image.

2.2 Animal Work

2.2.A Animal Handling and Environmental Conditions

Young male (200-250g) Wistar rats (Charles River Laboratories, Quebec, Canada) were housed at the animal facility in the Division of Comparative Medicine (DCM) (Faculty of Medicine, University of Toronto). For one week after the animals arrived there was no treatment of any kind to allow for acclimatization to the new environment. Throughout this period, animals were handled only by the author to minimize stress. During the acclimatization week, each rat was handled daily. When handling the rats, each animal was picked up with one hand around the back of the neck and the other hand underneath supporting the hind legs. The animal was held against the handler’s chest with a towel covering the head to create a dark, calming environment. Treatments began at the end of the acclimatization week. There were no restrictions on food and water during the study.

The animals were split into two groups: healthy control (C) and hyperglycemic (HG). All animals were treated and handled in a similar manner. Treatments commenced one week prior to the date of surgery. Each rat was weighed, their identification number was written on the tail using a permanent marker with a dull tip so as to not damage the tail, and the initial blood glucose was measured. For the glucose
measurement, the tail was dabbed lightly using a piece of gauss dampened with ethanol and a pinprick was made in a surface vein of the tail using a 23¼ gauge needle. The resulting drop of blood was applied to a single use FreeStyle Lite blood glucose test strip (Abbott Diabetes Care Inc., Alameda, CA, USA), which was pre-loaded into a conventional FreeStyle Lite blood glucose monitor, providing a consistent blood glucose measurement in units of mmol/L.

2.2.B Animal Injection Protocols

2.2.B.1 Control Group: Saline Injection
Animals in the control group were injected with 0.9% sterile saline (Medstore, University of Toronto, Canada) according to the protocol outlined below. Injections were dependent on the weight of the rat and a dosage chart was established for ease of practice (Appendix B).

2.2.B.2 Induction of Hyperglycemia: Streptozotocin Preparation and Injection Protocol
To induce hyperglycemia in the treated group, streptozotocin (STZ) powder (EMD Chemicals, Billerica, MA, USA) was delivered in 110mg/ml of 0.9% sterile saline according to the table in Appendix B, with injection volume once again dependent on the weight of the rat. A dosage of 65 mg/kg was used for this study, in accordance with previous work.\textsuperscript{50}

The appropriate amount of STZ was measured according to Appendix B using an analytical balance under a fume hood in a dark room, as light exposure degrades the drug.\textsuperscript{175} A separate aliquot was prepared for each animal and all vials were wrapped in aluminum foil, placed in an airtight box full of ice, and stored at -20°C until transportation to avoid exposure to environmental factors. Each aliquot was labelled in permanent marker with: animal number, amount of STZ in the vial, and amount of saline needed for the injection. Immediately prior to the injection, the STZ was mixed with the corresponding volume of saline, the rat was weighed once again to gauge the exact dosage, and the appropriate volume was injected according to the rat’s weight.

2.2.B.3 Issues when Preparing STZ Aliquots
As young rats are known to grow very quickly, each animal gained weight over the course of each day, complicating aliquot preparation. To compensate, 5g was added to the recorded morning weight measurement, as no animals were seen to gain more than 5g through the span of a single morning. Also, there was approximately 10µl of fluid remaining in the needle following an injection. To compensate, an additional 10µl of fluid was added.
For example, a rat found to weigh 372g in the morning was adjusted to 377g to compensate for weight gained through the morning. The altered weight of 377g corresponded to an injection volume of 22.7 units (227µl) on the table in Appendix B. This volume was adjusted to be 23.7 units of saline (237µl), in compensation for fluids remaining in the needle post-injection. This volume was considered the “final volume”, and the corresponding amount of STZ was then measured based on the table (25.67mg for this example). Thus, the final injection volume was 23.7U of saline for this example, which corresponded with 25.67mg of STZ. Each animal was weighed again immediately prior to injection, and the appropriate dosage was administered based on the weight at that time.

2.2.B.4 Post-Induction Measurements

Weight and blood glucose measurements were recorded 24-, 48-, and 72-hours post-injection, at the time of surgery, and at the time of sacrifice. A blood glucose level of 15mmol/L or above was considered hyperglycemic for the treated group. Hyperglycemic animals consumed significantly more water than the control group and urinated much more frequently. Cages for these animals were changed daily.

Certain rats in the HG group did not reach the desired blood glucose level after one injection. For such rats, the same injection procedure was repeated 7 days following the initial injection. This was repeated up to 3 times, after which all animals became hyperglycemic.

2.2.B.5 Injection Methodology: Intravenous Tail Injection

On the day of treatment, each animal was weighed in the morning between 9:00 and 10:00am and injections were conducted in early afternoon between 1:30 and 3:30 pm. Following the initial glucose measurement mentioned above, an intravenous (IV) tail injection was given to each rat according to the procedures outlined below. In preparation for the injection, each rat was placed under a heat lamp for approximately 20 minutes to bring the tail veins to the surface. The appropriate injection dosage was pre-loaded into a 1ml syringe with a 23¾ gauge needle attached. All air bubbles were removed.

The animal was then transferred to a plastic rat holder inside a fume hood, with a towel placed over the holder to create a dark environment. This setup immobilized the rat while providing unrestricted access to the tail (Figure 6). All injections were made into a vein in the distal tail, as this is an area of reduced sensitivity and decreases discomfort for the animal.
Figure 6: Rat immobilized inside plastic holder and covered in towel to create a dark, calming environment. The tail is accessible for IV injections.

To prepare the tail for injection, it was swabbed with a piece of gauss dampened with ethanol to remove surface contaminants. The needle was inserted into the tail vein parallel to the long axis of the tail, and confirmed to be inside the vein by observation of a flash of blood into the syringe, at which point the contents of the syringe were injected in a slow and controlled manner. Once the needle was removed, a piece of clean, dry gauss was applied to the injection site to stem bleeding, and the rat was placed back in its cage for recovery.

In the first group of STZ injections, necrosis of the tail was observed 4-7 days post-injection (Figure 7). As animals in the control group, which were injected with saline as explained earlier, did not display issues in the tail, the necrosis was thought to be caused by trace amounts of STZ remaining in the perivascular tissue post-injection. To solve this problem, the needle was kept inside the vein for 5 seconds after injecting the contents to ensure no leakage. A small amount of negative pressure was applied as the needle was removed to siphon any residual drug remaining around the injection site. This reduced necrosis in subsequent injection groups.

Figure 7: Necrosis in the rat tail due to trace amounts of STZ remaining in the perivascular tissue.
2.2.C Surgical Procedure: Bilateral Insertion of Implants into Distal Femurs

At 1 week post-injection, implants were placed bilaterally into the distal femoral metaphyses of the femora. This procedure was approved by the Ethics Committee of the Animals in Research Department at the University of Toronto. A total of 124 animals were used, half of which were hyperglycemic as described in Table 1. Each rat received both a grit blasted and a DCD-treated implant, with the side of implantation assigned by partial randomization. Rats were sedated using inhalation anesthesia administered through a nose cone: 4% isoflurane in 1L oxygen per minute for induction; 2% isoflurane in 1L nitrous oxide and 0.6L oxygen per minute for maintenance. Analgesic was administered pre- and post-operatively through a subcutaneous injection of 0.01-0.05 mg/kg buprenorphine.

To prepare the rat for surgery, each hind leg was shaved and the skin was cleaned with 10% betadine. To prevent hypothermia, a warm water circulation pad was placed underneath the anesthetized rat. An incision was made through the skin along the lateral aspect of the thigh to expose the muscle. The distal femur was exposed using blunt dissection to deflect the muscle bodies. The periosteum was removed and the femur was rotated laterally to expose the anterior aspect of the distal femur (Figure 8). Two bicortical holes were drilled 2.5mm apart along the midline of the femur using a 1.3mm dental burr attached to a dental hand piece (ImplantMED DU 900 and WS-75, W&H Dentalwerk, Austria). The holes were joined using a custom side-cutting burr (Biomet 3i, FL, USA) in a proximal-distal direction, forming the site for the implant (Figure 8). Saline irrigation was maintained by a surgical assistant through all drilling procedures in order to avoid overheating the tissue. Once finished drilling, a biodegradable suture was passed through the defect and the implant was threaded over the end of the suture and guided into the defect, where it was pressure-fitted, with the long axis of the implant oriented perpendicular to the long axis of the femur (Figure 8). A suture knot was tied on the lateral aspect of the femur to keep the implant in position during post-operative recovery. The muscle layers were then closed using biodegradable sutures and the skin was closed using 9mm wound clips.
Figure 8: After exposing the femur using blunt dissection and removing the periosteum (left), a bicortical slot was created using a 3-stage drilling procedure (middle), and the implant was press-fitted into place and supported with a biodegradable suture (right).

