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DISSERTATION

**THE IMPACT OF TYPE II DIABETES AND CHRONIC PERIODONTAL DISEASE ON  
PERIPHERAL BLOOD NEUTROPHIL APOPTOSIS**

by

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Submitted in partial fulfillment of the  
Requirements for the degree of  
Doctor of Science in Dentistry  
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**ABSTRACT**

**Background:**

Apoptosis plays a critical role in the host immune response and resolution of inflammation. A hyperactive or primed neutrophil phenotype has been associated with diabetes and chronic periodontitis. Delayed cell death prolongs chronic inflammation creating increased tissue damage. We tested the hypothesis that peripheral blood neutrophil spontaneous apoptosis is delayed in type 2 Diabetes Mellitus (T2DM), and co-expression with chronic periodontitis exacerbates the delayed spontaneous neutrophil apoptosis.

**Materials and Methods:**

73 individuals, including those with type 2 diabetes (DM) (n=16), chronic periodontitis (CP) (n=15), diabetics with chronic periodontitis (DM+CP) (n=21) and healthy volunteers (H) (n=21) were enrolled. Heparinized venous blood was

obtained and neutrophils isolated by density gradient centrifugation. Cells were maintained in RPMI-1640 supplemented with bovine fetal serum for 2-24 hours. Neutrophil apoptosis was determined by flow cytometry using TUNEL and Annexin V assays. Caspase 3, 8 and 9 activity was measured by colorimetric assay. Neutrophil surface death receptor quantification were stained with fluorescence conjugated anti-CD120a (TNFR1) and anti- CD95 (FasR) antibody. All samples were analyzed using a flow cytometer. Inflammatory Biomarker Analysis from serum samples were analyzed by multiplexed sandwich immunoassay using flowmetric Luminex™ xMAP technology. Statistical analysis was performed using Student's T-test and ANOVA with an LSD post-hoc test.

### **Results:**

In H, T2DM and T2DM+CP subjects, spontaneous neutrophil apoptosis reached 50% in 7.4, 8.5 and 9.4 hours, respectively. In 12 hours neutrophil apoptosis was  $85.3\% \pm 3.1$ ,  $67.3\% \pm 3.9$  and  $62.5\% \pm 5.4$ , respectively. Neutrophils from the T2DM and T2DM+CP groups showed a significant delay of apoptosis compared to the H group at 12 hours ( $p < 0.031$  and  $p = 0.003$ , respectively). Caspase-3 activity in the H group showed significantly higher activity compared to the T2DM ( $p = 0.018$ ) and T2DM+CP groups ( $p = 0.031$ ). Upstream caspase-8 (extrinsic pathway) activity from the T2DM+CP group was significantly decreased compared to the H group ( $p = 0.046$ ). The lack of caspase activity was possibly regulated by the reduction of cell surface Fas receptor in neutrophil from the T2DM ( $p = 0.01$ ) and T2DM+CP groups ( $p = 0.016$ ), the TNF receptor from the T2DM group ( $p = 0.005$ ) and

the level of serum Fas ligand ( $p= 0.035$ ) compared to the H group. To further investigate the mechanism, we mimicked T2DM+CP delayed neutrophil apoptosis in healthy donors. Our results showed that a high glucose condition alone did not affect neutrophil apoptosis. When hyperglycemia was combined with S100b (ligand for receptor for advanced glycation end production ligand;  $50 \mu\text{g/ml}$ ) and *P.gingivalis*, neutrophil apoptosis was delayed ( $p=0.002$ ).

**Conclusion:**

We showed that spontaneous apoptosis of the peripheral blood neutrophil was impaired in subjects with type 2 diabetes and chronic periodontitis. The mechanism underlying this finding were due to a lack of sFas ligand and its receptor expression on neutrophil cell surface. Furthermore, in T2DM patients, RAGE over activation is suggested to play a crucial role in delaying spontaneous neutrophil apoptosis.

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## VII. INTRODUCTION

### 1. Diabetes Mellitus

#### 1.1 Definition of Diabetes

Diabetes mellitus (DM) is a group of diseases characterized by chronic hyperglycemia and other metabolic abnormalities. Chronic hyperglycemia is a hallmark of DM regardless of its pathophysiology. Diabetes occurs when the pancreas does not produce enough insulin, or when the body cannot effectively use the insulin it produces. There are two main types of diabetes based on the primary cause of hyperglycemia. Type 1 diabetes results from autoimmune mediated destruction of the beta cells of the pancreas. Type 2 diabetes is characterized by resistance to the action of insulin and disorder of insulin secretion, either of which may be the predominant feature (1). According to the 2012 guidelines of the American Diabetes Association (ADA) and the International Expert Committee report of 2009, there are four criteria to diagnose diabetes (**Table 1**). First, it can be diagnosed by symptoms such as polyuria (excessive urination), polydipsia (excessive thirst), polyphagia (excessive hunger), hyperglycemic crisis with severe hyperglycemia (>600 mg/dl), hyperosmolarity, small ketones and casual plasma glucose concentration of more than 200mg/dl (11.1 mmol/l) (any time of day without regard to the time since the last meal). Second, a fasting plasma glucose level (FPG) more than 126 mg/dl (7 mmol/l) (no calorie intake for at least 8 hours)

suggests a diagnosis of diabetes. A third diagnostic characteristic is a 2-hour post load glucose more than 200 mg/dl (11.1 mmol/l) during an oral glucose tolerance test (OGTT). The last is a Hemoglobin A1c (HbA1c) level >6.5 % (normal values 4-6%) performed using a certified laboratory method. The ADA has adopted this criterion since 2010 (2). HbA1c is also a reliable monitoring test for long-term control of blood glucose over 2-3 months. It should be measured every 3-4 months in patients with ongoing diabetes, with an HbA1c value <7% indicating well controlled diabetes (3, 4). With only a single test, diagnosis cannot be confirmed. To diagnose diabetes, the laboratory value should be confirmed on a different day (5). The confirmation of chronic hyperglycemia is a prerequisite for diagnosis, and DM can be diagnosed when hyperglycemia meets the criteria for diabetic types confirmed on 2 or more occasions on separate days.

In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose $\geq 200$ mg/dl (11.1 mmol/l).
OR
<b>FPG <math>\geq 126</math> mg/dl (7.0 mmol/l).</b> Fasting is defined as no caloric intake for at least 8 h.
OR
<b>2-h plasma glucose <math>\geq 200</math>mg/dl (11.1mmol/l) during an oral glucose tolerance test (OGTT).</b> The test should be performed as described by the World Health Organization, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.
OR
<b>A1C <math>\geq 6.5\%</math></b> performed in a laboratory using a method that is NGSP certified and standardized to the DCCT assay.

**Table 1.** The criteria for the diagnosis of diabetes



## **1.2 Epidemiologic Considerations in Diabetes**

During the last twenty years, the prevalence of diabetes has increased dramatically in many parts of the world such that the disease has become a worldwide public health problem. The total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030 unless urgent action is taken (1, 2, 6). It is suspected that a major factor contributing to the increase in the prevalence of diabetes is lifestyle, including obesity and lack of physical activity. Nearly 3 million deaths per year are attributed to diabetes accounting for 5% of deaths globally. This number is expected to double by 2030. According to the American Diabetic Association approximately 8.3% of the total population of the US has diabetes. 26 million children and adults in the US have diabetes and 7 million among them are undiagnosed. With a rate of 1.9 million Americans ages 20 years and older being diagnosed each year. Furthermore, statistics have shown that the incidence of individuals being diagnosed with diabetes has nearly tripled from 1990 to 2010. The highest percentage of individuals with diagnosed and estimated undiagnosed diabetes by age group is senior citizens with around 26.9% of individuals considered to have diabetes. Each year, diabetes is listed as a primary cause of death for 71,382 American. The risk for death in a person with diabetes is twice of that compared to a person of similar age who does not have diabetes. Racial statistics within the United States for total estimated diagnosed and undiagnosed persons with diabetes show Non-Hispanic

whites have the lowest rate of diabetes with a prevalence of 10.2% versus 18.7% of Non-Hispanic blacks and 16.1% of American Indian and Alaskan Natives (7).

### **1.3 Types of Diabetes Mellitus**

There are two main types of diabetes. Type 1, previously called "insulin dependent diabetes", accounts for less than 20% of diabetes cases. In this type, 90% of cases are immune-mediated and characterized by destruction of pancreatic islet cells resulting in primary hypoinsulinemia and hyperglycemia. In less than 10% of type 1 diabetes cases there is no evidence of autoimmunity against  $\beta$ -cells to explain hypoinsulinemia and thus falls into the idiopathic cause. The onset of the disease is often abrupt, and patients with this type of diabetes are more prone to ketoacidosis and wide fluctuation in plasma glucose levels. Patients with type 1 diabetes need insulin therapy for survival. If untreated, these patients are likely to manifest the classic signs and symptoms of diabetes. These include polyuria, polydipsia and polyphagia, as well as pruritis, weakness and fatigue. Type 1 Diabetes usually presents itself in youth or late childhood (8) and has the highest incidence in northern European countries and US states populated with persons of Scandinavian descent such as Minnesota. In Scandinavia, the incidence in children aged 14 years or younger is 37 per 100,000/year. The US overall annual incidence is 15 per 100,000/year. Interestingly, ethnic genetic heterogeneity seems to play a key role in development of this type of diabetes (2, 9).

In contrast, Type 2 diabetes, previously called "non-insulin dependent diabetes", is responsible for more than 90% of the cases of diabetes worldwide. It is characterized by insulin resistance with relative insulin deficiency in one extreme to a predominantly secretory defect accompanied by insulin resistance in the other. In type 2 diabetic patients, the onset is generally more gradual than type 1 and it is quite rare to see ketoacidosis because insulin is produced. Insulin resistance stimulates insulin production at the early state of the disease, but as the condition progresses pancreatic insulin production is depleted. Although type 2 diabetes patients usually do not need exogenous insulin to survive, it might be taken as a part of treatment (10). Type 2 diabetic patients usually show obesity or have higher percentage of fat distribution in the abdominal region. Adipose tissue is a key factor to developing insulin resistance. Free fatty acid from adipose tissue contributes to insulin resistance by inhibiting glucose uptake, glycogen synthesis, and glycolysis and by increasing hepatic glucose production (11). In addition, the risk of type 2 diabetes increases with age and lack of physical activity, and this form of diabetes is more prevalent among people with hypertension or dyslipidemia and has a strong genetic component.

Besides these two main types of diabetes, another type is gestational diabetes which has its onset in the third trimester of pregnancy. Etiologically, many patients of this type of diabetes probably share common genetic susceptibilities with type 1 or type 2 diabetes, and the deterioration of glucose tolerance is

precipitated by the metabolic effect of pregnancy. Glucose intolerance during pregnancy is often normalized after delivery (5).

#### **1.4 Complications of Diabetes Mellitus**

Complications of diabetes can be categorized into 2 types: acute complications and chronic complications, based on the onset of symptoms.

The three most common acute complications of diabetes include hypoglycemic coma, ketoacidosis and hyperosmolar coma. Hypoglycemic coma is common in insulin-treated diabetic patients and also occurs occasionally in patients treated with the oral hypoglycemic sulfonylurea agents. Hypoglycemia may range from very mild lowering of glycemia with minimal or no symptoms, to severe hypoglycemia with very low levels of glucose and neurologic impairment. Diabetic ketoacidosis is one of the major acute diabetic complications. It usually occurs in the context of total insulin deficiency. Ketoacidosis is clinically defined by absolute insulin deficiency with hyperglycemia (glucose levels usually  $>250$  mg/dl), increased ketone production, hyperketonemia, and acidosis (arterial  $\text{pH}<7.3$ ). Precipitating factors for ketoacidosis in those with established diabetes include infection, other acute illnesses, lack of diabetes education and training, noncompliance, poor self-care, inadequate glucose monitoring, psychological problems. In individual who suffered from diabetic ketoacidosis, glucose levels are increased. Some of the excess circulating glucose are filtered into the urine and generate osmotic diuresis, which results in symptoms including, nausea, vomiting,

dehydration, pronounced thirst, excessive urine production and abdominal pain, confusion and occasionally coma. In severe case, breathing becomes labored and of a deep, gasping character which leads to hyperventilation. This is a state referred to as Kussmaul respiration pattern. Non-ketonic hyperosmolar coma is usually presented in type 2 diabetes with hyperglycemia (>600 mg/dl), dehydration, and stupor, progressing to coma if uncorrected, without the presence of ketosis or acidosis. These patients have sufficient circulating insulin to prevent lipolysis and ketosis. They respond well to hydration and small doses of insulin to correct hyperglycemia. Morbidity of Non-ketonic hyperosmolar coma consists of coma and impaired neurologic function with a predisposition to vascular occlusive disease from dehydration or poor perfusion. (1, 12).

The chronic complications of diabetes are related to long-term elevation of blood glucose concentrations or hyperglycemia. Hyperglycemia results in the formation of advanced glycation end products (AGEs), which have been linked to diabetic complications. Long-term complications may occur in both type 1 and type 2 diabetes. The classic complications of DM include: microvascular complications including retinopathy, nephropathy and neuropathy; macrovascular complications include coronary artery disease, cerebrovascular disease and peripheral vascular disease, inhibited wound healing, and periodontitis (13, 14). The pathogenesis of type-1 and type-2 diabetes is different, but both can lead to microvascular complications, if left untreated. According to the Diabetes Control and Complications Trial and the United Kingdom Prospective Diabetes Study, if hyperglycemia is well

controlled, the occurrence of retinopathy, neuropathy, and nephropathy is reduced, regardless of type. This suggests that diabetic hyperglycemia is responsible for diabetic microvascular complications(15). Diabetic retinopathy occurs in 75% of people with diabetes who have suffered for more than 15 years(16). One of the changes in the retina caused by hyperglycemia is the death of pericytes, predisposing to endothelial cell proliferation and the development of microvascular aneurysm(17). Almost 50% of people with diabetes have a certain degree of diabetic neuropathy. The most common form of diabetic neuropathy is polyneuropathy which produces a loss of peripheral sensation. Polyneuropathy can be combined with microvascular and macrovascular impairment leading to non-healing ulcers. Clinically, polyneuropathy manifests in paresthesia, dyesthesia, pain, impaired reflexes and decreased vibratory sensation (18). Diabetic nephropathy is characterized by glomerular hyperfiltration leading to glomerular damage. People with diabetic nephropathy, as it progresses, show pronounced proteinuria, decreased glomerular filtration rate and end-stage renal failure. Classically, people with diabetic nephropathy show the expansion of extracellular matrix in the mesangial area, with the increase of type I and type IV collagen and decrease of proteoglycans. This is associated with decreased glomerular filtration and glomerular surface area for filtration (14). Besides those complications, diabetic patients are 2 to 4-fold more prone to developing cardiovascular disease than non-diabetic patients. It is even more severe in poorly controlled diabetic patients. Several other factors, including hypertension, life style, and high cholesterol,

contribute to the development of this complication. Diabetic patients show a 3 to 4 fold higher tendency towards developing peripheral arterial disease when compared to non-diabetic patients. The abnormal metabolism of diabetic patients results in changes in the arterial state of function and structure which are prone to developing peripheral arterial disease (19).

### **1.5 Mechanisms of Diabetic Complications**

In both types of DM, the risk of micro- and macro-vascular complications are markedly increased. Microvascular complications, which originate from a dysfunction in the capillary vessels in tissues, include the retinopathy, nephropathy, and neuropathy. These complications eventually affect nearly all patients with diabetes. Macrovascular complications are due to atherosclerosis and remain the leading cause of death in diabetic patients. Four major pathogenic mechanisms have been proposed to be responsible for the development of long-term complications of DM. These include 1) increased polyol pathway flux, 2) increased advanced glycation end products (AGEs), 3) activation of protein kinase C (PKC), and 4) increased hexosamine pathway flux. All the pathways generally act intracellularly to modify cellular function, except for AGE. AGE binds to specific receptors called RAGE, which alter regular cell functions. In diabetic rats, it has been shown that accumulation of AGE and enhanced expression of RAGE lead to tissue destruction (20). RAGE is a multi-ligand member of the immunoglobulin family expressed on a wide range of cells including endothelial cells, fibroblasts, monocytes, neutrophils

and podocytes (21, 22). AGE/RAGE interaction initiates a cascade of signaling transduction events involving the production of reactive oxygen species (ROS) through at least in part the activation of NADPH oxidase (23). This, in turn activates the transcription factor NF-kappa B (24). Blockage of RAGE was shown to prevent the development of diabetic nephropathy and hyperglycemia-induced macrovascular complications supporting the role for AGE-RAGE axis in diabetes (25, 26). Recent evidence also showed that hyperglycemia-induced overproduction of reactive oxygen species (ROS) by the mitochondria's electron-transport chain is a critical mechanism. Hyperglycemia-induced overproduction of mitochondrial superoxide is responsible for an important reversible decrease in glyceraldehyde phosphate dehydrogenase (GAPDH) activity. Superoxide induces this effect either direct or indirect, via poly (ADP-ribose) polymerase (PARP) activation by oxidative lesions of mitochondria's DNA. Despite the modulation, superoxide GAPDH inhibition induces the above mentioned mechanisms (27-30).



## **2. Periodontal Disease**

### **2.1 The Periodontium**

The gingival tissue overlies the alveolar bone and forms a close collar around the teeth. The mucosal surface is keratinized and exhibits prominent rete ridges, and the epithelium includes scattered Langerhans cells, which act as antigen presenting cells. The connective tissue contains nerves, blood vessels, lymphatic vessels and fibroblasts. Healthy gingiva is clinically coral-pink in color and does not bleed on periodontal probing. Normally, a few lymphocytes are present and in healthy gingival tissue no large accumulations of plasma cells are observed. The gingival surface around the teeth forms a sulcus, which is less than three millimeters deep in healthy people. The sulcular epithelium is thin and non-keratinized, with no rete ridges. Junctional epithelium attaches to the cementum of the root at the base of the sulcus by means of hemidesmosomes. The connective tissue contains sets of specifically-oriented collagen fiber bundles called gingival fibers that attach to the cementum by means of Sharpey's fibers. Beneath this, the cementum is attached to the cortical bone of the socket by means of sets of specific collagen fibers called periodontal fibers. These fibers transverse a periodontal ligament space that also contains blood vessels, lymphatic vessels and nerve fibers for pain reception and proprioception (31).

## **2.2 Periodontal Disease**

Periodontal disease is a significant cause of tooth loss among adults. It is defined as a polymicrobial infection that stimulates an inflammatory response of the periodontal tissues resulting in a loss of supporting structures of the affected teeth (32, 33). This process is characterized by a dysregulated local inflammatory reaction and progressive destruction of periodontal supporting tissues as a result of breaking the gingival seal that protects against local invasion by periodontal pathogenic bacteria (32, 34, 35). Gingivitis is an initial stage to periodontitis, which is the presence of gingival inflammation without the loss of connective tissue attachment to the tooth, this process can be reverse and heal completely (36). Periodontal disease results from a failure of the immune system against infectious agents and an impaired restoration of homeostasis (37, 38).

## **2.3 Epidemiologic Considerations in Periodontitis**

According to the National Health and Nutrition Examination Surveys (NHANES III), loss of periodontal attachment is measured clinically by periodontal probing depth (PPD) defined as the distance from the gingival margin to the base of the crevice or pocket, and clinical attachment loss (CAL) defined as the distance from the cemento-enamel junction (CEJ) to the base of the crevice or pocket. Periodontitis is defined as a person who had at least 3 periodontal sites with 4 millimeters or more of attachment loss and 2 sites with 3 millimeters or more of pocket depth. In an analysis of the NHANES data collected between 1999 and 2004,

periodontal diseases were found to be more prevalent among African Americans than Caucasians or Hispanics in the United States. The prevalence of periodontitis was also found to increase with age, regardless of ethnicity. About 1.3% of African Americans and 0.7% of Caucasians aged 18 to 34 years met the definition of periodontitis. Among 35 to 59 year olds, the prevalence of periodontitis increased to 10.7% among African Americans and 3.6% among Caucasians. The prevalence of periodontitis further increased to 15.3% of African Americans aged 60 to 85 years, and 5.6% among Caucasians. When compared by sex, the prevalence of periodontal diseases is higher in men compared to women. It has been speculated that the differences in the prevalence of periodontal diseases between men and women may be due to higher levels of inflammation in response to infection or injury in men compared to women (39-41).

## **2.4 Classification of Periodontal Disease**

Classification of periodontal disease has changed, as we have learned more about the etiology of periodontal diseases. The most recent and widely accepted definition of periodontal diseases was developed at the 1999 International Workshop for a Classification of Periodontal Diseases and Conditions. Gingival diseases are classified into two main types: dental plaque-induced gingival diseases and non-plaque induced gingival lesions. Undisturbed dental plaque not only leads to the reversible condition known as gingivitis, but can ultimately lead to irreversible damage to the periodontal tissues known as periodontitis. Periodontitis

can be further classified into seven different categories, based on the etiology of the disease. Aggressive periodontitis (formerly Early Onset Periodontitis, EOP) is characterized by rapid loss of periodontal support. Aggressive periodontitis can also manifest as a part of systemic diseases such as acquired neutropenia, leukemias, and other genetic disorders including Papillon-Lefèvre syndrome and Chédiak-Higashi syndrome. Chronic periodontitis (formerly Adult Periodontitis) can develop as a result of inflammation of the gingival tissues due to dental plaque. Additionally, there are genetic and nongenetic factors that can influence the pathogenesis of chronic periodontitis. Chronic periodontitis can further be classified by the extent of the disease, depending on how many sites in the mouth are affected. Localized periodontitis refers to periodontitis affecting 30% or fewer of sites in the mouth, while generalized periodontitis is used to describe periodontitis affecting more than 30% of the sites in the mouth. Other causes of periodontitis that are independent of plaque accumulation include necrotizing periodontal diseases which may be linked to stress, poor diet, cigarette smoking, or HIV infection, as well as periodontitis resulting from developmental or acquired deformities of the teeth and gums (42).

## **2.5 Pathogenesis of Periodontal Disease**

The pathogenesis of periodontal diseases is the result of a complex interaction between plaque microorganisms and the host response to the presence of those microorganisms on tooth and gingival tissues. The gingival sulcus contains an inflammatory exudate that helps protect the junctional epithelium from plaque

bacteria byproducts and other irritants. Microbial plaque is considered to be the initiator of the disease process because it serves as a challenge to the host and host tissues (periodontal tissues). How the host responds to the plaque challenge determines the severity and extent of the tissue damage associated with that response. Below is a list of periodontal conditions and progression of the disease (31, 32, 34, 43-45).

1. Healthy periodontium: There is insufficient plaque challenge to elicit an inflammatory response that is clinically visible as a change in color, contour, or consistency of the gingival tissues. When clinically healthy periodontal tissues are viewed by histology there is usually some degree of gingival inflammation present. Perfect periodontal health is nearly impossible to achieve due to our inability to completely remove plaque from tooth and gingival surfaces. The lack of plaque challenge can be due to:

- a. Minimal amounts of plaque present because of excellent oral hygiene.
- b. A plaque that is made up primarily of gram-positive bacteria that do not promote a discernible host response.
- c. A combination of both characteristics.

2. Gingivitis: Caused by the changes in the composition of dental plaque and usually happens in 21-28 days. The pathological changes observed in gingivitis are characterized by changes in color, contour, and consistency of the gingival tissues that are frequently associated with increased redness, swelling, and bleeding on probing. These clinical and histological changes are due to the presence of an

increased inflammatory response that extends into and destroys cells and matrices of the gingival tissues but does not result in destruction of periodontal ligament and bone. By elimination of irritants, tissue health will restore gingival homeostasis back to healthy stage.

3. Periodontitis: The pathological changes in periodontitis are the same as those that occur in gingivitis except that the inflammation and tissue destruction extend from the gingival tissues into the periodontal ligament and alveolar bone, resulting in an irreversible destruction of periodontal tissues. The extent and severity of periodontal destruction reflects the extent and severity of the inflammatory process. The extent and severity of the inflammatory response can be influenced by:

a. The failure to remove plaque from tooth and gingival surfaces, resulting in a chronic challenge to the host.

b. Environmental and/or genetic factors that may enhance the host response to the plaque challenge, resulting in an increase in the extent and severity of periodontal tissue damage.

c. A combination of factors 1 and 2.

## **2.6 Characteristics of the Host Response in Periodontal Disease**

1. Cells of the host response: Neutrophils, monocytes/macrophages, mast cells, and dendritic cells are considered to be cells of the innate immune response that protects us from birth. Lymphocytes are considered part of the specific immune

response, and these cells develop antigen-specific responses throughout life. T cells, B cells, and plasma cells are the major cells of the specific response.

a. Neutrophils (polymorphonuclear leukocytes; PMN): These migrate from the blood vessels of the subepithelial vascular plexus into the periodontal pocket where they interact with plaque microorganisms. The primary role of neutrophil is to protect the body from infection. However, they are also considered to be an important cell in the destruction of the periodontal tissues. Neutrophil move from blood vessels toward sites of infection by a process of directed locomotion (chemotaxis) along a gradient of powerful chemotaxins such as C5a, IL-8, LTB<sub>4</sub>, and the bacterial protein N-fMLP. Neutrophil are capable of internalizing microorganisms by a process of phagocytosis and, once internalized, they can kill and digest the microorganisms using a powerful mixture of oxygen radicals (H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub><sup>-</sup>) and granule enzymes (myeloperoxidase) that form the biological equivalent of commercial bleach. Abnormalities in neutrophil function (neutropenia, agranulocytosis, Chediak-Higashi syndrome, Papillon-Lefevre syndrome, leukocyte adhesion deficiency) make the host more susceptible to infection.

b. Monocytes/macrophages: Monocytes are also part of the leukocyte family but live much longer in the tissues than neutrophils. They are responsible for ingesting antigens (such as bacteria) and presenting them to the cells of the specific immune response. They are also very important in regulating the immune response through the release of chemical signals called cytokines.

c. Mast cells: are important in immediate inflammation and are responsible for creating vascular permeability and dilation. They are important cells in anaphylaxis and allergic responses.

d. Dendritic cells: are distributed throughout the tissues and are important in antigen processing and presentation to cells of the specific immune response.

e. Lymphocytes: The predominant lymphocytes are B cells and T cells. B cells differentiate into plasma cells and are responsible for the production of antibodies. T cells (derived from the thymus) fall into two major groups: T-helper cells (CD4 cells), which help in the production of antigen specific antibodies by B cells and plasma cells, and T-cytotoxic cells (CD8 cells), which are important in controlling intracellular antigens such as bacteria, fungi, and viruses. Natural killer (NK) cells are T cells that can recognize and kill tumor and virally infected cells.