2.2.D Animal Sacrifice, Sample Harvesting and Preparation
Animals were euthanized by exposure to carbon dioxide (CO$_2$) gas followed by cervical dislocation. Femora were harvested (Figure 9) and stored in 30% sucrose buffer during preparation for mechanical testing. This sucrose buffer helped maintain tissue hydration at the bone-implant interface during specimen preparation and transport.

Figure 9: Femora were harvested from sacrificed animals. The rectangular implant is visible in the distal femur. Bone surrounding the implant on the proximal and distal sides was removed using a high speed dental drill to create the final test specimen.
In preparation for mechanical testing, the bone was trimmed to the width of the implant using a cylindrical diamond burr (Brasseler, GA, USA) connected to a high speed air-powered dental drill (Handpiece: KaVo Dental Corporation, IL, USA; Handpiece control: DCI International, OR, USA). All bone was removed on the side of the implants. The final test specimens consisted of two arches of bone attached to each face of the implant (Figure 10). Prior to testing, each specimen was stored in a 30% sucrose buffer solution to prevent dehydration. This solution was prepared by dissolving 3g of 99.5% Ultra Pure sucrose (BioShop Canada Inc., Burlington, ON, Canada) for every 10ml of Dulbecco’s Phosphate Buffer solution (DPBS) (Gibco, Life Technologies, Burlington, ON, Canada).

Figure 10: Final test specimen with both a medial a lateral arch on either side of the implant.
2.3 Mechanical Testing – Disruption Test

A mechanical disruption test was conducted on each specimen to investigate the strength of the bone-implant interface. The test measures the force required to disrupt the model and assesses the ability of a surface treatment to render an implant bone-bonding.\(^3\) Values are indicative of stability at the bone-implant interface as well as peri-implant bone maturity, which can be linked to the speed at which osteoconduction and de novo bone formation occur. There were two potential results for this test: the specimen did not exhibit bone-bonding and the intact arch detached from the implant surface; or the specimen exhibited bone-bonding at the bone-implant interface and the model was disrupted in the peri-implant region. This test was conducted at 5, 7, and 9 days post-surgery (Table 1).

2.3.A Previous Tensile Models

Numerous biomechanical testing models have been developed in an attempt to quantify the strength and quality of the bone-implant interface. Past models include shear models, in the form of pull-out or push-out tests\(^{15}\), torque measurements\(^{15,176,177}\), and tensile tests.\(^3,178,179\) Practical issues limit many push-out tests, as the exact alignment of the plug is difficult to ascertain, and it is difficult to establish an appropriate jig without pre-stressing the surrounding bone. As such, the tensile model has emerged as the most appropriate for such investigations.\(^3,179,180\)

2.3.B The New Tensile Model

The tensile model used in this study was adapted from a model developed in 1985, where rectangular ceramic blocks were inserted into the tibiae of rabbits.\(^{178}\) The latter focused mainly on later time points – 2 weeks and onward – which neglected the effect of their surfaces and materials on the early stages of bone healing critical to the long-term stability of an implant. The model has since been adapted in ensuing investigations, including important work that defined the ability of surface topography to render titanium surfaces bone-bonding.\(^3\) However, due to the physical design of the test, bias was introduced into previous models due to geometrical asymmetry among the test arches, indicating uneven distribution of the applied force through the test specimen. Combined with the inability to accurately quantify the cross-sectional area at the fracture site to allow for normalization of the data, this model failed to properly isolate the peri-implant region, leading to unpredictable fracture planes along the cortical arch of the test specimens. A new model has been developed for the work presented herein, which accurately isolates the region of peri-implant bone and is described below.

While some studies report mechanical testing values as the “tensile strength” of the interface\(^{180,181}\), this is impossible to accurately calculate since the true contact area of the bone is unknown and, on surfaces of topographical complexity, the fracture plane lies in the arch itself.\(^3\) In addition, there are shearing
interactions present when the healing bone interdigitates within undercuts present on the implant surface. The results of such tests can only be accurately reported as the “failure load” or the “force to disrupt the model”, which is in itself an indication of the bone-implant interface and the maturity of the surrounding bone. The work presented herein will refer to mechanical testing values as the force required to disrupt the model, in accordance with previous work in the Davies lab.

2.3.C Design of Components

A new disruption model was developed to address concerns over previous models, which failed to properly isolate the peri-implant bone. A custom breakaway mould was designed to pot each specimen, similar to more traditional orthopaedic testing models, creating a repeatable and accurate method of preparing samples for mechanical testing. Conceptualization and design of the new model was completed by the Bone Interface Group, while design revisions and manufacturing were conducted by the Technology Development division of Biomet 3i (Palm Beach Gardens, FL, USA) (Figure 11). See Appendix D for complete engineering drawings. The design allowed for 0.5mm of peri-implant bone to be isolated for testing across all groups, creating a consistent testing zone for more accurate comparison. In addition, the implant was centred and held completely horizontal during the potting process, thus ensuring uniform distribution of force through the test region, irrespective of geometric asymmetries. This design removed bias due to geometrical asymmetry between specimens and effectively isolated the peri-implant bone to assess and compare the stability of the bone-implant interface.

Figure 11: Custom breakaway mould design. Full technical drawings are found in Appendix D.
2.3. D Potting of the Specimens in Preparation for Mechanical Testing

Prior to testing, each specimen was removed from the sucrose buffer solution and gently blotted dry using a paper towel. Each specimen was positioned in the custom mould, with a pin fitting through the hole down the middle of the implant to fix the specimen in place. The bottom of the implant was 0.5mm above the top of the mould, thus exposing exactly 0.5mm of bone for the mechanical test. A lateral squaring block was fitted against the sidewall of the implant to ensure horizontal alignment and to keep the specimen centered. The base of the mould was completely filled with flowable dental composite (Filtek™ Supreme Ultra Flowable Restorative, 3M ESPE, St Paul, Minnesota, USA) and cured for 60 seconds using a Sapphire Plasma Arc high intensity curing light (Den-Mat Holdings, Santa Maria, CA, USA) (Figure 12). Normal cycle times for this curing light in clinical practise range from 5 to 30 seconds, but due to the depth of the mould and the amount of composite used for each test, the exposure time was lengthened to ensure complete curing. The mould was then broken apart by unscrewing the outer walls and the hardened specimen block was ready for testing, with a consistent peri-implant testing region and horizontal alignment of the implant (Figure 13).

Figure 12: Each specimen was fixed in the mould with a pin and a squaring block. The base of the mould was then filled with flowable dental composite and cured with a high intensity light.
2.3.E Intron Testing and Data Collection

All testing was conducted at the Mechanical Testing Lab (Dental Research Institute, University of Toronto, Canada) using an Intron 4301 operating at a speed of 30mm/minute. Before each session, a vice was centred on the middle of the Intron using a prefabricated test block replica. Each specimen was secured into the centred vice and a nylon line (Red Wolf 10lb fishing line, IA, USA) was threaded through the implant and attached to the moving crosshead (Figure 13). The lateral side was always tested first, and a thin black line was drawn in permanent marker on the lateral side during specimen preparation for identification. Testing was conducted, with the setup allowing for uniform distribution of applied force through the test region. Following testing of the lateral side, the process was repeated for the medial side. Both of the finished pots were labelled in permanent marker and, along with the tested implant, stored in 10% neutral buffered formalin solution (Sigma-Aldrich Co. LLC., Canada) for future observation.

Figure 13: Final test setup. The specimen was centered in the Intron, and a nylon line was threaded through the implant and attached to the moving crosshead.
2.3. F Visual Assessment of Implant Surfaces Following Mechanical Testing

Implant surfaces were qualitatively analyzed following mechanical testing using a Leica Wild M3Z Stereozoom dissecting microscope at 0.63 objective (Heerbrugg, Switzerland) to assess the amount of residual bone on the implant surface following mechanical testing. Images were acquired using a QImaging Micropublisher 5.0 RTV digital camera coupled with QCapture 2.90.1 acquisition software (QImaging, Surrey, BC, Canada).

The height of residual bone on the implant surface was measured using a Fowler & NSK Max-Cal electronic digital caliper (Fred V. Fowler Company, Inc., Newton, MA, USA) to assess the effectiveness of the new mechanical testing model in accurately isolating 0.5mm of the peri-implant region.

2.4 Statistical Analysis

Blood glucose values for STZ-treated animals were compared to assess the effect of multiple injections. All data after 72 hours post-injection was pooled together for each of the three injection groups: rats that required 1 injection to become hyperglycemic compared to those that required 2 injections and 3 injections. A one-way ANOVA followed by post-hoc Tukey’s HSD test for multiple comparisons was conducted to assess differences in final blood glucose levels among the injection groups.

Statistical analysis was conducted on mechanical testing data to determine significant trends. A one-way ANOVA was used to test for differences between medial and lateral test data. Post-hoc analysis using Tukey’s HSD test for multiple comparison was used to check for differences between the medial and lateral test data at p<0.05.