2. Controlling the bacterial challenge: Neutrophils are the most important cells involved in controlling the bacterial challenge. They migrate from blood vessels under the gingival epithelium (subepithelial vascular plexus), into the periodontal pocket, where they form a barrier to protect the body from periodontal bacteria. They phagocytose and kill bacteria and also release large quantities of oxygen radicals and enzymes (myeloperoxidase, lysozyme and collagenase) into the extracellular environment.

3. Tissue destruction in periodontal disease: Periodontal cells and tissues are destroyed by cells and proteins of the immune response. Matrix metalloproteinases (MMPs) are considered the most important proteinases involved in the destruction



of periodontal tissues. They are produced by most cells of the periodontal tissues, but neutrophil produce large quantities of MMP-8 (collagenase) that is responsible for destroying collagen of the periodontal connective tissues and periodontal ligament. Oxygen radicals (superoxide and hydrogen peroxide) produced by inflammatory cells (neutrophil and macrophages) are also toxic to cells of the periodontium having a direct effect on cell functions and DNA.

4. Cytokines are important signaling molecules released from cells: The cytokine IL-1 is important in bone resorption; IL-8 is important in attracting inflammatory cells (chemotactic); and tumor necrosis factor (TNF) is important in activating macrophages.

5. Prostaglandins are produced from arachidonic acid of cells membranes in response to cyclooxygenases (COX-1 and COX-2). They have widespread proinflammatory effects but can be inhibited by non-steroidal anti-inflammatory drugs (aspirin and other NSAIDs) (45).

### ***2.7 Porphyromonas gingivalis***

By utilizing cluster analysis, community ordination and checkerboard DNA-DNA hybridization, Socransky et al. have grouped the microorganisms that colonize the subgingival plaque into 5 color-coded complexes based on similarities between pairs, associations and clustering of species including, red complex (*Bacteroides forsythus*, *Porphyromonas gingivalis* and *Treponema denticola*), orange complex (*Fusobacterium nucleatum/periodonticum* subspecies, *Prevotella intermedia*,

*Prevotella nigrescens* and *Peptostreptococcus micros*), yellow complex (*Streptococcus sanguis*, *S. oxalis*, *S. mitis*, *S. gordonii* and *S. intermedius*), green complex (*Capnocytophaga* species, *Campylobacter concisus*, *Eikenella corrodens* and *Actinobacillus actinomycetemcomitans serotype*) and purple complex (*Veillonella parvula* and *Actinomyces odontolyticus*). *Porphyromonas gingivalis* (*P. gingivalis*) is most closely associated with chronic periodontitis and is a member of the red complex of bacteria. It is a nonmotile, gram negative pleomorphic rod. It grows anaerobically and becomes darkly pigmented on blood agar plates. It also can invade epithelial and endothelial cells. *P. gingivalis* has routinely been reported as a major member of the pathogenic microbiota in various periodontal diseases characterized by loss of periodontal attachment and crestal alveolar bone (46-49). In vitro, growth of *P. gingivalis* and analysis of its various components (i.e. lipopolysaccharide, outer membrane proteins (proteases), fimbriae and end-products of metabolism) reveals it to produce a substantial array of putative virulence factors (50, 51). *P. gingivalis* is frequently isolated from individuals with chronic periodontitis, diabetes-associated periodontitis, and periodontal breakdown around endosseous implants (33, 48, 52) (48, 53).

Specific virulence factors include:

1. Fimbriae and adherence.
2. Presence of a capsule.
3. Proteases that cleave immunoglobulins and complement components.
4. Proteases that cleave other tissue-associated host proteins.

5. Collagenase.
6. Alpha hemolysin.

## **2.8 Periodontal Disease as a Diabetic Complication**

Several complications are caused by chronic hyperglycemia in diabetes, such as heart disease, high blood pressure, blindness, kidney disease, nervous system disease, foot ulcers leading to amputation, and periodontal disease. One third of patients with diabetes suffer from severe periodontal disease (54). Periodontal disease has been closely associated with diabetes. In fact it has been reported as the sixth major complication of diabetes along with neuropathy, nephropathy, retinopathy, and micro and macrovascular diseases (55, 56). Numerous studies have found a higher prevalence of periodontal disease among diabetic patients than among healthy individuals (57-66). In a large cross-sectional study, Grossi and others showed that diabetic patients were twice as likely as nondiabetic subjects to have attachment loss. Periodontal attachment loss was defined as the distance from the cemento-enamel junction (CEJ) to the bottom of the pocket/sulcus around the examined tooth and was calculated as the sum of the probing depth and gingival recession measurements. Gingival recession was defined as the distance from the cemento-enamel junction (CEJ) to the free gingival margin (67). Firatli monitored type 1 diabetic patients and healthy controls for 5 years. People with diabetes had significantly more clinical attachment loss than controls (62). In another cross-sectional study, Bridges and others found that diabetes significantly affected all

periodontal parameters including bleeding scores, probing depths, and loss of attachment and missing teeth (68). In fact, one study has shown that diabetic patients are 5 times more likely to be partially edentulous than nondiabetic subjects (69). Glycated hemoglobin (HbA1c) is frequently used to monitor overall glycemic control level in people with diabetes, as it is a good marker providing blood glucose level over time (6). Glycemic control as measured by HbA1c affects the progression of periodontal disease in terms of bleeding on probing and pocket depth. Poorly controlled diabetic patients show increased bleeding on probing and pocket depth (70). Other studies have revealed that there is a positive correlation between HbA1c and severity of periodontal disease (68, 71). Treatment of periodontal disease with systemic antibiotic administration for a month reduced HbA1c level 3 months later, but after the cessation of antibiotics, the level of HbA1c got worse, suggesting that controlling bacterial infections, including periodontal disease, has a positive effect on metabolic control of type 2 diabetes (72). In a large epidemiologic study in the United States, adults with poorly controlled type II diabetes had a 2.9-fold increased risk of having periodontitis as compared to non-diabetic adult subjects; conversely, well-controlled diabetic subjects had no significant increase in the risk of periodontitis (73). In a longitudinal Pima Indian study, poor glycemic control of type 2 diabetes was associated with an 11 fold increased risk of progressive bone loss compared to non-diabetic controls, whereas well controlled diabetic subjects had no significant increase in risk (74). Thus, people with type 1 and type 2 diabetes appear equally susceptible to periodontal disease and tooth loss. Recent investigations have

attempted to determine if the presence of periodontal disease influences the control of diabetes. Data from 4343 persons aged 45-90 years from NHANES III, with type 2 DM and with glycosylated hemoglobin > 9% or poorly controlled diabetes, revealed a significantly higher prevalence of severe periodontitis than those without diabetes [odds ratio=2.90]. In contrast, in better-controlled diabetes, there was a less tendency for prevalence of severe periodontitis [odds ratio=1.56] (73). In another study that followed diabetic patients and non-diabetic controls for 3 years, the level of periodontal health in diabetic patients with good or moderate control of their condition was similar to that in the non-diabetic controls (75). Those with poor control had more attachment loss and were most likely to exhibit recurrent disease. Several researchers proposed that a two-way relationship between the mechanism of periodontal disease and diabetes is feasible and is based on a dysregulated inflammatory response manifested both locally and systemically in a diabetic individual with periodontal disease (76-81). Numerous mechanisms by which diabetes associated with deterioration of periodontitis have been suggested include impaired immune function, microvascular alterations and changes in structural and biochemical support of the surrounding cells (77). In a poorly controlled diabetic individual with a healthy periodontium, gingival levels of inflammatory cytokines were found to be elevated compared to the well-controlled and non-diabetic groups (82-84). Several recent studies have also found a positive correlation between periodontal disease and polymorphisms in IL-1 gene cluster in healthy and diabetic patients (85-87). One of the important host factors involved in periodontal diseases

is the family of matrix metalloproteinases (MMPs). MMPs are responsible for collagen and extracellular matrix degradation of the periodontal tissues. A recent study has reported a significant increase in MMP-8 and 9 in diabetic patients compared to the healthy control (84). In Streptozotocin (STZ)-induced diabetes in mice the level of gingival tissue albumin-AGE was increased, which led to the suggestion that AGE might play a role in pathogenesis of periodontal disease in individuals with diabetes (88). In support of this observation, a cross-sectional study including 97 type 2 diabetes patients with and without periodontitis has shown a significant association between serum AGE and severity of periodontal disease (89). In addition, Grossi and Genco (90) proposed a dual pathway model, whereby periodontal disease increases the severity of diabetes through upregulation of cytokine synthesis by periodontal microorganisms and thereby exacerbates the intensity of AGE mediated cytokine upregulation in diabetes. TNF- $\alpha$  is a cytokine secreted in acute inflammation and highly expressed in type 2 diabetic patients with obesity, releasing free fatty acids from adipose tissues and impairing insulin signaling leading to insulin resistance. The level of TNF- $\alpha$  in periodontal disease is increased, which in turn exacerbates insulin resistance already existing in obese people. Treatment of periodontal disease with antibiotics significantly decreases the level of circulating TNF- $\alpha$  and of HbA1c, thereby improving metabolic control for type 2 diabetes. This indicates that circulating TNF- $\alpha$  plays an important role in mediating the two-way relationship between diabetes and periodontal disease (91, 92).

### **3. Neutrophils**

#### **3.1 General Concept**

Neutrophils are the first line of host defense of the innate immune system. When there is infection or injury, they can be easily mobilized to the invading or injurious site where they localize invading microorganisms and clear dead host cells and debris (93). These cells are the most abundant (90%) of the leukocytes found in the peripheral human blood although not in the murine blood where lymphocytes dominate. The neutrophils are small cells, about 9-19  $\mu\text{m}$  in diameter and possess a multilobulated nucleus (2-5 lobes). This feature contributes to the elasticity of the cell and its ability to squeeze through the tight junctions between the endothelial cells.

There is considerable evidence to suggest that neutrophils and monocytes share a common progenitor cell in the bone marrow (94). This is called the granulocyte-macrophage colony forming unit, (CFU-GM) because of its ability to give rise to colonies of neutrophils and monocytes in semi-solid marrow cultures. The neutrophil begins its 2 weeks lifespan in the bone marrow, with the commitment of a hematopoietic stem cell to myeloblastic differentiation (95, 96).

The structure of the neutrophil is uniquely adapted to perform the cells' numerous functions (97). Perhaps the most important structural components of the cells are the cytoplasmic granules. These granules are distinct and adapted to perform specific functions. They have been broadly classified into three categories

based on their ultrastructural and cytochemical characteristics: primary or azurophil granules, secondary or specific granules, and tertiary or secretory granules. Granule secretions are used as markers for neutrophil activity. The azurophil granules are characterized by their content of myeloperoxidase, beta-glucuronidase enzymes and alpha-defensins (98). Markers for secondary granule activity include lactoferrin and vitamin B12 binding protein. The granules are released into the extracellular environment during cell movement or in response to specific stimuli, and they form the secretory component of the neutrophil. Tertiary granules are the most readily and rapidly secreted. These granules contain alkaline phosphatase and cytochrome b and are believed to play an important role in cell adhesion. Their function is the replenishment of cell surface receptors. Secretory granules contain the enzyme gelatinase, and it has been reported that the release of this enzyme may be related to increased expression of the adhesion-promoting glycoprotein Mo1 that functions as the receptor for complement component C3bi and mediates neutrophil binding (107).

### **3.2 General functions**

In spite of its short life span, neutrophil has numerous antimicrobial effector mechanisms. Neutrophils are recruited from the circulation to the infection sites by macrophages and mast cells by producing cytokines and chemokines that have encountered pathogens. Neutrophils are accumulated at the infection site and start phagocytic activity and neutralizing pathogens. Upon arriving at the inflammation



site, receptors on neutrophils recognize microorganisms and engulf them by a process called phagocytosis. The innate immune system recognizes microorganisms by identifying specific chemotactic formylated peptides expressed on prokaryotic cells. Neutrophils detect chemotactic stimuli gradients across their surface and migrate towards these stimuli in a concentration dependent movement (99). On initiation of an inflammatory process, the neutrophil responds to the stimulus by a series of well-coordinated functional responses. The neutrophil response to microbial invasion can be categorized as follows (93, 95, 96):

#### 1. Adherence and diapedesis

Neutrophils are recruited and activated to resolve infections, via phagocytosis and the release of antimicrobial molecules to the inflammation site. Neutrophil can be recruited from the vasculature by mast cells and macrophages that reside in the tissue. Pro-inflammatory signals such as interferon- $\alpha$  and interleukin-1 from these cells lead to the expression of adhesion molecules such as selectins and integrins on the surface of endothelial cells near the wound. TNF- $\alpha$  signaling also leads to an increase in capillary permeability and slow flow of blood as well as the recruitment of neutrophils to endothelial cells. This process involves margination and attachment of the neutrophil to vascular endothelium via specific molecules present on the surface of the neutrophil and the endothelial cell (97). Then the neutrophils roll along the endothelial wall, an interaction mainly mediated by p-selectin on the leukocyte surface and p-selectin glycoprotein ligand-1 (PSGL-1)

on the endothelial surface. Neutrophil rolling leads to a more firm neutrophil attachment to the endothelium, mediated by integrins such as Lymphocyte function-associated antigen 1 (LFA-1) and macrophage adhesion ligand 1 (Mac-1) on leukocytes and intercellular adhesion molecule-1 (ICAM-1) on the endothelial surface. Transmigration through endothelial cells into the infected tissue (also called diapedesis or extravasation) is mediated by platelet endothelial cell adhesion molecule (PECAM).

## 2. Chemotaxis

The neutrophils travel to the site of microbial injury in response to specific chemical agents (chemoattractants) (97). The term chemotaxis is used to describe the directed movement of cells against a concentration gradient in response to a chemoattractant. Numerous chemoattractants have been identified for neutrophils. These include complement fragment C5a, fMLP (a synthetic bacterial peptide), Platelet-activating factor (PAF), Interleukin-8 (IL-8) and the arachidonic acid metabolite LTB<sub>4</sub>. Each chemoattractant has a specific receptor that couples to a G-protein (guanyl nucleotide binding protein), which interdigitates and binds with hetero trimeric G-proteins. Occupancy of receptors with a ligand induces binding to G-proteins, exchange of bound GDP for free GTP, dissociation of heterotrimer, and release of activated subunits. Released alpha subunit of G protein activates phospholipase C, which in turn cleaves phosphatidyl inositol-bis-phosphate (PIP<sub>2</sub>) to produce inositol triphosphate IP<sub>3</sub> and diacylglycerol (DAG). IP<sub>3</sub> binds IP<sub>3</sub>

receptors on SER (smooth endoplasmic reticulum) such as calcisomes to release intracellular  $Ca^{++}$ . Elevated intracellular  $Ca^{++}$  and DAG bind protein kinase C which phosphorylates many membrane and cytosolic proteins in an ATP dependent manner (e.g. a kinase) (100). Upon chemoattractant signaling, actin monomers are polymerized to form F-actin with corresponding changes in the cytoskeleton and cellular shape, thus enabling cell mobility during neutrophil chemotaxis (101-103). In the extracellular matrix, the area of the cell that first contacts the chemotactic factor is the area that polarizes in the shape of protrusion. When neutrophil starts moving by a chemotactic factor, a protrusion is formed in the forward direction and adheres to the surface. The neutrophil will move forward, using the protrusion as an anchor. Finally, the neutrophil moves forward in the direction of chemotactic substrate by the action of actomyosin network (104-106). These directed movements occurring through adhesion and de-adhesion series are controlled by signaling systems that are mediated by adhesion molecules such as integrins. Maintaining the direction of the cell and the formed protrusion towards the chemoattractant chemical is regulated by the feedback loop between the adhesions and surface of the cell (107).

### 3. Phagocytosis

The invading organism is coated with molecules (such as IgG and C3b) called opsonins that are plasma proteins. This process of recognition and coating is referred to as opsonization. Opsonization enhances adhesion and renders the

organism more susceptible to phagocytosis. Phagocytosis is a receptor-mediated process between opsonin ligands IgG, C3 (complement component and fragments) and the receptor (FcR, C3R), internalizing a target particle or organ. The plasma membrane of a cell extends toward its target and initiates a process, eventually creating a phagosome, a membrane bound vacuole (108). Phagocytosis is the engulfment of a particle, which requires cytoskeletal rearrangement and membrane trafficking during active actin polymerization and the formation of a membrane-bound structure known as the "phagosome" or "phagocytic vesicle" (109). Neutrophils carry an array of receptors that enable them to recognize, bind and engulf pathogens. Thus, phagocytosis receptors, and receptor-ligand interactions must occur over the entire surface of the target particle. It also involves intraphagolysosomal secretion and isolation of the target organism within an extremely stringent environment. The next step is the fusion of lysosomes with the phagosome. The result is a phagolysosome. Lysosomes are derived from the Golgi apparatus, much like secretion vesicles, but their contents are focused on destroying microorganisms (110-112).

#### 4. Bacterial killing

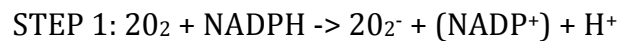
Neutrophils use an oxidative burst involving degranulation of secretory vesicles and subset of granules to kill microorganisms (108). The mechanism of bacterial killing and periodontal tissue destruction can be broadly classified into oxidative and non-oxidative killing(97). Non-oxidative killing involves secretion of

cytoplasmic granules into the phagosome by fusion of the phagosome with lysosome, forming the phagolysosomal complex. Granular contents can also be released extracellularly, in which the case the process is termed extracellular killing. The known chemicals which are secreted from the granules include lysosomal enzymes, peptides, and proteins including lysozyme; and permeability-inducing proteins such as defensins, and lactoferrin (113, 114). Oxidative killing involves the production of the potentially toxic reactive oxygen species (ROS) including superoxide anion ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical ( $OH^\cdot$ ), and hypochlorous acid (HOCl). Oxidative radicals have been shown to destroy proteins, lipids, carbohydrates and nucleic acids (115). Superoxide-mediated tissue damage can include cytotoxicity for host cells, induction of neutrophil apoptosis (116), induction of fibroblasts apoptosis (117), and connective tissue degradation. The basic mechanism is outlined below. This oxidative killing mechanism uses the ROS in an oxidative burst to kill the engulfed pathogen (118, 119). Superoxide production begins when nicotinamide adenine dinucleotide phosphate (NADPH) oxidase reduces oxygen to yield two oxygen anions, a proton, and oxidized  $NADP^+$ . When superoxide is released, it causes a signal transduction followed by a cellular response (119) and results in the formation of hydrogen peroxide when superoxide dismutase (SOD) metabolizes the two oxygen anions and proton. Peroxide and an oxygen anion are then converted to two hydroxide anions and oxygen. Myeloperoxidase (MPO) then metabolizes peroxide and chloride anion to generate HOCl and water. Both hypochlorous acid and nitric oxide are produced by MPO and

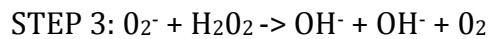
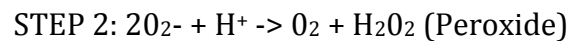
nitric oxide synthase in leukocytes (120). It is also important to note that malfunctions in this pathway are associated with disease.

### *Superoxide production mechanism*

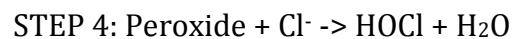
#### NADPH Oxidase



#### SOD



#### MPO



### 5. Elimination of neutrophils

If there are no specific anti-apoptotic signals generated by cytokines, neutrophils undergo apoptosis which mediates clearance of dying neutrophils from the inflammatory site, followed by subsequent ingestion by macrophages to clear neutrophils and promote resolution of inflammation (121). It has been proposed that circulating apoptotic neutrophils are cleared from circulation by macrophages located in the liver (~29%), spleen (~31%) and the bone marrow (~32%), suggesting that these three tissues contribute equally to neutrophil clearance from the circulation (122, 123). Tissue neutrophils, which migrate to tissues during

infection, are removed by local macrophages that secrete anti-inflammatory cytokines TGF- $\beta$  and IL-10 upon phagocytosis of neutrophils (124). For normal homeostasis to take place and in order to keep normal counts of neutrophils in the circulation ( $2.5-7.5 \times 10^9/l$ ), neutrophil turnover must be tightly balanced between granulopoiesis and neutrophil apoptosis/clearance. Neutrophil turnover is estimated to be  $\sim 10^{11}$  cells per day in the average adult human aged over 21. Delayed neutrophil apoptosis has been associated with several acute and chronic inflammatory diseases (125, 126).

### **3.3 Abnormal neutrophil Functions in Diabetes (DM)**

Functional abnormalities of neutrophil have been observed in several systemic disorders and might be associated not only with altered response against the microbial invasion, but also play a role in neutrophil-mediated tissue injury (127). In DM, neutrophil dysfunction has been noted in assays of neutrophil chemotaxis, adherence, and phagocytosis (128) and this dysfunction could lead to impaired host resistance to infection. A significantly lower chemotaxis has been found in neutrophil of diabetic patients (type 1 and type 2) than in those of controls (101, 129, 130). Conflicting data have been reported about the *in vitro* adherence of diabetic neutrophil without stimulation (131-133) as well as increased adhesion (134). In contrast, no differences in adherence have been found between diabetic and control neutrophils after stimulation (128). Neutrophils of diabetic patients have shown a lower phagocytic capacity compared to neutrophils of controls (135).

Bactericidal activity of diabetic neutrophils in general is lower than that of control neutrophils (136). Patients also have increased superoxide production (137) and reduced receptor expression (138). Finally, leukocytes in patients with diabetes also have impaired LTB<sub>4</sub> and signal transduction abnormalities (139).

Although the effects of hyperglycemia on ROS production in neutrophils from diabetic patients or normal controls in vitro have been studied by many authors, the results are still equivocal. ROS production in neutrophils seems to be impaired by hyperglycemia when the cells are activated by fMLP (140-142) whereas activation by PMA (141-145) particulate stimuli (80, 146-148) does not seem to be affected by elevated glucose concentrations in human neutrophils. Hyperosmolarity (149) and protein glycosylation (140) were also suggested to impair neutrophil ROS production. The exact mechanisms by which hyperglycemia affects ROS production in human neutrophils, and the intracellular signaling pathways sensitive to glucose, are still unclear and further investigation is needed.

### **3.4 Abnormal neutrophil Functions in Periodontitis**

Neutrophils are key actors in acute inflammatory reactions to pathogens. Arrival and accumulation of neutrophils in the tissue is part of the inflammatory process. Disorders that affect the neutrophil number or function strongly predispose individuals to infection. Neutrophil disorders can be divided into quantitative defects in neutrophils i.e., those that affect neutrophil numbers, and qualitative disorders i.e., neutrophil physiological functions. Individuals with



diminished neutrophil counts are usually involved with systemic diseases including agranulocytosis in which neutrophil count is less than 500/mm<sup>3</sup> of blood (150-153), neutropenia where the neutrophil count is less than 1500/mm<sup>3</sup> of blood, cyclic neutropenia (154-159), chronic benign neutropenia (160), chronic idiopathic neutropenia (161, 162), and familial benign chronic neutropenia (163-166), which are all associated with aggravation of periodontal disease.

Those with normal neutrophil counts (4000-8000/mm<sup>3</sup> of blood) but who exhibit functional neutrophil impairments are also predisposed to periodontitis. One example is disorders of the recruitment process of circulating neutrophils called leucocyte adhesion deficiency syndrome (LAD) (167) which exhibits abnormalities of adherence-dependent leucocyte function. Clinical features of LAD patients include rapidly progressive attachment and bone loss in the primary and permanent dentitions leading to premature tooth loss (168-171). Another inherited form of periodontitis is found in the dipeptidyl peptidase I deficiency or Papillon-Lefevre syndrome; caused by a mutations in the cathepsin C gene which produced a lysosomal protease called Cathepsin C, which is an important activator of neutrophil-derived serine proteases and an essential element in the pathway of normal physiological defense in periodontitis (172, 173). Other diseases of abnormal neutrophil function involving periodontitis are A1AT-deficiency: (114-116), Granulomatous disease (defective NADPH oxidase system) (107, 121-122), NA2 polymorphism in Fc RIIb (a neutrophil-specific antibody receptor) (123-124) and Multiple single nucleotide fMLP-R polymorphisms (3, 125-129).

On the other hand, hyperreactivity of neutrophils was also suggested to contribute to disease pathogenesis. Some studies have found elevated neutrophil chemotactic responses in aggressive periodontitis patients (174). *In vitro* functional studies indicate a hyperreactivity of the neutrophil in response to *A. actinomycetemcomitans*, which may be one of several pathways leading to more severe periodontal breakdown (175). Nicu et al. (176) showed that hyperreactive neutrophils in patients with periodontitis that are homozygous for FcγRIIa 131 H/H genotype have more severe bone loss than those with the H/R or R/R genotype. Periodontal attachment loss and tissue damage in the periodontium is due to a hyper-responsiveness of the neutrophil (139). There is also evidence that peripheral blood neutrophils of periodontitis patients are already in a hyperfunctional or primed state (177, 178), so their potential to cause host tissue damage by secretion of reactive oxygen species is enhanced. Apoptosis of these activated neutrophils is therefore a crucial step in the resolution of inflammation and the prevention of further damage caused by necrotic cell lysis and the release of cytotoxic granule proteins (179-181).

## **4. Apoptosis**

### **4.1 Definition of apoptosis**

Apoptosis, also termed programmed cell death, is a specific type of cell death that is crucial for maintaining an appropriate number of cells as well as the organization of tissue. The term 'apoptosis' was proposed in 1972 by Kerr and colleagues to differentiate naturally-occurring developmental cell death from the necrosis that results from acute tissue injury. They adopted the Greek word for the process of leaves falling from trees or petals falling from flowers (182).

Cells usually die resultant of two primary processes: necrosis or apoptosis. Cell death resulting from necrosis usually follows major pathological perturbations such as hypoxia, hyperthermia, and viral invasion, exposure to various exogenous toxins, or attack by complement. Necrosis is characterized by early mitochondrial swelling and rupture of the cell membrane with release of cell contents including proteases and lysozymes into the surrounding tissue. This in turn induces an inflammatory response with cytokine release by the surrounding macrophages (183).