Data from mechanical testing was not normally distributed due to the large number of zero values, specifically at the 5 day time point, which invalidated conventional parametric statistical methods. As a result, a non-parametric Kruskal-Wallis test was conducted to assess the difference between groups. The test indicated there were significant differences across groups (p<0.0001), and a post-hoc Wilcoxon rank sums tests for multiple comparisons was conducted to assess where differences occurred within the data. To avoid inflation of type I errors in the analysis due to multiple comparisons, statistical significance was set at p<0.01, with p<0.0001 considered extremely significant.
2.5 Histology

2.5.A Resin Embedding
To prepare for block-face staining, samples were rinsed in water, dehydrated in increasing concentrations of alcohol (75%, 95%, 100% v/v) and embedded in polymethyl methacrylate (PMMA) (Osteobed, Polysciences, PA, USA) according to manufacturer’s specifications. Once the PMMA was fully cured, the specimens were trimmed parallel to the side walls of the implant to remove excess resin using an Exakt 300 CL band saw system (Exakt Technologies, Inc., Oklahoma City, OK, USA). Each specimen was ground by hand using the Exakt 400 CS micro-grinding system (Exakt Technologies, Inc., Oklahoma City, OK, USA) using 1000 and 1200 grit paper for 5 minutes at each level. This restored transparency to the resin walls through which it was possible to visually confirm the implant’s orientation. The sidewall of each specimen was mounted to a back-up slide using Technovit 7210 VLC UV-curing adhesive (Exakt Technologies, Inc., Oklahoma City, OK, USA). The opposite, unattached side was then ground parallel to the slide using 1000 grit paper for 5 minutes and mounted to another backup slide using the same Technovit 7210 VLC UV-curing adhesive. At this point, the sample was situated between two parallel slides.

Contrary to more traditional histological approaches for light microscopy, where thin sections of 100-400 microns are cut from a sample to create each slide, it was desired to cut each specimen directly in half down the long-axis of the implant and then examine the two faces of each block directly. As such, backup slides were used for mounting, as they are more robust. Once the sandwich was created, a laser sight was aligned on the band saw, to ensure an exact cutting path directly down the long axis of the implant, and the samples were cut in half. The exposed face of each half was then ground and polished to remove markings from the band saw using 1200 (2 minutes), 2000 (5 minutes), and 4000 (5 minutes) grit paper.

2.5.B Block Face Staining
To provide a qualitative analysis of maturity in the peri-implant bony region, the faces of each block were stained with 1% toluidine blue solution, placed in an oven at 60°C for 5 minutes, and then rinsed in water to remove extra stain. A cover slip was placed on the block using a drop of water, and the face was observed using a Leica Wild M3Z Stereozoom microscope at 1.0 objective (Heerbrugg, Switzerland). As in the previous section, images were acquired using a QImaging Micropublisher 5.0 RTV digital camera coupled with QCapture 2.90.1 acquisition software (QImaging, Surrey, BC, Canada). Toluidine blue is known to stain nucleic acids and polysaccharides, found in uncalcified bone, blue and purple, respectively, leaving calcified tissue unstained (white). Thus, by analyzing the amount of unstained,
calcified white bone in the peri-implant region, it was possible to make qualitative comparisons between each group.

2.6 Backscattered Electron Imaging (BSE)

The same specimens used for block-face staining at 2, 5, and 7 day time points were assessed under backscattered electron (BSE) imaging to gain a higher resolution view of the peri-implant regions in each group. Stained specimens were re-polished to remove stain from the surface before BSE imaging. As the polishing technique removes upwards of ten of microns, specimens were imaged at similar, but slightly different regions. Imaging was conducted at 25x magnification using a Hitachi SEMS-570 scanning electron microscope (SEM; Hitachi, Tokyo, Japan) operating at 20kV and a working distance of 25mm, and URSA V.2.81230 analysis software (Mektech Inc., Toronto, Ontario, Canada).

2.7 Study Design

Statistical analysis of previous work has suggested 14 rats per treatment group at each time point for the mechanical test in order to achieve the desired study power of 80%. This was calculated using repeated measurements analysis of variance (ANOVA) assuming constant correlation among observations in each rat. From previous studies, the intra-class correlation (ICC) was estimated to be 0.3 and the study was designed to detect a difference of one standard deviation or larger at the level of $\alpha=0.05$. In addition to the disruption test, 4 additional rats were sacrificed at each time point for histological analysis and BSE imaging. Time points leading up to 9 days post-op were selected to assess the effect of hyperglycemia on early stages of healing. The study was originally designed to include specimens for quantitative BSE analysis, but due to hardware issues with the required SEM, this section was excluded from the analysis presented herein.
Table 1: Project outline, showing the various time points for each respective aspect of the analysis. GB = grit-blasted surface (microtopography); GB-DCD = grit blasted surface with calcium phosphate nanoparticles (nanotopography). (*): The study was originally designed to include quantitative backscattered electron imaging (qBSE) at all 5 time points. Due to hardware failures with the required SEM, these time points were neglected for the analysis presented herein.

<table>
<thead>
<tr>
<th>Test</th>
<th>Disruption Test</th>
<th>BSE and Histology Samples</th>
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</thead>
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<td>Timeline</td>
<td>5 days</td>
</tr>
<tr>
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<td>14</td>
</tr>
<tr>
<td>Control GB-DCD</td>
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<td>14</td>
</tr>
<tr>
<td>Diabetic GB</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Diabetic GB-DCD</td>
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<td>14</td>
</tr>
<tr>
<td>TOTAL</td>
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<td>56</td>
</tr>
<tr>
<td>TOTAL SAMPLES</td>
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<td></td>
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<tr>
<td>TOTAL ANIMALS</td>
<td>124</td>
<td></td>
</tr>
</tbody>
</table>

BSE and Histology
- Only BSE
Chapter 3.0 Results

3.1 Implant Surface Analysis

3.1.A Grit Blasted Surface Analysis
To assess the microtopographically complex implant surfaces, a qualitative assessment was conducted using SEM imaging, following which observation of the grit-blasted implants confirmed all CaP particles had been effectively removed during the cleaning process (Figure 14). Quantitative analysis using a surface profiler and optical interferometer was then conducted, and surface roughness (Sₐ) values were calculated and averaged together for each group of implants (Table 2).

Table 2: Final surface roughness values for each implant group.

<table>
<thead>
<tr>
<th>Implant Group</th>
<th>Surface Roughness (Sₐ) Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grit Blast (GB)</td>
<td>1.89 µm</td>
</tr>
<tr>
<td>Grit Blast with DCD (GB-DCD)</td>
<td>1.86 µm</td>
</tr>
</tbody>
</table>

3.1.B DCD-Treated Surface Analysis
Field emission scanning electron microscopy (FE-SEM) was necessary to assess the nanotopography on the DCD-treated group. Images were taken at 25X, 100X, 1000X, 10 000X, and 30 000X to provide an objective assessment of the surfaces from multiple perspectives (Figure 15). These SEM observations confirmed an absence of debris and agglomerations, indicating a consistent distribution of particles and a surface free of environmental contaminants. The superimposed nanotopography was clearly visible on the DCD-treated implants at the higher magnifications (Figure 16).

An analysis of 15 randomly selected implants was then conducted at 30,000x magnification to quantify the overall coverage of nanoparticles along the surface (Figure 15). The average surface coverage was 63±1.5% (mean±SD).
Figure 14: SEM images of implants following grit-blasting. It is evident at the lowest magnification that all CaP blast particles were removed from the implant surface during the cleaning stage. As the magnification increases, the undercuts and surface roughness become more evident. Magnification (L to R): 30x, 100x, 300x 500, 1000x, 2000x.
Figure 15: FE-SEM images of the nanotopographically complex implants. The CaP nanocrystals can clearly be seen as the magnification increases. Bottom right: A single image at 30 000x was used to quantify overall coverage of CaP nanoparticles along the surface. Magnification (L to R): 100x, 1000x, 5000x, 10 000x, 30 000x, 30 000x.
Figure 16: SEM image of a GB implant (left) compared to a DCD-treated implant (right), clearly showing the distribution of CaP nanoparticles across the surface of the GB-DCD implant. Magnification: 50 000x.

3.2 Induction of Hyperglycemia

Blood glucose levels and weight of each rat were measured on the day of induction, 24-, 48- and 72-hours post-injection, on the day of surgery and at sacrifice. This provided confirmation of successful induction of experimental hyperglycemia, and allowed for the continued monitoring of the health of the animals.

3.2.A Blood Glucose Measurements

Experimental hyperglycemia was successfully induced in the treated group, as can be seen in Figure 17. At 48 hours post-injection, blood glucose readings in the STZ-treated HG group were 19.75±4.31mmol/L (mean±SD), with all of the rats above the minimum cut-off of 15mmol/L. At the same time point, blood glucose levels in the Control group were 5.73±0.98mmol/L (mean±SD), which was not significantly different than the baseline level of 5.31±0.75mmol/L (mean±SD) at the time of induction, confirming an absence of effects from the saline injection. At the time of induction, there was no significant difference between blood glucose levels in the two animal groups, indicating a comparable healthy baseline for all rats.
Figure 17: A comparison of blood glucose measurements for the two animal groups (n=62 for each group). All measurements in the HG group were above 15mmol/L at 48 hours post-injection, thus confirming the successful induction of experimental hyperglycemia. There was no significant change in the healthy Control group, confirming the absence of any effects from the saline injections.

3.2.A.1 The Effect of Multiple Inductions

Several rats in the HG group required more than one STZ injection for successful induction, thus it was necessary to determine whether there was a correlation between the number of injections and the final blood glucose levels. All animals that were not hyperglycemic (ie. blood glucose not greater than 15mmol/L) at the 48-hour cut-off point were re-injected the following week on the same schedule, with this being repeated a maximum of three times per rat. Statistical analysis of blood glucose values for each of the three injection groups (rats that required 1 injection to become hyperglycemic compared to those that required 2 and 3 injections) indicated no significant difference between groups at p<0.05. As such, blood glucose data from each of the groups was combined at each respective time point. All rats used in the HG group for this study had a blood glucose level above 15mmol/L, and there were no significant differences in the post-48 hour data between rats with a single injection and those with multiple injections.
3.2.B Weight Measurements

As expected, the Control group gained weight at a greater rate than the HG group immediately following the STZ injection (Figure 18). Using an unpaired two-tailed t-test, there was no significant difference seen at the time of induction (p<0.05), indicating a comparable initial healthy weight for all rats. From 24-hours post-injection onward, the control group animals were significantly heavier at each time point than the HG group.

Figure 18: The Control group is seen to gain weight at a significantly higher rate than the hyperglycemic group over time (n=62 for each data point).
3.3 Mechanical Testing Results

3.3.A Overall Results

Femora were extracted from each animal and prepared for mechanical testing, conducted using an Instron machine at an extraction rate of 30mm/min as described in Section 2.3.E. Each group had 14 femurs, with a medial and lateral component on every femur. Statistical analysis indicated no significant difference at p<0.05 between medial and lateral data comparing at each time point, between surfaces, and with all of the data combined. Thus, medial and lateral data was pooled together to allow for 28 data points per mechanical testing group at each time point. A zero value was assigned to specimens that fell off the implant during preparation. From 336 total overall test arches, there were 71 zero values, with 50 at the 5 day time point (Table 3). Raw data is plotted in Figure 19. Due to non-normality of the mechanical testing data – a result of the high number of zero values at the early time points – a non-parametric Kruskal-Wallis test was conducted to assess the difference between groups, as explained in Section 2.4.

Table 3: Distribution of zero values for mechanical testing. A zero value was assigned to any arch of bone that fell off the implant surface during preparation, thus failing to make it to mechanical testing.

<table>
<thead>
<tr>
<th>Metabolic Group</th>
<th>GB</th>
<th>GB-DCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 5d</td>
<td>24</td>
<td>6</td>
</tr>
<tr>
<td>HG 5d</td>
<td>19</td>
<td>1</td>
</tr>
<tr>
<td>Control 7d</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>HG 7d</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Control 9d</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>HG 9d</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>61</td>
<td>10</td>
</tr>
</tbody>
</table>
Figure 19: Raw disruption test values (mean±SD). (*)=p<0.01; (**)=p<0.0001.

Overall, disruption force values were significantly greater in the GB-DCD surface compared to the GB surface at 5 days (Control p<0.0001; HG p<0.0001), 7 days (Control p<0.0001; HG p<0.0001) and 9 days (Control p<0.0001; HG p<0.0001). As well, comparing the GB-DCD surface at successive time points in both metabolic groups, there was a significant increase in disruption force values from 5 days to 7 days (Control p<0.0001; HG p<0.0001) and 7 days to 9 days (Control p=0.002; HG p=0.0004). There was an increase with the GB surface from 5 days to 7 days (Control p<0.0001; HG p=0.0003) but no significant change between 7 days and 9 days.

Comparing within each surface treatment, there was a significantly greater disruption force in the Control group than the HG group with the GB-DCD surface at 7 days (p<0.0001) and 9 days (p=0.0042), while no difference was present at 5 days. Interestingly, there was no significant difference between the Control GB-DCD at 7 days and the HG GB-DCD at 9 days. There were no differences seen between metabolic groups with the GB surface at any time point.
Figure 20: Comparing both surfaces overall showed the GB-DCD was more likely to produce mechanical testing results above the overall median than the GB surface, irrespective of time or animal group, thus demonstrating the superior performance of the GB-DCD surface.

Importantly, the GB-DCD surface in the HG group performed significantly better than the GB surface in Control animals at 5 days (p<0.0001), 7 days (p<0.0001) and 9 days (p<0.0001). To further analyze the performance of each surface, all mechanical testing data from both metabolic groups and all time points was combined, the absolute median from all mechanical testing data was found (3.418 N), and the percentage of values falling above and below the absolute median was calculated for each surface (Figure 20). This showed the likelihood of a surface to produce a disruption force value above the median, which would indicate superior performance, or below the median, indicating inferior performance. There was a significantly greater chance of producing mechanical testing values above the median when using the GB-DCD surface, regardless of time or metabolic group.
There was a significant increase in the disruption force values with the DCD-treated surface for both metabolic groups at 5 days (Figure 21), which was not expected. Compared to the GB surface, there was a considerable improvement of 1384% and 799% in the control and HG groups, respectively, after only 5 days of healing (Figure 22).
To further assess the performance of each surface, mechanical testing data was distributed into quartiles. All data points were pooled together and regions were calculated as 1st quartile (Q1=0-0.08N), 2nd quartile (Q2=0.08-3.4N), 3rd quartile (Q3=3.4-8.6N), and 4th quartile (Q4=8.6+N) distributions. Each group of data consisted of 28 measurements, and the number of data points in each distribution was counted for each group and expressed as a percentage of the total group (Figure 23). The percentage of Q4 data points increased steeply with time for the GB-DCD groups in both metabolic groups, while there was almost no Q4 data for any of the GB groups. While the Control GB-DCD group at 7 days showed considerably more Q4 data than the HG GB-DCD group, the two groups were very similar at 9 days. As well, the distribution of data for the HG GB-DCD group at 9 days was comparable to that seen in the Control GB-DCD group at 7 days. Both of these trends were similar to the overall trends seen in Figure 19. As expected, the Q1 data declined steeply with time.

![Quartile Distribution of Tensile Data](image)

*Figure 23: Distribution of categorical force data into quartiles. As time passed, the percentage of 4th quartile data for the Control and HG GB-DCD groups increased, indicating a higher force to disrupt the model. In addition, while the Control GB-DCD group at 7 days had a significantly larger percentage of 4th quartile data, the HG GB-DCD group was comparable to the control GB-DCD by 9 days. There was almost no 4th quartile data for the GB groups at any time point.*
3.4 Visual Assessment of Implant Surfaces

Following mechanical testing, each implant surface was observed using a dissecting microscope to assess residual bone on the implant surface. Representative images from each group are found in Figure 24. There was a clear variance among specimens, as could be expected from the large standard deviations in mechanical testing data (Figure 19). Comparing the GB and GB-DCD surfaces at each time point, there was substantially more bone remaining on the GB-DCD surfaces at all time points, confirming trends seen in the mechanical testing data (Figure 19). However, more important than the overall surface coverage of residual bone was the distance from the implant surface at which the bone fractured. Residual bone on the GB-DCD surfaces fractured further from the implant surface, leaving visibly larger and thicker sections of residual bone. In addition, there was a clear increase in residual bone at each time point. Comparing across animal groups, there was more bone on the Control GB-DCD surfaces than the HG GB-DCD surfaces at 5 and 7 days, but it was difficult to discern a visual difference at 9 days, analogous to the visual trend seen in quartile distribution of the mechanical testing data (Figure 23). The residual bone on the GB-DCD surface was considerably greater than the GB surface at 5 days, which was unexpected and matched the trend seen in Figure 22. Comparing the GB surfaces at each time point, there was a much smaller amount of residual bone, both in terms of surface coverage and depth of residual bone, than that remaining on the GB-DCD surfaces. There was little visual difference among the GB surface images comparing both across metabolic groups and across time points.

3.4.A Residual Bone Height

The height of the residual bone from the implant surface was measured using a digital caliper, as discussed in Section 2.3.F. From 336 total mechanical tests, 309 specimens, representing 92% of all test specimens, had residual bone within the height designed by the new potting arrangement. Of the 27 remaining specimens with residual bone heights greater than 0.5mm, 20 came from the lateral section of test specimens (Table 3). From the 10 highest mechanical testing values, only 4 of these specimens had residual bone greater than 0.5mm.
Table 4: Distribution of test specimens with residual bone at a height less than 0.5mm. 92% of all test specimens fractured in the targeted peri-implant region.