Alternatively, cells undergoing apoptosis are individually destroyed while the integrity and architecture of surrounding tissue is preserved. It is characterized by well-defined morphologic changes. The early stage of apoptosis is characterized by compaction and margination of nuclear chromatin, condensation of cytoplasm and degrading DNA into small fragments of oligonucleosomes (184-186). This

results in disruption of the cytoskeleton, cell shrinkage, and membrane blebbing to form apoptotic bodies. Alterations of the plasma membrane include flipping of the anionic phospholipid phosphatidylserine from inside to the outside which signals neighboring phagocytic cells to recognize and engulf them. Apoptotic bodies are finally degraded within lysosomes of neighboring cells which prevents injury of surrounding tissues from the discharge of deleterious cytoplasmic contents (187-189).

#### **4.2 Apoptotic Process**

Apoptosis can be triggered by specific signals. One of the mechanisms of inducing apoptosis is through activation of receptors with "death domains" that belong to the TNF receptor family. They are activated through Fas and TNF receptor ligands binding, which leads to the activation of caspases. Apoptotic caspases exist in normal cells as inactive zymogens, analogous to the zymogens involved in the regulation of blood clotting with a prodomain of variable length followed by a large subunit (p20) and a small subunit (p10). When cells undergo apoptosis, these caspases become activated through one or two sequential proteolytic events (190-193). Caspases involved in the process of apoptosis have been divided into two subgroups based on their function:

1. Upstream or initiator caspases are those that are responsible for initiating the caspase cascade by becoming aggregated upon receipt of a pro-apoptotic stimulus and start of proteolytic activity (caspase-2, -8, -9 and -10). They tend to

have long N-terminal regions (prodomains) with caspase recruitment domain motifs (CARDs), and death effector domains (DEDs) that are also present in molecules such as FADD and apoptotic protease activating factor-1 (Apaf-1) which promote their aggregation (192, 194-197).

2. Downstream, or effector, caspases (caspase-3, -6, and -7) are thought to be responsible for the actual destruction of the cell and have a propensity to have short or absent prodomains. Active effector caspases promote apoptosis in several ways: by activating other destructive enzymes such as DNases by promoting mitochondrial cytochrome c release via Bcl-2 family proteins like BID and by degrading key structural and regulatory proteins within the cell (190, 196, 198, 199).

### **4.3 Apoptosis Signaling Pathways in Neutrophils**

In general, apoptotic pathways can be sub-divided into two categories: extrinsic apoptotic signals initiated by ligand engagement of cell surface receptors, and intrinsic pathways activated by signals emanating from cellular damage sensors or developmental cues. The pathways activated by extrinsic and intrinsic signals can overlap to some extent. Nonetheless, recent studies have shown non-caspase pathways also exist (200).

#### **1. Intrinsic pathway**

Mitochondria play an important role in the intrinsic pathway of apoptosis. Intrinsic signals can be induced by a variety of stimuli including DNA damaging

agents, kinase inhibitors, and activation of the cell surface death receptor (201, 202). These signals usually operate by triggering the release of three key mitochondrial proteins from the intramembrane space of the mitochondria to the cytosol: cytochrome c (cyt c), Smac/DIABLO, and apoptosis inducing factor (AIF) (203). Most notable among these is cytochrome c (204). Once released from the mitochondria, cytochrome c works together with the other two cytosolic protein factors, Apaf-1 and procaspase-9, to form the apoptosome and activate caspase-3. At the same time, Smac/DIABLO neutralizes IAPs and allows caspase activation to proceed. Biochemical analysis reveals a multistep reaction leading to caspase-3 activation. First, Apaf-1 binds ATP/dATP and hydrolyzes it to ADP and dADP. Second, the formation of a multimeric Apaf-1/cytochrome c complex is fully functional in recruiting and activating procaspase-9 (205, 206). Then, caspase-9 subsequently activates effector caspases such as 3, 6 and 7 (207) **(Fig. 1)**. This process is regulated by various proteins and molecules. Mcl-1 is a key Bcl-2 family protein in constitutive apoptosis. As neutrophils undergo apoptosis, levels of Mcl-1 fall rapidly suggesting a pro-survival role of this protein. Another antiapoptotic protein in neutrophils is the Bcl-2-A1 (Bfl1) gene product, which is largely cytoplasmic. Some data indicates that Bcl-2-A1 may function alongside Mcl-1 in neutrophils to control cell function (126). Neutrophils possess very few mitochondria as well as expressing low amounts of cyt c and Smac/DIABLO. Nevertheless, these amounts are sufficient to induce apoptosis. The tendency of

neutrophils towards spontaneous apoptosis is inversely correlated with Bcl-2 expression (208).

## 2. Extrinsic pathway

Caspase Activation by Cell Surface Death Receptors: extracellular signals can be triggered by engagement of cell surface death receptor (DR) with their specific death ligands which play a central role in apoptosis. Cell surface death receptors are a family of transmembrane proteins that belong to the TNF receptor superfamily. Mammalian death receptors include Fas (also called CD95) and TNFR1 (209, 210). Additional death receptors such as DR3 (death receptor 3), DR4 and DR5 are also structurally related to the TNF receptor superfamily (211). These receptors share a conserved cysteine-rich repeat at their extracellular domains. Although the regions of greatest sequence homology between superfamily members are extracellular, Fas and TNFR1 share a region of homology at the cytoplasmic face termed the death domain. This domain is required for apoptotic signaling by both Fas and TNFR1. The activating ligands for these DR are structurally related molecules that belong to the TNF gene superfamily (195). For example, Fas/CD95 ligand (FasL) binds to Fas, TNF binds to TNFR1, Apo3 ligand (Apo3L) binds to DR3, and Apo2 ligand (Apo2L, or TRAIL) binds to DR4 and DR5 (211). Previous studies also showed that neutrophil cell surfaces express both Fas and FasL which may contribute to an increased spontaneous apoptosis compared to other leukocytes (126, 212). When the Fas receptor binds its ligand, this recognition event is translated into intracellular

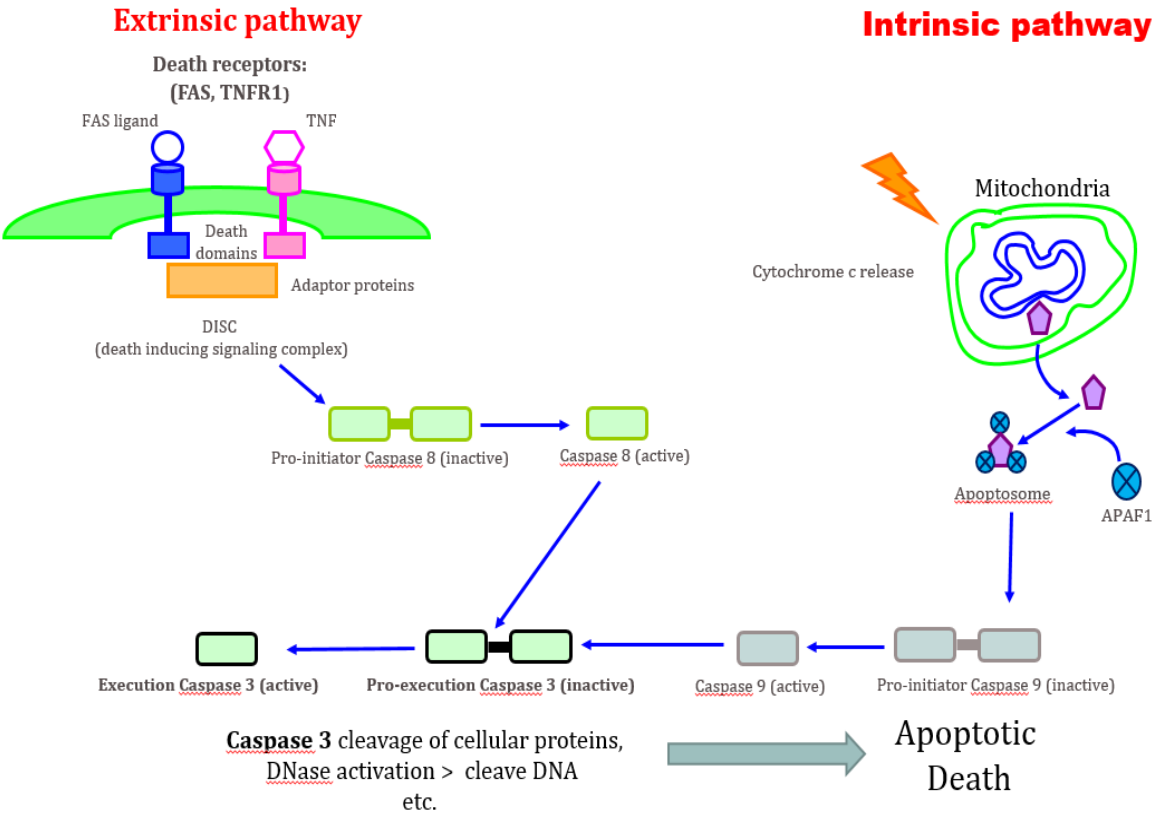
signals that eventually lead to caspase activation. In particular, there are three distinct steps: ligand-induced receptor trimerization, the recruitment of intracellular receptor-associated proteins, and the initiation of caspase activation. After binding of FasL to Fas receptors and trimerization, the cytoplasmic region of Fas, which contains a death domain (DD), recruits a death domain-containing adaptor molecule designated FADD. FADD also contains a death domain at its C terminus and binds to Fas via interactions between the death domains. Several other novel proteins that contain homologous death domains have subsequently been identified which include TRADD (TNF receptor associated death domain) and RIP (receptor interacting protein) (213). Whereas the death domain of FADD is necessary for physical association with the ligand bound-death receptors complex (the death-inducing signaling complex, or DISC), the N terminus of FADD, which is termed the death effector domain (DED), is critical for recruiting the upstream procaspases such as procaspase-8 and/or procaspase-10. Procaspase-8 contains two DED domains at the N-terminal region through which it binds FADD. Caspase-8 is then dimerized by FADD and proteolytically processed to the active forms that consist of large and small catalytic subunits. Once activated, caspase-8 can process and activate other 'effector' members of the caspase family, leading to cellular destruction (193, 214-216) **(Fig. 1)**.

As mentioned above, the extrinsic and intrinsic (cytosolic and mitochondria) pathways are not entirely distinct. Indeed, caspase 8 activation by FADD promotes cleavage and activation of a mitochondrial cytochrome c-releasing factor (Bid)



leading to engagement of the Apaf-1 /caspase 9 pathway. This pathway is regulated by members of the Bcl-2 family, which may be either pro- or anti-apoptotic or act on the mitochondria to regulate apoptosis. The cleavage of Bid, a Bcl-2 family member by caspase-8, serves as a link between the death receptor (cytosolic pathway) and mitochondrial death pathway (203, 217).

**Figure 1.** Caspase dependent pathway of apoptosis



**Figure 1.** Caspases are present as inactive pro-enzymes that are activated by proteolytic cleavage. Caspases 8, 9 and 3 are situated at pivotal junctions in apoptosis pathways. Caspase 8 initiates disassembly in response to extracellular apoptosis-inducing ligands and is activated in a complex associated with the cytoplasmic death domain of many cell surface receptors for the ligands. Caspase 9 activates disassembly in response to agents or insults that trigger the release of cytochrome c from mitochondria and is activated when complexed with apoptotic protease activating factor 1 (APAF-1) and extra-mitochondrial cytochrome c. Caspase 3 appears to amplify caspase 8 and caspase 9 initiation signals into full-fledged commitment to disassembly. Caspase 8 and caspase 9 activate caspase 3 by proteolytic cleavage and caspase 3 then cleaves vital cellular proteins or other caspases.

### 3. Caspase-independent pathway

Apoptosis-inducing factor (AIF) is a mitochondrion-localized flavoprotein. It is normally located in the intermembrane space which translocate from mitochondria to the cytosol and nucleus when apoptosis is induced. Caspase inhibitors cannot block the DNA fragmentation and chromatin condensation mediated by AIF, thus considered a caspase-independent pathway. Until now, the study on mechanism of caspase independent apoptosis in neutrophils is still lacking. AIF does not leave the mitochondria so it does not contribute to cell death in neutrophils (126).

#### **4.4 Substrate Specificity and Synthetic Peptide Inhibitors of Caspases**

Caspases are specific cysteine proteases that cleave their respective substrates at highly specific sites (218). For example, caspase-3 recognizes Asp-Glu-Val-Asp (DEVD) and cleaves poly (ADP-ribose) polymerase at this site. Because of the highly specific nature of the substrate site it is possible to design peptide-based reporter systems to monitor caspase-activity. Peptide-based caspase inhibitors are ideal for studying the apoptotic pathway because they are highly specific and nontoxic. In addition, they can be modified in order to bind irreversibly (219). Peptide inhibitors have been successfully used to examine apoptotic pathways under a variety of conditions in vivo and in vitro. A pan-caspase inhibitor usually is the initial blocker of choice because it enables one to dissect the pathways of the cell

death under certain conditions and investigate if it is dependent upon caspase-activity or not (220-227).