<table>
<thead>
<tr>
<th>Metabolic Group</th>
<th>Surface</th>
<th>Number of Specimens with Residual Bone &lt;0.5mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 5d</td>
<td>GB</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>GB-DCD</td>
<td>24</td>
</tr>
<tr>
<td>HG 5d</td>
<td>GB</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>GB-DCD</td>
<td>26</td>
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<tr>
<td>Control 7d</td>
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<td>28</td>
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<tr>
<td></td>
<td>GB-DCD</td>
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</tr>
<tr>
<td>HG 7d</td>
<td>GB</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>GB-DCD</td>
<td>22</td>
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<tr>
<td>Control 9d</td>
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<td>28</td>
</tr>
<tr>
<td></td>
<td>GB-DCD</td>
<td>24</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td>309</td>
</tr>
</tbody>
</table>
Figure 24: Representative images showing residual bone on implant surfaces following mechanical testing. As time passed in both metabolic groups, peri-implant bone on the DCD surfaces was seen to break further from the surface of the implant, indicating a progressive increase in bone maturity surrounding DCD-treated surfaces. There was no visually evident trend with the GB surface.
3.5 Histological Assessment

3.5.A Block Face Staining

Animals were sacrificed at 2, 5, and 7 days for histological staining. Samples were collected and processed in resin and polished, and block faces were stained using 1% toluidine blue as described in Section 2.5.B. By assessing the amount of white tissue – indicative of calcified bone – in the peri-implant region, it was possible to qualitatively assess the amount and quality of calcified peri-implant bone. Representative images of each group are found in Figure 25. It should be noted that observations from this technique were restricted due to both the limited resolution of images and the variance seen among groups.

As expected, there was very little calcification of tissues in the peri-implant region at 2 days. Any bone present in the peri-implant region at this time point was very likely original trabecular bone or bony debris from the surgical drilling procedure. At 5 days, it was difficult to evaluate the amount of calcified bone in the peri-implant region due to the low resolution of this observational technique; however the trabeculae present in the Control groups appeared thicker and more developed than in the HG group. By 7 days, there was significantly more calcified tissue in the peri-implant region of specimens with the GB-DCD surface than the GB surface in both metabolic groups. In addition, trabeculae in the Control groups were noticeably more developed than the HG groups at 7 days.
Figure 25: Histological images from block face staining of 2, 5, and 7 day specimens. There was a noticeable increase in calcified peri-implant bone at each successive time point, as evidenced by the increase in white tissue along the implant surface. There was also more calcified bone in the peri-implant region of GB-DCD surfaces than GB surfaces.
3.6 Backscattered Electron (BSE) Imaging

3.6.1 Qualitative BSE Analysis

The same specimens used for block-face staining at 2, 5, and 7 day time points, were assessed under backscattered electron imaging (BSEI) to gain a higher resolution view of the peri-implant regions in each group, as discussed in Section 2.6. The study was originally designed to accommodate quantitative backscattered electron imaging (qBSE) as a means to quantify the mineral density of bone surrounding the implant, however, due to technical issues with the requisite microscope, qualitative BSEI was conducted instead on a different machine. Representative images of each group are found in Figure 26, with all imaging conducted at 25X magnification and the lateral side of the specimen oriented to the right. There was a considerable amount of variance seen in the BSE images, just as there was at previous stages of the investigation.

Implants were seen by BSEI to favour the lateral side of the defect, leaving a larger gap on the medial side in most cases. This was due to the surgical protocol, in which implants were pressure-fitted into the prepared defect in the distal femur and a suture was used to fix the implant to the lateral side of the defect to stabilize it during the initial healing stages. The same trend was noted with residual bone, as there were more lateral test specimens with residual bone greater than 0.5mm following mechanical testing. As noted earlier, there was no difference in mechanical testing results between the medial and lateral components, and, therefore, this visual effect had no discernible impact on the disruption test.

As expected, there was little calcified tissue in the peri-implant region at 2 days using BSEI, confirming observations from the stained sections. Any peri-implant bone visible at this time point was likely due to leftover bony debris from the surgical procedure. For both metabolic groups at 5 and 7 days, there was noticeably more calcified bone in the peri-implant region of the GB-DCD surface than the GB surface. As well, it was clear using BSEI that trabeculae in the Control animals were much thicker and denser than those in the HG group, confirming peri-implant bone in healthy animals to have increased maturity compared to that seen in a hyperglycemic animal, as reported in previous sections.
<table>
<thead>
<tr>
<th></th>
<th>2 Days</th>
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<th>7 Days</th>
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</thead>
<tbody>
<tr>
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<td><img src="GB.png" alt="Image" /></td>
<td><img src="GB.png" alt="Image" /></td>
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<tr>
<td>Hyperglycemic</td>
<td><img src="GB-DCD.png" alt="Image" /></td>
<td><img src="GB-DCD.png" alt="Image" /></td>
<td><img src="GB-DCD.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure 26: BSE images of specimens from 2, 5, and 7 days. All images are oriented with the lateral side, to which the implant was sutured during surgery, to the right. Magnification: 25X (scale bar = 2mm in all images).
3.6.B Comparing Block Face Staining and BSE

Backscattered electron imaging provided a much more detailed view of the peri-implant region, allowing for increased magnification of the specimens and more effective isolation of the calcified tissue due to thresholding of greyscale levels during image processing. Side-by-side comparison of stained specimens with BSEI specimens at certain time points clearly demonstrated trabeculae in the control groups to be much thicker and more developed than those in hyperglycemic animals (Figure 27). It was difficult to determine a visual difference in the quantity of peri-implant bone between the two metabolic groups at 7 days with either imaging technique.

![Figure 27: Specimens used for toluidine blue staining were cleaned and polished, and viewed using BSE to attain a higher resolution image of the peri-implant region. In side-by-side comparisons, it was clear that trabeculae in the control groups (right) were much thicker and more developed than those in hyperglycemic animals (left) along the GB-DCD surface.](image)
Chapter 4.0 Discussion

4.1 Hyperglycemia causes a delay in the peri-implant healing response

The Davies lab has focused on elucidating the mechanisms involved in bone healing for almost two decades, with a specific emphasis on the early phases of healing responsible for osteoconduction and de novo bone formation. Through the successful establishment of a simple animal model of common systemic disease, which allowed for a controlled and reproducible onset of hyperglycemia, the use of a standard drill-defect bone healing model, and the development of a new model for mechanical testing, it was possible to investigate the effect of untreated systemic disease on bone healing mechanisms while setting a standard for comparison in future work. The presence of uncontrolled hyperglycemia was shown to affect the early stages of bone healing corresponding with osteoconduction, confirming previous work in the Davies lab and elsewhere. The delayed healing is a crucial observation in the analysis of diabetic pathologies as it is relevant to implant-based rehabilitation strategies in both dental and orthopaedic applications.

Our results showed a significantly lower disruption force in the hyperglycemic GB-DCD group compared to the healthy control GB-DCD group at both 7 and 9 days (Figure 19). While no significance was detected at 5 days, this could be due to the magnitude of the difference being smaller than the standard deviations in the data, which were large at all time points. A previous report from a removal torque test also describes a wide variance in mechanical testing values of topographically complex implants compared to smooth implants. The same trend was not observed in the GB surface, as there was no significant difference at any time point between metabolic groups, potentially for the same reason. Regardless, as the nano-surface performed considerably better than the micro-surface, differences in healing capacity between the two metabolic groups were amplified as a result, exacerbating the delayed healing in hyperglycemic animals.

The effects of hyperglycemia on cell function are notable, leading to impaired formation, function and secretory ability of osteoblasts. This is supported by decreased levels of serum osteocalcin and alkaline phosphatase in both humans and animals, which are used as markers for bioactivity of osteoblasts. Furthermore, it has been reported that insulin control resulted in increased peri-implant bone formation and BIC compared to uncontrolled diabetic animals, suggesting that, in the absence of glycemic control, hyperglycemia compromises the quantity and quality of bone surrounding an implant. Although BIC was still reduced compared to non-diabetic control animals in this study, it is likely that hyperglycemia, or at least elevated glycaemia, was still present at the time of surgery, suggesting the presence of elevated glucose levels impacted the early stages of healing, thus leading to compromised...
osteoconduction and, as a result, reduced BIC. Our results confirm these observations, with BSE images showing a decreased amount of peri-implant bone in hyperglycemic animals. In addition, thinner trabeculae were present, especially at early time points. This has been reported in previous work, which showed inferior development and lower trabecular numbers in hyperglycemic animals, likely as a result of compromised cellular function and mineralization, another notable effect of hyperglycemia which has been reported previously.

Uncontrolled hyperglycemia has been shown to promote increased and prolonged inflammation of peri-implant tissue in diabetic subjects, which adversely affects the differentiation of MSC populations. Additionally, a delay in the formation of the fracture callus and subsequent bone resorption during early healing in a bone defect model has been described. This matches literature reports of slower bone healing in diabetic models, primarily in the early phase of healing as demonstrated by delayed bone formation, remodeling and mineralization. Compromised mineralization in early stages of bone formation was observed in the healing of large bone defects created in uncontrolled diabetic animal models, resulting in compromised apposition, formation, and timing of mineralization, none of which presented in diabetic animals with sufficient glycemic control. As the disruption force values in our data were significantly lower in hyperglycemic animals, while trabeculae appeared to be visually less developed, it is likely that hyperglycemia compromised mineralization of the peri-implant bone, leading to a reduction in mechanical properties and decreased quality of the bone.

While hyperglycemia impacts cellular function to a significant degree, arguably more important is its effect on the healing compartment immediately following placement. There is evidence that hyperglycemia causes compromised capillary function due to injured perivascular cells, which would affect the number and quality of MSCs, and thus osteogenic cells, available for recruitment to the implant surface. Platelets are hyperactive in diabetic patients, becoming stimulated more easily, likely as a combination of changes to the surrounding environment as well as the platelets themselves. Furthermore, there is evidence of compromised clotting in diabetic patients, as evidenced by decreased clot permeability, likely resulting in decreased migration of cells through the clot, as well as decreased lysis time, which suggests compromised fibrin attachment. These effects would severely impact osteoconduction, leading to reduced BIC and decreased implant stability at early time points.

Previous reports have pointed to delayed fracture healing and reduced BIC, among the effects mentioned above, yet it has been established that peri-implant bone formation is highly dependent on the early stages of healing. Therefore, it is logical that, in a diabetic patient with poorly controlled glycaemia, compromised physical properties of peri-implant bone are in fact repercussions from delays in the early healing process.
stages of bone formation, rather than long-term pathologies independent of early healing. While clinicians conduct a thorough medical history with each patient at the outset of implant-based treatments, this will be for naught if the patient themselves is unaware of their hyperglycemic condition. This investigation confirms hyperglycemia to have a significantly negative impact on peri-implant bone healing, leading to decreased contact osteogenesis and, therefore, reduced bone quality. Our work supports the growing trend in literature suggesting uncontrolled hyperglycemia to be a contraindication for the long-term success of implants.

4.2 Contact osteogenesis is greater on nanotopographically complex surfaces, even in an environment of uncontrolled hyperglycemia, than on microtopographically complex surfaces in a normoglycemic environment

Contact osteogenesis is the term used to describe two separate, measurable phenomena. The first component is osteoconduction, known as the recruitment and migration of osteogenic cells to the implant surface.\(^1\) As no bone is present on the implant surface upon insertion, it is logical that osteogenic cells must first arrive to the surface before bone matrix may be laid down. The second constituent is \textit{de novo} bone formation, where recruited osteoblasts deposit a collagen-free matrix directly onto the implant surface, establishing a bone-bonding interface.\(^{29}\) Together, these phenomena have been elucidated as the early stages of peri-implant bone healing crucial to the long-term success of an implant.\(^{29}\)

Our results show that the addition of nanotopographically complex surface features, with undercuts, to an already microtopographically complex surface can result in bone-bonding in both healthy and hyperglycemic animals, as shown by the abundance of residual bone on nano-surfaces following mechanical testing. These findings confirm our previous work, which has shown deposition of calcium phosphate nanoparticles on an underlying microtopography to render an implant bone-bonding.\(^3\) Such surfaces, in addition to providing increased surface topography for fibrin attachment leading to enhanced clot retention, are known to increase platelet activation, which strengthens the chemotactic pathways used to recruit cells to the healing compartment.\(^{30}\) Of particular importance is the increased release of PDGF and TGF-b from activated platelets, which are recognized chemotactic signalling factors for osteogenic cells.\(^{31}\) The increased presence of these two factors, among others, greatly strengthens the recruitment of osteogenic cells to the implant surface, thus increasing osteoconduction. Furthermore, it has been shown that coating biomaterials with a calcium phosphate layer allows for increased protein adsorption to the surface – a factor that also contributes to increased fibrinogen binding and further increases platelet activation.\(^{29}\) Therefore, through increased protein adsorption and platelet activation, nano-surfaces create
a stronger gradient of chemotactic factors, leading to further activation of platelets, increased fibrin attachment to the implant surface, and, ultimately, a significant increase in osteoconduction.

The presence of CaP nanocrystals on the surface of a biomaterial promotes adsorption of non-collagenous bone proteins, which, in addition to affecting the recruitment of cells as described above, creates a collagen-free organic matrix with nucleation sites for calcium phosphate mineralization.29 This de novo bone deposited on the implant surface becomes interdigitated with the complex nanofeatures, creating a collagen-free cement line which then becomes interwoven with the collagenous matrix of new bone.1 In essence, the de novo bone represents the collagen-free transition zone between the implant surface and the collagen compartment of newly formed bone. Together, osteoconduction and de novo bone formation, collectively known as contact osteogenesis, are responsible for the bone-bonding phenomenon.

Our results showed a significantly greater performance in mechanical testing, regardless of time or metabolic status of the animal, when using the nano-surface. Mechanical testing data, especially that from tensile testing, which is viewed as the most accurate method to assess the bone-implant interface,179,180 provides an indication of stability at the bone-implant interface and the overall bone-bonding ability of an implant surface.178 An increase in mechanical testing data is correlated to the speed at which osteoconduction and de novo bone formation occur at the surface.3 Nowhere was this more evident than at 5 days, which showed a substantial difference between mechanical testing data of nano- and micro-surfaces in both metabolic groups (Figure 22). As implants differed only in their nanotopography, it could only be through the increased osteoconduction and acceleration of the early stages of the bone healing cascade, and the subsequent increase in de novo bone formation, that led to superior performance of the nano-surface. The existence of a difference at such an early time point proves there must be accelerated bone formation on the DCD surface since, without bone formation, mechanical testing values would be zero.

Implant surfaces used in this study were specifically chosen to address the effect of sub-micron surface topography with undercuts on the early stages of the bone healing cascade. Both surfaces had the same underlying microtopography, a grit-blasted treatment known to be osteoconductive but not bone-bonding,21 with the DCD-treated group having an additive nanotopography superimposed onto the surface. Thus, all observable changes could only be attributed to the additive submicron topography with undercuts, providing further proof for mechanical interdigitation of de novo bone as the driving force behind the bone-bonding phenomena.29

It is important to understand that the DCD surface was not able to overcome the negative effects of hyperglycemia; rather, the effects of hyperglycemia on healing were notable, as described above, and the
control group outperformed the hyperglycemic group. Despite this, disruption force values of the nano-surface were far superior to that of the micro-surface, with a greater performance even in the presence of hyperglycemia than that of the micro-surface in an environment of normoglycemia. Recent evidence points to improved cell adhesion onto the nanotopographical features of implant surfaces, leading to increased proliferation efficiency of MSC populations,\textsuperscript{57} as well as enhanced osteoblast adhesion and increased protein interaction.\textsuperscript{59} Additionally, the nano-surface used in this study promotes angiogenesis,\textsuperscript{29} which leads to further expression of VEGF in the healing compartment, and an increased population of perivascular cells in the area. While the effect of hyperglycemia on the migration and differentiation capacity of perivascular cells is notable, as discussed above, it is possible that such effects from the nano-surface nullify, or at least reduce, the compromised migration and cellular function in hyperglycemic subjects. This would contribute further to the increased performance of nano-surfaces in a hyperglycemic environment, although this has not been investigated to the author’s knowledge. Regardless, such evidence demonstrates increased proliferation and activity of osteogenic cells, providing further support for increased contact osteogenesis along nanotopographically complex surfaces.

The acceleration of contact osteogenesis on an implant surface is of significant clinical interest, as it necessarily leads to an increased BIC and greater stability at early time points, thus decreasing recovery time and increasing stability.\textsuperscript{29} The results of this study provide support for the implementation of nanotopographically complex implants in environments of compromised healing, as, while the nano-surface was not seen to truly overcome the effects of hyperglycemia, its performance was considerably better in an environment of uncontrolled hyperglycemia than that of the microtopographically complex surface in a healthy animal. The continued development of an implant surface capable of creating a bone-bonding interface, regardless of a patient’s metabolic state, is an invaluable clinical tool that holds promise to become the status quo as implant-based therapies continue to evolve.
4.3 Peri-implant bone matures more rapidly on nanotopographically complex surfaces, even in an environment of uncontrolled hyperglycemia, compared to a microtopographically complex surface in a normoglycemic environment

Residual bone on the GB-DCD surfaces fractured further from the implant surface during mechanical testing, leaving visibly larger and thicker sections of residual bone compared to the micro-surface – a strong indication of increased bone maturity in the peri-implant region. Furthermore, there was a clear increase in residual bone at each time point on the DCD surface, both in terms of surface coverage and depth of the bone, showing a progressively increasing maturity in peri-implant bone surrounding nano-surfaces over time. To the author’s knowledge, this is the first discussion of fracture planes and residual bone patterns following tensile testing of the bone-implant interface.