#### **4.5 Apoptosis and Diabetes**

Diabetes-induced cell death has been observed in multiple organs *in vivo* (228-230) and in cells *in vitro* (231-233). Insulin itself is anti-apoptotic (234). Many studies of diabetes-associated apoptosis have concentrated on the death of beta cells in the pancreas. However, a body of evidence has emerged that apoptosis plays an important role in several diabetic complications. For instance, the incidence of apoptosis increases in the heart of patients with diabetes and STZ-induced diabetic animals (235, 236). Apoptosis of neuronal cells occurs in diabetic neuropathy (237) and apoptosis of mesangial cells occurs in diabetic nephropathy (238, 239). Notwithstanding, the mechanism of diabetes-enhanced apoptosis is not well-understood. One factor might be a prolonged inflammatory response (240). A persistent infiltration of inflammatory cells coupled with advanced glycation end product (RAGE) axis caused by indirect effects of hyperglycemia could lead to sustained production of cytokines such as IL-1, IL-6 and TNF (241). Increased TNF can amplify apoptosis by caspase-3 activation. Another mechanism of diabetes increasing apoptosis is by the production of reactive oxygen species (ROS). Persistent inflammation and hyperglycemia could cause the cellular accumulation of ROS. Oxidative stress has been shown to induce apoptosis in various types of cells including fibroblasts (242, 243) especially for cells in areas of active proliferation

(244). ROS have been shown to cause mitochondrial cytochrome c release and activation of caspase-3 (245). Although relatively little is known about the specific caspases that are responsible for the increased apoptosis associated with diabetes in vivo, it has been reported that caspase-3 inhibitors block high glucose enhanced apoptosis in vitro (229, 231, 246). Mohr and colleagues (247) reported that the onset of diabetes is associated with the induction of several caspases (caspases-1, -2, -6, -8 and -9) and as diabetes progresses, the activities of the executioner caspases (caspase-3, -6) become more prominent.

#### **4.6 Apoptosis in Periodontal Disease**

Periodontal disease is characterized by the loss of connective tissue and bone resorption leading to loss of tooth support and, if untreated, tooth loss (248). In subjects with gingivitis or periodontitis, cell death was found to be significant in the most apical part of the gingival sulcus (249). Periodontal pathogens cause tissue destruction by a number of mechanisms including production of cell death-inducing factors such as leukotoxins, lipoproteins, and lipopolysaccharides (LPS) as well as by induction of inflammatory cells to secrete tissue destructive cytokines and lysosomal enzymes (250-258).

It is noteworthy that the loss of fibroblasts is one of the most distinctive cellular changes that occur in progressing periodontal disease (259). One mechanism by which fibroblasts may be lost is through stimulation of apoptosis. Interestingly, it has been reported that fibroblastic cells in patients with

periodontitis have the highest rate of apoptosis of the various cells in the gingival tissue (260). In addition, these apoptotic fibroblasts are observed predominantly in areas where inflammatory cells have been recruited (260, 261). Although bacterial products have been shown to induce apoptosis in fibroblast or osteoblast cell lines (205, 262-264), most studies of apoptosis in the periodontium were focused on apoptosis of leukocytes rather than matrix-producing cells (260, 265-268). Additionally, very little is known about how higher levels of apoptosis of matrix producing cells could limit tissue repair or regeneration.

#### **4.7 Abnormal resolution of inflammation in Diabetes and Periodontitis**

Resolution of inflammation is a tightly regulated active process that follows successful removal of foreign material. It consists of switching off proinflammatory pathways and clearing local tissue debris, ultimately leading to complete restoration of homeostasis. Essential signals from front line neutrophils are needed to initiate the resolution processes. Once it has accomplished its mission, the neutrophil triggers self-destruction mechanisms in a non-phlogistic manner by initiating apoptosis (269-271). Aberrant and prolonged activation of neutrophils can lead to tissue destruction with ineffective clearance of bacteria leading to chronic infection, inflammation, scarring and possibly even systemic complications (272, 273).

To date, research on neutrophil apoptosis, which plays a crucial role in resolution of inflammation in patients suffering with diabetes, still presents controversial results. A group from Japan found a decrease in caspase 3 enzyme in

diabetes subjects when compared to a healthy group. This was reinforced by another study which found an increase in amounts of anti-apoptosis cytokines produced from neutrophil in diabetes patients (274, 275). Studies in mice and rats suggest that there was no significant difference between spontaneous neutrophil apoptosis in diabetic and non-diabetic groups. Yet, in the presence of infection by *Staphylococcus aureus*, significantly fewer apoptotic cells were found among neutrophils from diabetic as opposed to nondiabetic mice (276). Similarly, a study in humans demonstrated that diabetic neutrophils undergo normal spontaneous apoptosis. But after co-incubation with *Escherichia coli* LPS, these neutrophils did not demonstrate an inhibition in apoptosis as compared to the non-diabetic neutrophil, and apoptosis was relatively increased (277, 278). Many studies have concluded that there is a decrease in neutrophil apoptosis in subjects with periodontal disease through a variety of approaches. The methods used include measurement of anti-apoptotic signaling molecules in the periodontal biopsy and gingival crevicular fluid, neutrophil extraction and *ex vivo* culture from infected periodontal biopsy or *in vitro* co-culture of HL60-derived neutrophils with *Porphyromonas gingivalis*; a well-known periodontal pathogen (255, 268, 279, 280).

Although a considerable amount of research has been dedicated to investigate the alteration in neutrophil apoptosis and resolution of inflammation in subjects with diabetes and chronic periodontal disease, it remains unclear whether peripheral blood neutrophils from these patients demonstrate defects in apoptosis. As a consequence, we hypothesized that in type 2 diabetes circulating neutrophil



exhibit delay in apoptosis, which may contribute to the persistent inflammation and compromised wound healing associated with diabetes. The delay in type 2 diabetes neutrophil apoptosis phenotype can be exaggerated by the presence of periodontal disease, which would further prolong inflammation and host tissue destruction by neutrophils.

## **VIII. HYPOTHESIS**

Based on the available data in the literature, we hypothesized that peripheral blood neutrophil spontaneous apoptosis is delayed in T2DM and that co-expression with chronic periodontitis exacerbates this delayed spontaneous neutrophil apoptosis.

## **IX. OBJECTIVE**

To explore the impact of type II diabetes and periodontal disease on peripheral blood spontaneous neutrophil apoptosis.

## **X. SPECIFIC AIMS**

1. To investigate and compare the nature of neutrophil apoptosis between people with type 2 diabetes and/or chronic periodontitis and healthy individuals.
2. To investigate and compare the mechanism of neutrophil apoptosis between people with type 2 diabetes and/or chronic periodontitis and healthy individuals.
3. To mimic type 2 diabetes with chronic periodontitis neutrophil apoptosis in healthy donors *in vitro*.

## **XI. MATERIALS AND METHODS**

### **1. Reagents**

S100B was purchased from EMD-Biosciences (San Diego, Cam, USA). Roswell Park Memorial Institute (RPMI) 1640 medium and fetal bovine serum (FBS) were obtained from American Type Cell Culture (Manassas, VA, USA). All other materials *were obtained from Sigma Chemical Co. (St. Louis, MO, USA).*

### **2. Subject recruitment**

In total, 73 individuals including those with type 2 diabetes (T2DM) (n= 37), moderate to severe chronic periodontitis (CP) (n= 15), and healthy volunteers (H) (n= 21) were recruited at the Clinical Research Center of Forsyth Institute. Healthy control subjects were normoglycemic individuals with no systemic or local infections (e.g., periodontitis). All subjects signed an informed consent documents and the study was approved by the Forsyth Institutional Review Board. Patients with T2DM were selected according to the criteria of the American Diabetes Association(2) with no diabetic complications, and patients with CP were selected according to the criteria of the American Academy of Periodontology(35). Demographic data for all patient groups including diabetes status, age, gender, race, smoking status, and BMI were recorded (**Table 2**).

**Table 2. Demographic characteristics of the study population.**

Sample size (n)		H (21)	CP (15)	T2DM (16)	T2DM+CP (21)
Age (mean in y $\pm$ S.D; median)		38 $\pm$ 11 (37)	47 $\pm$ 9 (48)	57 $\pm$ 7 (59)	57 $\pm$ 11 (59)
Gender	Male	11	9	7	15
	Female	10	6	9	6
Ethnicity	African-American	5	6	5	13
	Caucasian	14	8	9	5
	Asian	2	-	1	3
	Hispanic	-	1	1	-
BMI (mean $\pm$ S.D; median)		27.0 $\pm$ 4.7 (27.7)	27.3 $\pm$ 4.5 (27.4)	32.5 $\pm$ 5.9 (32.2)	32.8 $\pm$ 6.9 (31.5)
Smoking status	Non-Smoker	20	7	12	11
	Former smoker	1	4	4	7
	Smoker	-	4	-	3
Control of DM	Good (<7.5%)			10	11
	Moderate (7.5-8.5%)			3	5
	Poor (>8.5%)			3	5
HbA1c (mean in % $\pm$ S.D; median)				7.3 $\pm$ 1.4(6.8)	7.4 $\pm$ 1.2(7.15)
FBG (Mean in mg/dl $\pm$ S.D; median)				204.1 $\pm$ 93.6 (189.5)	178.0 $\pm$ 53.4 (160)
Duration of T2DM (mean in y $\pm$ S.D; median)				9.1 $\pm$ 5.9(8)	7.3 $\pm$ 4.4(7)

### **3. Neutrophil isolation**

In order to prepare the neutrophils to be used for the experiments, neutrophils were isolated and purified from the whole blood by “density gradient centrifugation”. This is a technique that allows the separation of particles on the basis of their size, shape, and density. A density gradient is typically created by layering media of increasing density in a centrifuge tube. When a sample is layered on top of a density gradient and centrifuged, the various particles move through the gradient at different rates. The particles appear as bands or zones in the gradient with the denser and larger particles migrating furthest. Peripheral venous blood was collected into vacutainer tubes containing 10 U/ml heparin. Neutrophils were isolated using a discontinuous gradient system as previously reported (281). Briefly, 3 ml MonoPoly™ 1119 and 2 ml Histopaque™ 1077 were layered in 15 ml polystyrene culture tubes. Peripheral blood (4.5 ml) was layered on the separating medium, and the tubes were centrifuged at 1,000 g for 15 min. The neutrophil-enriched layers were collected, and contaminating erythrocytes were lysed with a hypotonic NH<sub>4</sub>Cl buffer (155 mM NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, 120 mM EDTA, pH 7.4). The isolated cells were washed twice with PBS. Cell preparations were routinely 99% neutrophil with ≥95% viability, as determined by trypan blue exclusion.

#### **4. Quantitative TUNEL assay**

In order to quantify the amount of neutrophil apoptosis in this study, the TUNEL assay originally developed by Gavrieli et al. was used. (282) The biochemical hallmark of apoptosis is degradation of DNA by endonucleases, which produce double-stranded oligonucleosomal DNA fragments. These DNA fragments are 180-200 bp in size and can be separated into a ladder-like pattern on agarose gel electrophoresis. Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay has been designed to detect apoptotic cells that undergo extensive DNA degradation during the late stages of apoptosis. This method relies on the template-independent identification of blunt ends of double-stranded DNA breaks by TdT. The enzyme catalyzes the addition of labeled dUTPs to a 3' hydroxyl termini of DNA ends. In order to analyze the DNA fragmentation of apoptotic death in neutrophil, a TUNEL assay was performed. Briefly, neutrophils ( $1 \times 10^6$ ) were fixed with 1% (w/v) paraformaldehyde for 1 hour on ice, washed in PBS, pH 7.4 and post fixed in 70% (v/v) ethanol for 30 minutes on ice. Cell apoptosis was assessed by terminal deoxynucleotidyl-mediated dUTP nick-end labeling (TUNEL) assay. TUNEL staining of cells was performed with the APO-DIRECT Flow Cytometry Kit for Apoptosis (CHEMICON INTERNATIONAL, Cat.No: APT110,). Cells were re-suspended with 1.0 ml of wash buffer, and the process repeated 1 more time. Cells were then centrifuged and supernatants were aspirated. Cells were then incubated in the DNA Labeling Solution for 60 min at 37°C. After incubation, 1.0 ml of Rinse Buffer was added to each tube and centrifuged to wash the cells. In the end, the cell

pellet was re-suspended in 0.3 ml of the PI/RNase Staining Buffer and were incubated in the dark for 30 min at room temperature, followed by flow cytometric analysis to quantify the percentage of cells undergoing apoptosis. Cells were analyzed by flow cytometer (FACSCAN™, BECKTON-DICKINSON), using the CELLQUEST™ software (BECKTON-DICKINSON, Franklin Lakes, NJ, USA).

## **5. Annexin V Expression on neutrophil**

In order to quantify the amount of neutrophil apoptosis in this study, a special protein called “Annexin V” one of a family of calcium-dependent phospholipid-binding proteins, binds to phosphatidylserine (PS) to identify apoptotic cells. In healthy cells, PS is predominantly located along the cytosolic side of the plasma membrane. Upon initiation of apoptosis, PS loses its asymmetric distribution in the phospholipid bilayer and translocates to the extracellular membrane, which is detectable with fluorescently labeled Annexin V. In early stages of apoptosis, the plasma membrane excludes viability dyes such as propidium iodide (PI) and 7-AAD; therefore cells which display only Annexin V staining (PI/7-AAD negative) are in the early stages of apoptosis. During late-stage apoptosis, loss of cell membrane integrity allows Annexin V binding to cytosolic PS, as well as cell uptake of PI and 7-AAD. An Annexin V-fluorescein isothiocyanate (FITC)/PI apoptosis assay kit (Pharmingen, BDSciences) and flow cytometry were used to measure the Annexin V expression of neutrophil. The experiment was performed by following the manufacturer's instructions, with minor changes. Briefly, after isolation or

incubation with cell culture media, neutrophils ( $1 \times 10^6$ ) were washed twice with ice-cold PBS and then resuspended in binding buffer. Annexin V-FITC and PI were added into the culture tube and incubated for 15 minutes in the dark. Neutrophils were analyzed by flow cytometry within 1 hour of Annexin V-PI labeling. Viable neutrophils were defined as those found to be negative for Annexin V-FITC and PI staining; apoptotic neutrophil were defined as those positive for Annexin V-FITC staining but negative for PI staining. Cells positive for both Annexin V and PI staining were considered necrotic cells. Cell survival/apoptosis was expressed as a percentage of neutrophils relative to the total number of counted neutrophils.