The fracture plane has been described previously following removal torque (RMT) testing in machined and laser-etched surfaces,\(^{16}\) during which bone was seen to fracture consistently at the bone-implant interface of smooth surfaces and deeper into the mineralized bone with a surface of increased complexity. It was further reported in the same study that the micro-surface was associated with significantly greater amounts of mineralized bone in comparison to the smooth implant surface. Recent work describes nanomechanical properties of bone surrounding implants of micro- (sand-blasted and acid-etched) and nanotopography (sand-blasted and acid-etched with an additive nano-scale hydroxyapatite), in which bone in the threaded region of the implant was subjected to nanoindentation, showing a significant increase in the elastic modulus and rank hardness of bone surrounding the nanotopographically complex surface.\(^{186}\) As the modulus of elasticity and mineral content of bone have a positive relationship,\(^{187}\) these results indicate increased mineralization in the peri-implant region of nanotopographically complex surfaces – a conclusion supported by the enhanced osteogenic markers, specifically alkaline phosphatase and osteocalcin, seen with the same nano-surface.\(^{188}\) Such observations confirm the effectiveness of topographically complex surfaces in increasing osteoconduction and accelerating bone formation at early time points, as discussed above, while also demonstrating that implant surfaces of increasing complexity lead to elevated mineralization levels and, thus, maturity, of peri-implant bone.

It was not possible to calculate the absolute tensile strength of the peri-implant bone, as the true contact area at the bone-implant interface and the exact cross-sectional area of the fracture plane were not known. However, during mechanical testing, fracture planes tend to occur through regions of weakness. Thus, as developing bone is structurally weaker than mature bone due to its reduced mineral content,\(^{189}\) it was initially expected that fracture planes would occur in regions of newly formed peri-implant bone. This
was seen with the micro-surface, yet not the nano-surface, which had fracture planes occurring through bone away from the implant at all time points. Such a pattern indicates that, not only did peri-implant bone mature significantly faster than that of the micro-surface, it also healed to become at least as strong as the surrounding bone. As a Baud curve was observed along implant walls, it is likely that the cross-sectional area of the fracture planes with the nano-surface, which were observed away from the implant surface, were smaller than that of the newly formed bone in the peri-implant region. Therefore, since a material will break at the weakest point, and a material with similar mechanical properties will break at the smallest cross-sectional area during loading, this demonstrates peri-implant bone along the nano-surface had strength, and therefore, maturity, comparable to that of the surrounding bone. This study was initially designed to incorporate quantitative BSE (qBSE) imaging, which permits analysis of greyscale levels – directly related to mineral content of a tissue – using a precise image calibration protocol, and allows for accurate comparison of mineralization, and thus bone maturity, between groups. However, due to hardware issues with the required SEM, qBSE imaging was not possible, preventing a quantitative analysis of peri-implant bone maturity.

As mentioned in the previous subsection, nanotopography has a distinct cellular effect, leading to increased proliferation of MSC populations\(^57\) and increased adhesion of osteoblasts to the implant surface.\(^59\) In addition, reports of increased osteocalcin and alkaline phosphatase\(^188\) are indicative of increased osteoblastic function in response to a nanotopographically complex surface. It logically follows that, in response to augmented bone deposition and increased activation of osteogenic cells, mineralization occurs at a faster rate in the peri-implant bone of nano-surfaces, thus leading to increased bone maturity at early time points, as seen in our results. While hyperglycemia is reported to compromise MAR\(^50\) and affect late stages of osteoblast differentiation\(^116\) leading to immature mesenchymal tissue,\(^101\) it is possible that the impact of nanotopographically complex features on cellular function is able, at least in part, to abrogate these effects and increase mineralization in the peri-implant region. Furthermore, it is possible that such surfaces, through increased release of growth factors as mentioned above, may help osteogenic cells to differentiate fully despite the hyperglycemic environment, leading to increased mineralization. Indeed, our observations from BSE images, which show increased mineralization along nano-surfaces even in the presence of hyperglycemia, suggest this may be possible, although additional experiments, including qBSE to accurately quantify mineral content, would be required to test this assumption.

Interestingly, while performance expectations were low for early time points for the micro-surface, it was unexpected to see a sustainably poor performance at ensuing time points, with only a small change in mechanical testing data, and very little visual change in residual bone. Visual analysis of BSE images
shows an increase in peri-implant calcified tissue, confirming the grit-blasted surface to be osteoconductive, while the lack of residual bone and poor mechanical testing demonstrates its inability to create a bone-bonding interface.

Osteoconductive surfaces, namely acid-etched and grit-blasted surfaces, result in bone-implant contact, and can provide limited early implant stability, especially in shear tests, yet fail in this model where the applied load is perpendicular to the implant surface. The effectiveness of such surfaces in providing implant stability has been known for some time, yet much of the literature has emphasised surface roughness values, a singular limited parameter measured with technologies that cannot accurately analyze the topographical features and, in many cases, can’t even discern the submicron topography found on modern surface treatments, while failing to elucidate the true biological relevance of such surface topographies. Recently, the biological relevance of varying grades of topographical complexity has been demonstrated, with the establishment of two crucial terms to differentiate between apparent effects in peri-implant healing: the “true” interface and the “functional” interface. Indeed, it is known that submicron topography, when applied to a smooth implant surface, can render an implant bone-bonding, despite not being able to withstand high forces. This observation forms the basis for the “true” interface, which is the mechanical interlocking of the collagen-free cement line with the submicron topography and is precisely what is seen at naturally remodeling bone sites, where the cement line interdigitates with the complex nano-features of exposed collagen in topographically complex osteoclast resorption pits. The “true” interface follows the exact contour of the implant, essentially representing the border between implant material and peri-implant bone and depends on the availability of undercuts, without which the bone-bonding mechanism fails. The “functional” interface is then provided by the high-order micron and coarse-micron topographies, found in natural remodeling sites as individual osteoclast resorption lacunae and osteoclast resorption tracts, respectively. This interface may extend over several orders of magnitude, as is common at remodeling sites where osteoclast resorption pits can extend for several hundred microns. Thus, the bone bonding mechanism will occur due to interdigitation of the cement line with submicron features, forming the “true” interface, and stability is enhanced by features in the micron scale-range, which form the “functional” interface and correspond to the dimensions of individual resorption lacunae.

Results from this study corroborate the roles of different scale ranges of surface topographies on the stability of the bone-implant interface. The submicron topography created by the DCD treatment allows for interdigitation of the cement line, while the micron-scale topography created by grit blasting adds additional stability to the implant over time. Furthermore, it shows that, in the absence of submicron topography, the true interface, while still present, is not interdigitated with the implant surface, rendering
the implant significantly less effective in withstanding loads perpendicular to the implant surface, as evidenced by the poor mechanical testing performance of grit-blasted implants. In addition, our results indicate that a surface with topography in the same scale-range as that seen during normal remodeling has the ability to stabilize an implant more effectively, even in an environment of uncontrolled hyperglycemia, leading to increased peri-implant bone maturity at much earlier time points.

4.4 Newly developed model for mechanical testing allows for more effective isolation of the peri-implant region

Development of a new mechanical testing model was imperative to reduce bias resulting from geometrical asymmetry in the test specimens. The design of a new method to accurately pot each specimen in the same orientation and centred over the same location during Instron testing allowed for force to be applied in a directly vertical manner for all tests, reducing shear and torque at the bone-implant interface. The design presented herein was very effective, with 92% of all test specimens fracturing through the targeted region of peri-implant bone. Our results demonstrate this new protocol provided much more effective isolation of the peri-implant region than previous iterations of the model.3,178

Potting of bone specimens is a standard method to prepare such samples, and is commonly done using PMMA, plaster of paris, epoxy, or similar cement-like materials.70,194 As the goal for this project was to test specimens in near in vivo conditions by maintaining tissue hydration and mechanical properties of the interface, many conventional potting materials were rejected due to long curing times, which occasionally necessitate frozen specimens, and the exothermic nature of certain materials, which compromises the surrounding tissue. Flowable dental composite was selected as the final potting material due to its fluid properties, which allowed for the mould to be completely filled while also maximizing contact with the test specimen, and its fast curing time which prevented dehydration of the specimen during preparation. This appears to be the first study to use a flowable dental composite for potting bone samples in preparation for tensile testing.

The mechanical properties of bone have been investigated since the 1960’s. Early work correlated the ash content of bone to the modulus of elasticity, static strength, work to failure, and impact strength using rabbit metatarsal bones.195 A three-point bending test was used in conjunction with impact testing to determine a positive correlation between ash content and mechanical properties, showing a swift increase in static strength over a small range of mineral content, with a sharp cut-off in performance at higher mineral levels, at which point bone becomes increasingly brittle. The mechanical properties of healthy
bone have been used over the ensuing decades as baseline values for which to compare and analyze the effects of disease on bone.