## **6. Caspase activity in neutrophil**

In order to investigate the mechanism of neutrophil apoptosis, internal neutrophil apoptosis executing enzymes were first inspected. Pro-apoptotic signals activate the enzymatic cascade resulting in the cleavage of protein substrates, leading to the disassembly of the cell. Caspases (cysteine proteases) are categorized in two groups: the initiators (caspases 8 and 9) and the effector caspases (caspases 3). The initiator caspases 8 are involved with the extrinsic apoptosis pathway that originates upon activation of cell surface death receptors. Caspases 8 are monomers that bind to death receptor proteins through their death effector domain (DED) structure. Initiator caspase 9 is involved in the intrinsic apoptosis pathway that results from the mitochondrial release of cytochrome c. The caspase 9 monomer binds other proteins through their caspase activation and recruitment domain



(CARD). These initiator caspase-protein interactions result in dimerization of the initiator caspases that leads to their activation. The activated initiator caspases 8 and 9 then cleave effector pro-caspases at specific aspartic acid residues to yield large (20 kDa) and small (10 kDa) subunits that then assemble into the heterotetrameric, catalytically active forms of the caspase effector enzymes. Caspase 3, 8, and 9 protease activity was measured using the ApoTarget™ caspase3, 8, and 9 protease assay kit (BioSource International Inc., Camarillo, CA), according to the manufacturer's instructions. The kit allows detection of caspase proteolytic activity in lysates of mammalian cells. The caspase enzyme activities recognize the amino acid sequences DEVD (caspase-3), IETD (caspase-8), and LEHD (caspase-9). The individual substrates provided for measuring the activity of these caspases are synthetic peptides labeled at their C-termini with para-nitroaniline (pNA). Upon cleavage of the substrates by caspases, absorption of light by free pNA can be quantified using a spectrophotometer. In brief, neutrophils ( $1 \times 10^6$ ) were collected, washed in PBS, and suspended in 50  $\mu$ l of lysis buffer. Lysates were then centrifuged for 1 min at 10,000 g. The supernatant was incubated with substrate-pNA and reaction buffer for 2 h at 37°C. Levels of the chromophore pNA released by caspase activity were spectrophotometrically quantified at 405 nm using a microplate reader (Spectra MAX 340, Molecular Devices Co, and Sunnyvale, CA, USA). The data were normalized for protein concentration by using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA).

## **7. Neutrophil surface death receptor quantification**

In the extrinsic pathway of apoptosis, signal molecules such as Fas ligand (FasL) and Tumor necrosis factor alpha (TNF- $\alpha$ ), which are released by other cells, bind to transmembrane death receptors on the neutrophil to induce apoptosis. The binding of the FasL to Fas receptors or TNF- $\alpha$  to TNF receptors (a death receptor) on the neutrophil cell will trigger multiple receptors to aggregate together on the surface of the target cell. The aggregation of these receptors recruits an adaptor protein known as Fas-associated death domain protein (FADD) on the cytoplasmic side of the receptors. FADD, in turn, recruits caspase-8, an initiator protein, to form the death-inducing signal complex (DISC). Through the recruitment of caspase-8 to DISC, caspase-8 is activated and is able to directly activate caspase-3 to initiate degradation of the cell (200). In order to analyze the expression of neutrophil surface death receptor, neutrophils ( $1 \times 10^6$ ) were fixed with 1% paraformaldehyde at 4°C until the cells are used. Cells were incubated for 30 min at 4°C with 1% BSA in PBS to reduce non-specific binding of antibodies and fluorochrome reagents. The cells were then stained with FITC conjugated mouse anti-human CD120a (TNFR1) antibody (Pierce, Rockford, IL, USA) and APC conjugated mouse anti-human CD95 (FasR) antibody (Biolegend, San Diego, CA, USA) for 30 minutes in the dark as recommended by the manufacturer. All samples were analyzed using a flow cytometer (FACSCAN™, BECKTON-DICKINSON), using CELLQUEST™ software (BECKTON-DICKINSON, Franklin Lakes, NJ, USA).

## **8. Multiplex Cytokine and Inflammatory Biomarker Analysis**

In order to further investigate the mechanism of the extrinsic pathway of apoptosis inhibition in neutrophils signal molecules, including Fas ligand (FasL) and TNF- $\alpha$ , which are released by other cells and bind to transmembrane death receptors on the neutrophil to induce apoptosis, have to be measured. Multiplex arrays have been recently developed from traditional Enzyme-Linked ImmunoSorbent Assay (ELISA) with the purpose of measuring multiple cytokines in the same sample at the same time. They are available in several different formats based on the utilization of flow cytometry, chemiluminescence, or electrochemiluminescence technology. Flow cytometric multiplex arrays, also known as bead-based multiplex assays, represent probably the most commonly used format at the present time. The Luminex multi-analyte profiling (xMAP) technology from Luminex employs proprietary bead sets which are distinguishable under flow cytometry. Each bead set is coated with a specific capture antibody, and fluorescence or streptavidin-labeled detection antibodies bind to the specific cytokine-capture antibody complex on the bead set. Multiple cytokines in a biological liquid sample can thus be recognized and measured by the differences in both bead sets, with chromogenic or fluorogenic emissions detected using flow cytometric analysis(283).

The binding of the FasL to Fas receptors or TNF- $\alpha$  to TNF receptors (a death receptor) on the neutrophil cell will trigger multiple receptors to aggregate together

on the surface of the target cell. The aggregate of receptors recruits an adaptor protein known as Fas-associated death domain protein (FADD) on the cytoplasmic side of the receptors. FADD, in turn, recruits caspase-8, an initiator protein, to form the death-inducing signal complex (DISC). Through the recruitment of caspase-8 to DISC, caspase-8 will be activated and it is now able to directly activate caspase-3 to initiate degradation of the cell (200). In order to measure amount of serum FasL and TNF- $\alpha$ , 10 ml of whole blood was collected for serum separation in a non-heparinized tube. The sample was centrifuged at 1,500 g for 15 min at 4C and the serum layer was aliquoted and frozen at -80 C. Multiplexed sandwich immunoassays, based on flowmetric Luminex™ xMAP technology, were conducted at The Forsyth Institute (Cambridge, MA). Assays were carried out on a Bio-Plex 100™ platform. Immediately prior to the initiation of study measurements, the Bio-Plex platform underwent a complete on-site maintenance cycle and operational qualification by Forsyth Institute technicians. Assay kits were purchased from EMD Millipore Corp (Darmstadt, Germany).

## **9. Mimicking type 2 diabetes with chronic periodontitis delayed neutrophil apoptosis in healthy donors**

To further investigate the mechanism of neutrophil apoptosis, we used a model mimicking diabetes modified from Omori et al. (284) by culturing healthy subject neutrophil in diabetic conditions consisting of high glucose with or without S100B, a ligand for Receptor for Advanced Glycation Endproducts (RAGE).

Neutrophil ( $1 \times 10^6$ /ml) were cultured for 8 hrs in RPMI supplemented with 5.5 mM glucose (normoglycemic, NG) or 25 mM glucose (hyperglycemic, HG), with or without RAGE ligand, S100B.

In order to induce an inflammatory response similar to T2DM+CP, *P. gingivalis* (MOI 20) was co-incubated with the neutrophils ( $1 \times 10^6$ /ml) for 8 hrs in RPMI supplemented with 25 mM glucose (hyperglycemic, HG) and RAGE ligand, S100B 50 ug/ml.

*P. gingivalis* A7436 or W50 were grown on anaerobic blood agar plates (Becton Dickinson Microbiology System, Cockeysville, MD) in an anaerobic chamber with 85% N<sub>2</sub>, 5% H<sub>2</sub>, and 10% CO<sub>2</sub>. After incubation at 37°C for 2-3 days, the bacterial cells were inoculated into Blood Heart Infusion broth supplemented with 0.5 M Hemin, 0.1 M Menadione, and 0.75 M L-Cysteine, and grown for 1-2 days. Sterility of the bacterial cells was confirmed by gram staining. The bacterial cells were harvested by centrifugation at  $8,500 \times g$  (15 minutes at 4°C) and the pellet was washed 3 times with sterile PBS (pH 7.2). After washing, cells were opsonized with normal human serum for 30 minutes before being suspended in fresh PBS. Cultures were adjusted to an A<sub>660</sub> of 1.0 as read on a spectrophotometer (Beckman DU 7500, Canada). An OD reading of 1.0 nm corresponded to approximately  $10^9$  CFU/ml(285). Heat-killed *P. gingivalis* was generated by heating the bacteria suspension in a water bath (Precision Waterbath 184) at 60°C for 20 minutes. Viability of the bacteria was checked before and after heat-killing the bacteria. The purity of the cultures and the number of *P. gingivalis* were ascertained by plating.

## **10. Statistical analysis**

All data presented are representative of the average of at least three experiments with their standard error of mean except for demographic data, which used standard deviation. For isolated values, Student's t test was applied for significance, and ANOVA was used with LSD post-hoc analysis for multiple condition experiments.

## XII. RESULTS

**Specific aim 1:** To investigate and compare the nature of neutrophil apoptosis between people with type 2 diabetes and/or chronic periodontitis and healthy individuals.

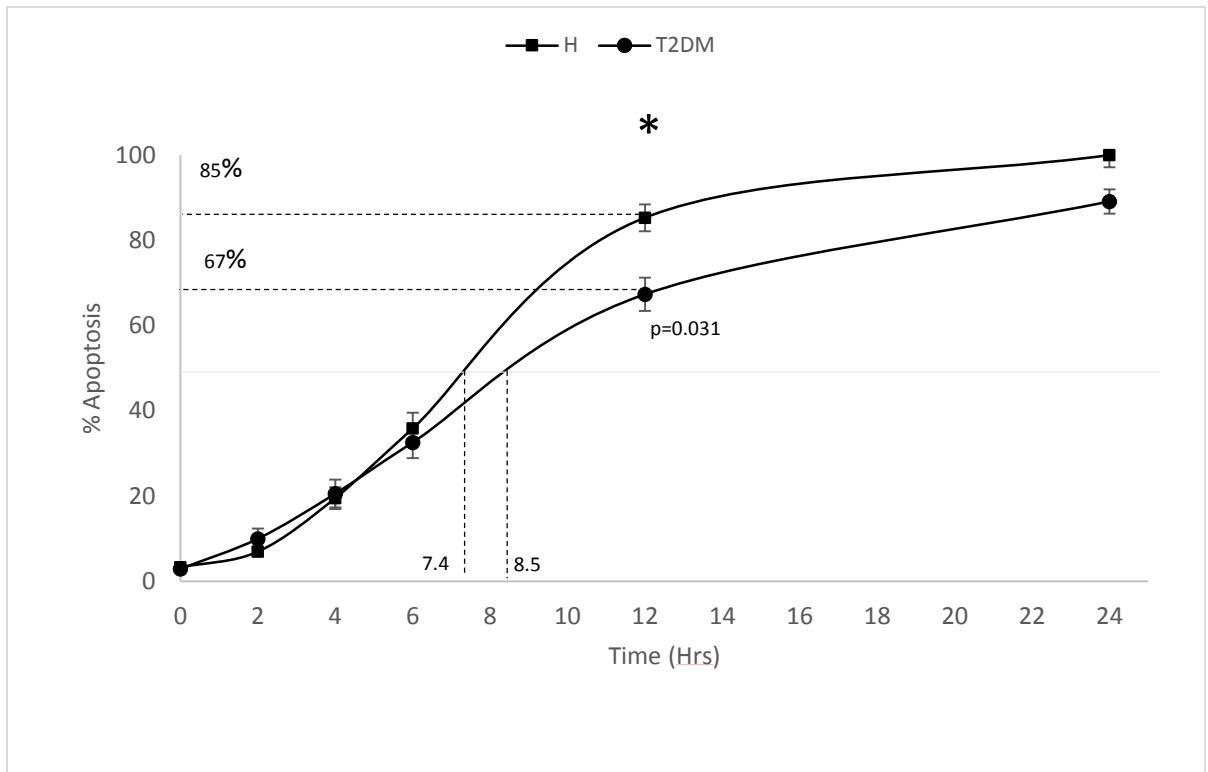
### 1. Spontaneous neutrophil apoptosis is delayed in type 2 diabetes subjects.

To compare spontaneous neutrophil apoptosis between type 2 diabetes (T2DM) and healthy subjects (H), individuals with of type 2 diabetes (T2DM) (n= 16) and healthy volunteers (H) (n= 21) were recruited at the Clinical Research Center at the Forsyth Institute. Neutrophils were isolated from whole blood using a discontinuous gradient system as previously reported (281). Neutrophils ( $1 \times 10^6$  cells/mL) were resuspended and cultured in RPMI-1640 supplemented with bovine fetal serum at 37°C in a 5% CO<sub>2</sub> atmosphere and were collected at time points 0, 2, 4, 6, 12, and 24 hrs. The collected neutrophils were analyzed by TUNEL assay. Spontaneous neutrophil apoptosis profiles were established for both groups. In healthy subjects, spontaneous neutrophil apoptosis reached 50% in 7.4 hrs and 85.3% ( $\pm 3.1$ ) in 12 hrs. In T2DM, spontaneous neutrophil apoptosis reached 50% in 8.5 hrs; and 67.3% ( $\pm 3.9$ ) in 12 hrs. In comparison to neutrophils from healthy volunteers, circulating neutrophils from the T2DM group showed a significant delay of apoptosis in *in vitro* culture after 12 hrs ( $p= 0.031$ ) (**Fig. 2**). This result was confirmed by Annexin V assay (data not shown).

Correlations between neutrophil apoptosis and T2DM characteristics, such as glycemic control, duration of diabetes, and obesity control were evaluated. Spontaneous neutrophil apoptosis in T2DM groups based on glycemic control, where HbA1c levels  $\leq 7.5\%$  represent good control and  $>7.5\%$  represent poor control, (286) showed a significant delay in neutrophil apoptosis in the poor glycemic control group compared to the good control group after 12 hrs ( $p=0.041$ ) (**Fig. 3A**). Grouping by duration of diabetes, ( $\leq 5$  yrs, 6-9 yrs, and  $\geq 10$  yrs) suggested a delay caused by increased disease duration which was not significant (**Fig. 3B**). When subjects were grouped by BMI as a measure of obesity, where a BMI  $\leq 30$  is ideal-overweight and  $>30$  is obese, (287) a significant delay in spontaneous neutrophil apoptosis was seen in the obese group compared to the ideal - overweight group at 12 hrs ( $p=0.006$ ) (**Fig. 3C**). The effect of smoking on neutrophil apoptosis was also analyzed, but no significant result was found (data not shown).

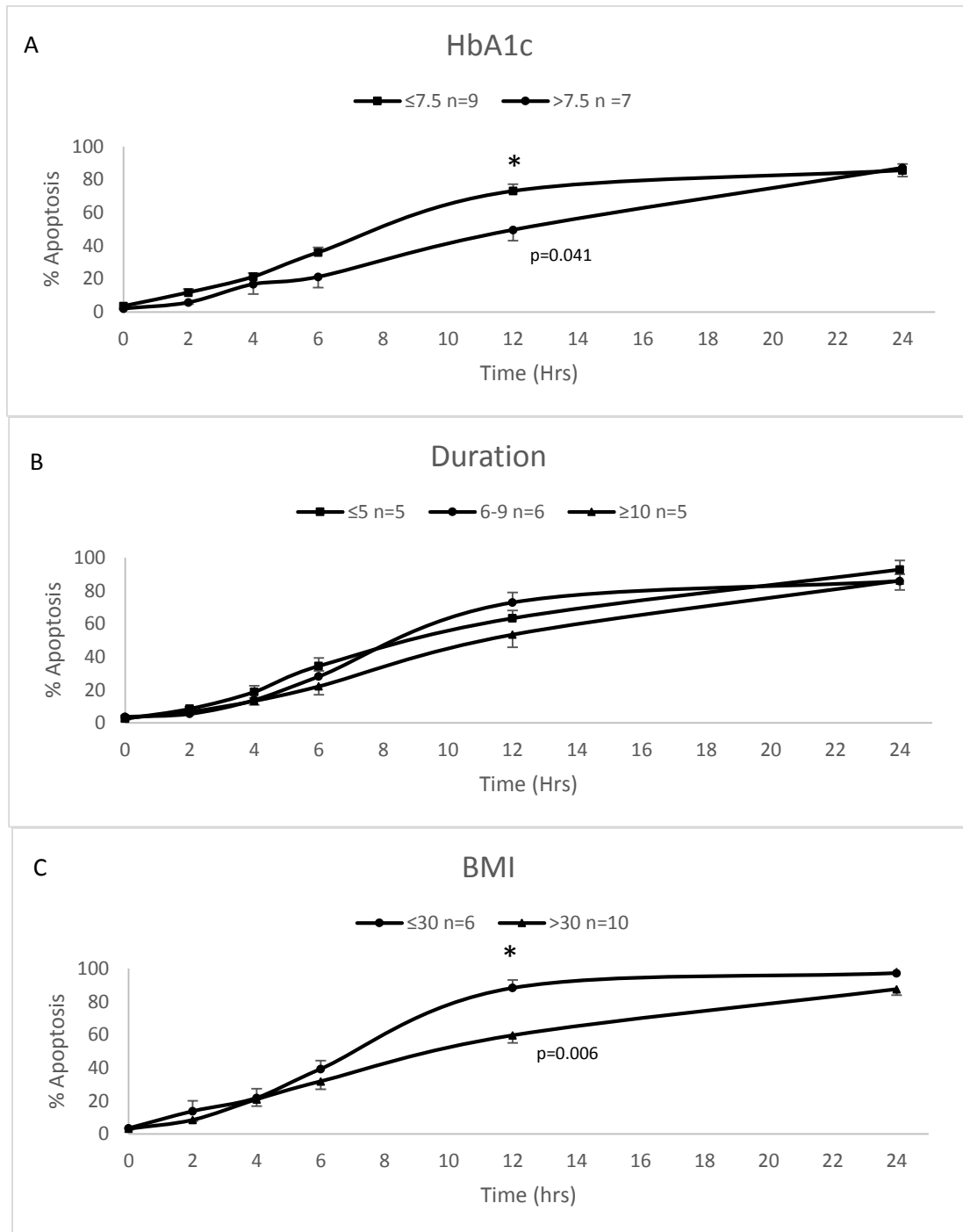


**Figure 2.** Comparison of spontaneous neutrophil apoptosis between H and T2DM subjects.



**Figure 2.** Spontaneous neutrophil apoptosis of healthy and T2DM subjects. Neutrophil ( $1 \times 10^6$  cells/mL) were resuspended and cultured in RPMI-1640 supplemented with bovine fetal serum at 37°C in a 5% CO<sub>2</sub> atmosphere and were collected at time points 0, 2, 4, 6, 12, and 24 hrs. The collected neutrophils were analyzed by TUNEL assay. A significant delay was seen in the T2DM group compared to the healthy group at the 12 hour time point. \*indicates  $p < 0.05$  vs healthy neutrophil at 12 hrs time point by Student's t test.

**Figure 3.** Correlations between neutrophil apoptosis and T2DM characteristics.



**Figure 3.** Neutrophils from T2DM groups ( $1 \times 10^6$  cells/mL) were resuspended and cultured in RPMI-1640 supplemented with bovine fetal serum at 37°C in a 5% CO<sub>2</sub> atmosphere and were collected at time points 0, 2, 4, 6, 12, and 24 hrs. The collected neutrophil were analyzed by TUNEL assay. (3A) Spontaneous neutrophil apoptosis based on glycemic control. A significant delay was seen in the group with HbA1c >7.5 compared to ≤7.5 group at the 12 hour time point. (3B) The effect of duration of diabetes on the spontaneous neutrophil apoptosis. Trends suggested a delay caused by increased disease duration, but the change was not statistically significant. (3C) Effect of body mass index (BMI) on spontaneous neutrophil apoptosis. A significant delay was seen in the group with BMI >30 compared to ≤30 group at the 12 hour time point.

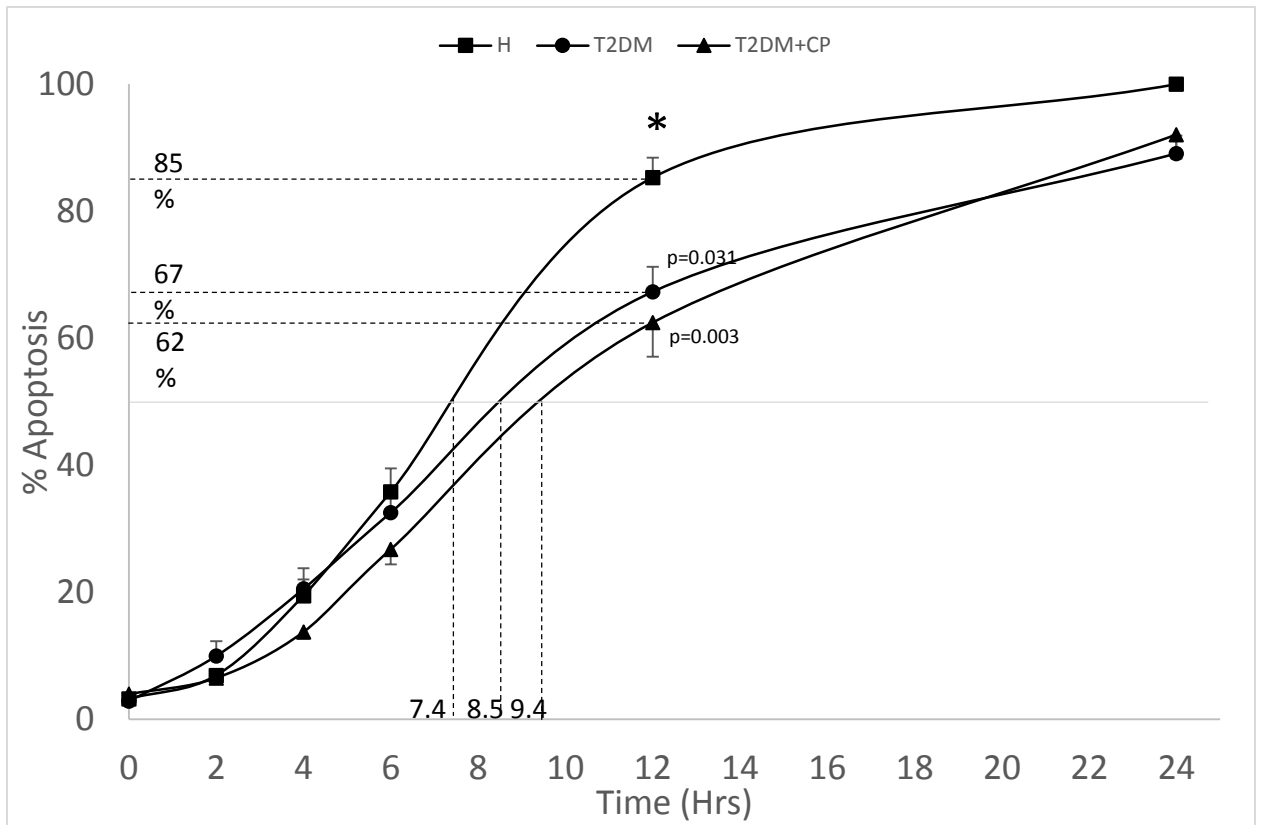
\*indicates  $p < 0.05$  at 12 hrs time point by Student's t test.

## **2. Spontaneous neutrophil apoptosis is delayed in type 2 diabetes subjects with chronic periodontitis**

Several studies have reported delayed neutrophil apoptosis in chronic periodontal disease (CP) (255, 280). Based on the delay in spontaneous apoptosis of neutrophil in T2DM, we investigated further to see if local infection and inflammation would play a role in changing the neutrophil apoptosis profile in the presence of both T2DM and CP.

In total, 73 individuals consisting of T2DM (n= 16), CP (n= 15), (T2DM+CP) (n= 21) and H (n= 21) were recruited. Neutrophils were isolated from whole blood using a discontinuous gradient system as previously reported (281). Neutrophils ( $1 \times 10^6$  cells/mL) were resuspended and cultured in RPMI-1640 supplemented with bovine fetal serum at 37°C in a 5% CO<sub>2</sub> atmosphere and were collected at time points 0, 2, 4, 6, 12, and 24 hrs. The collected neutrophils were analyzed with TUNEL assay. We established the spontaneous neutrophil apoptosis profile for each group, measured by TUNEL assay (**Fig. 4**). Spontaneous neutrophil apoptosis from CP and T2DM+CP reached 50% in 9.4 hrs, compared to 7.4 hrs in a healthy group. In 12 hrs, neutrophils from the CP and T2DM+CP groups showed a significant delay in spontaneous apoptosis by apoptosis rates of  $62.9\% \pm 3.5$  and  $62.5\% \pm 5.4$ , respectively, compared to an apoptosis rate of  $85.3\% \pm 3.1$  ( $p= 0.003$ ) in the healthy neutrophil.

**Figure 4.** Comparison of spontaneous neutrophil apoptosis between H, T2DM, and T2DM+CP subjects.



**Figure 4.** Neutrophil ( $1 \times 10^6$  cells/mL) were resuspended and cultured in RPMI-1640 supplemented with bovine fetal serum at 37°C in a 5% CO<sub>2</sub> atmosphere and were collected at time points 0, 2, 4, 6, 12, and 24 hrs. The collected neutrophils were analyzed with TUNEL assay. A significant delay in apoptosis is seen in the T2DM+CP group as compared to the healthy group at 12 hours. Healthy neutrophils demonstrated 85.3%  $\pm$  3.1 apoptosis after 12 hours. Meanwhile, T2DM+CP neutrophils reached only 62.5%  $\pm$  5.4 apoptosis after 12 hours.

\* indicates  $p < 0.05$  vs healthy neutrophil by one-way ANOVA.

**Specific aim 2:** To investigate and compare the mechanism of neutrophil apoptosis between people with type 2 diabetes and/or chronic periodontitis and healthy individuals.

### **3. Decreased caspase activity in type 2 diabetes with chronic periodontitis subjects**