The effect of diabetes on mechanical properties of bone has been investigated using torsional loading, where rat femurs were potted on either end using epoxy cement, and torsion was applied to the specimen resulting in a spiral fracture. This study demonstrated decreased torsional strength, and energy absorption, while stiffness was seen to be greater per volume of tissue, indicating more brittle bone. Other reports confirm a significant decrease in energy absorption, maximum load, ultimate stress, and toughness in diabetic bone. Recently, a three-point bending test demonstrated a decreased moment of inertia ($I_{\text{min}}$), rigidity and peak moment values in bone from a rat model of Type 1 diabetes. While there is still discrepancy in the literature regarding the true impact of diabetes on bone and its physical properties, it is generally agreed that mechanical properties are decreased in the presence of hyperglycemia.

Many biomechanical models, including three-point bending, are impractical to assess the bone-implant interface, as they cannot accurately isolate the interface as the breaking region. Several models have been developed in an attempt to quantify the strength and quality of the bone-implant interface, including torque tests, pull-out and push-out tests, and tensile models. However, practical issues limit the effectiveness of these tests, with pull-out and push-out tests limited by the alignment of the implanted plug, and torque tests limited by the thread design on implants. Furthermore, it is difficult to conduct testing with these models without pre-stressing the surrounding bone, since the specimen needs to be fixed in some way during mechanical testing. As such, the use of a tensile model has emerged as the most accurate method for investigating stability of the bone-implant interface.

Results from our study demonstrate the newly designed mechanical testing model is an effective means to isolate the peri-implant region, while also demonstrating the effect of hyperglycemia on peri-implant healing. While studies have been conducted to assess both the effect of diabetes on bone, as noted above, as well as the effect of implant surface design on biomechanical properties of the surrounding bone, this appears to be the first study to design a mechanical testing model that effectively isolates peri-implant bone to investigate the effect of surface design and the ability of a nanotopographically complex surface to abrogate the effects of hyperglycemia.
4.5 Future Work

The project described herein is the latest in almost two decades of work from the Davies lab in the area of early bone formation and peri-implant bone healing, providing an important foundation supporting the use of nanotopographically complex implant surfaces in patients with uncontrolled hyperglycemia. The results of this study prove the efficacy of nanotopographically complex implant surface designs in an environment of uncontrolled hyperglycemia, but, as discussed, much work remains to be done before results can be extrapolated directly to the target human population. While it was decided to isolate hyperglycemia exclusively from other diabetic symptoms and pathologies, as a result, conclusions cannot be directly applied to the targeted human population, the majority of which suffer from T2DM. Future work should be conducted in more precise animal models of T2DM to confirm the advantages of such a surface in a model more closely related to the target population.

The study design used herein addressed only one possible contraindication for the small number of endosseous implant failures – that of hyperglycemia – yet nanotopographically complex surface designs hold potential to drastically improve the healing response in patients with a compromised healing capacity, regardless of origin. As such, future studies should address the effectiveness of such surface designs in the presence of different environments of compromised healing, such as those receiving or recovering from radiation, as well as those suffering from other systemic and autoimmune diseases.

Based on qualitative results presented herein, there was an increased maturity in bone surrounding DCD-treated surfaces. This is an important observation and suggests increased osteoconduction at early time points. As discussed earlier, quantitative confirmation of the increased bone maturity was not achieved in the current work due to malfunctioning hardware, but would add strength to the qualitative observations described herein. A method such as quantitative backscattered electron (qBSE) imaging of embedded samples and subsequent statistical analysis would allow for accurate, quantitative analysis of greyscale levels in the peri-implant region, which are directly representative of the mineral content in the bone and thus are correlated to maturity of peri-implant bone. Such an analysis would provide quantitative support for visual observations. Furthermore, future studies could be designed to include BIC measurements, which provide a more direct clinical interpretation of data and allow for quantitative comparison of the bone-implant interface above and beyond disruption force values.
Chapter 5.0 Conclusions

Overall, it was evident that hyperglycemia caused a delayed response in the early stages of the peri-implant endosseous healing response, corroborating previous findings. Contact osteogenesis was increased on nanotopographically complex surfaces, even in an environment of uncontrolled hyperglycemia, compared to microtopographically complex surfaces in a normoglycemic environment. The DCD-treated surfaces outperformed the grit-blasted surfaces in both healthy and hyperglycemic animals, showing peri-implant bone matured more rapidly on nanotopographically complex surfaces, even in an environment of uncontrolled hyperglycemia, compared to a microtopographically complex surface in a normoglycemic environment. Finally, the newly developed model for mechanical testing allowed for more effective isolation of the peri-implant region compared to previous iterations of the model.
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Appendix A: Genetic and Spontaneous Animal Models of Diabetes

The animal model of STZ-induced diabetes used in this study was chosen for several reasons, most importantly cost-effectiveness and predictable onset and severity of symptoms, in addition to previous experience with the model. As mentioned in Section 1.4, there are numerous other animal models used in diabetic research, some of which are presented below, but they were not selected for several reasons. The variance in onset of symptoms in such models was not conducive to a complex surgical schedule, and the inconsistency in severity of symptoms would have obscured the number of animals required to create a controlled level of hyperglycemia in the treated animal group. Despite these shortcomings, a discussion of other relevant animal models is still valid and has been included below. Large animal models have been excluded from the discussion due to the high cost of maintaining such a model, while mice models have been excluded due to their miniscule skeletal structure, which cannot support the weight-bearing implants used in this investigation.

Spontaneous animal models of T1DM have been created by inbreeding under laboratory conditions and selecting for hyperglycemia. One of the most common animal models is the BB rat, discovered in Ottawa in 1974 at Bio Breeding Laboratories, which undergoes weight loss, polyuria, polydipsia, hyperglycemia, and insulinopenia around the time of puberty. Similar to T1DM in humans, pancreatic islets are subject to a spontaneous immune attack featuring T cells, B cells, macrophages, and natural killer cells leading to pancreatic insulitis. BB rats with untreated, chronic hyperglycemia display the long-term pathologies seen in T1DM and accurately represent the effects on the skeletal system. However, these animals are expensive to maintain and are highly inbred, with development of diabetes genetically determined unlike the heterogeneity in humans.

A vast body of work in the last two decades has been dedicated to the development of an accurate model of non-insulin-dependent diabetes; not surprising since T2DM accounts for over 90% of diabetic patients worldwide. The Zucker Diabetic Fatty (ZDF) rat is one of the more popular models of T2DM. This is an inbred rat model with a homozygous functional defect in the leptin receptor, allowing for spontaneous development of Type 2 diabetes. The model is characterized by a lack of symptoms at 4-5 weeks, followed by hyperinsulinaemia and normoglycemia around 6-7 weeks, and finally insulin-resistance and hyperglycemia by 9-15 weeks, although the timeline varies significantly. ZDF rats develop hyperinsulinaemia and insulin resistance, mild glucose intolerance and hyperglycemia, and hypertension, thus presenting symptoms comparable to that of non-insulin-dependent diabetes (T2DM). Downregulation of beta cell GLUT2 transporters in combination with impaired insulin synthesis is thought to create hyperglycemia in the animals.
An additional model of T2DM derives from spontaneously hypertensive rats (SHR). The obese spontaneously hypertensive rat (SHROB), also known as the Koletsky rat, is genetically hypertensive and obese due to a mutation affecting the leptin receptor, which occurs at a different location than in ZDF rats.\textsuperscript{201,202} The animals present with obesity, hypertension, hyperinsulinaemia, hyperlipidemia, nephropathy and premature vascular disease over time,\textsuperscript{199} thus presenting similar phenotypic features to metabolic syndrome X\textsuperscript{202} – a collective term referring to insulin resistance associated with compensatory hyperinsulinaemia, impaired glucose tolerance, dyslipidemia, and hypertension.\textsuperscript{203}

Finally, the Goto-Kakizaki (GK) rat is a non-obese Wistar rat model of lean T2DM created through selective inbreeding of Wistar rats with abnormal glucose tolerance.\textsuperscript{198,204} These animals are characterized by their lean stature and normolipidemia, moderate fasting hyperglycemia, hypoinsulinaemia, impaired glucose tolerance, and early onset of diabetic pathologies.\textsuperscript{154} In the adult GK rat, total pancreatic beta cell mass is decreased by 60%.\textsuperscript{198} As a result of comparable pathologies in the absence of obesity, the GK rat is an effective model for the study of T2DM, especially as it relates to changes in beta cell mass and diabetic complications in non-insulin-dependent diabetes mellitus.
# Appendix B: Streptozotocin (STZ) Dosage Chart

## Streptozotocin Chart

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Appendix C: Technical Drawings for Implant
Appendix D: Technical Drawings for Tensile Test Breakaway Mould
4. ALL FILLETS 0.002 - 0.005 R.A.O.
3. ALL DIAMETERS CONCENTRIC WITHIN 0.001.
2. BORE ALL HOLES 0.001 - 0.003.
1. ALL SURFACES 16 R.U.S. OR BETTER
NOTES, UNLESS OTHERWISE SPECIFIED: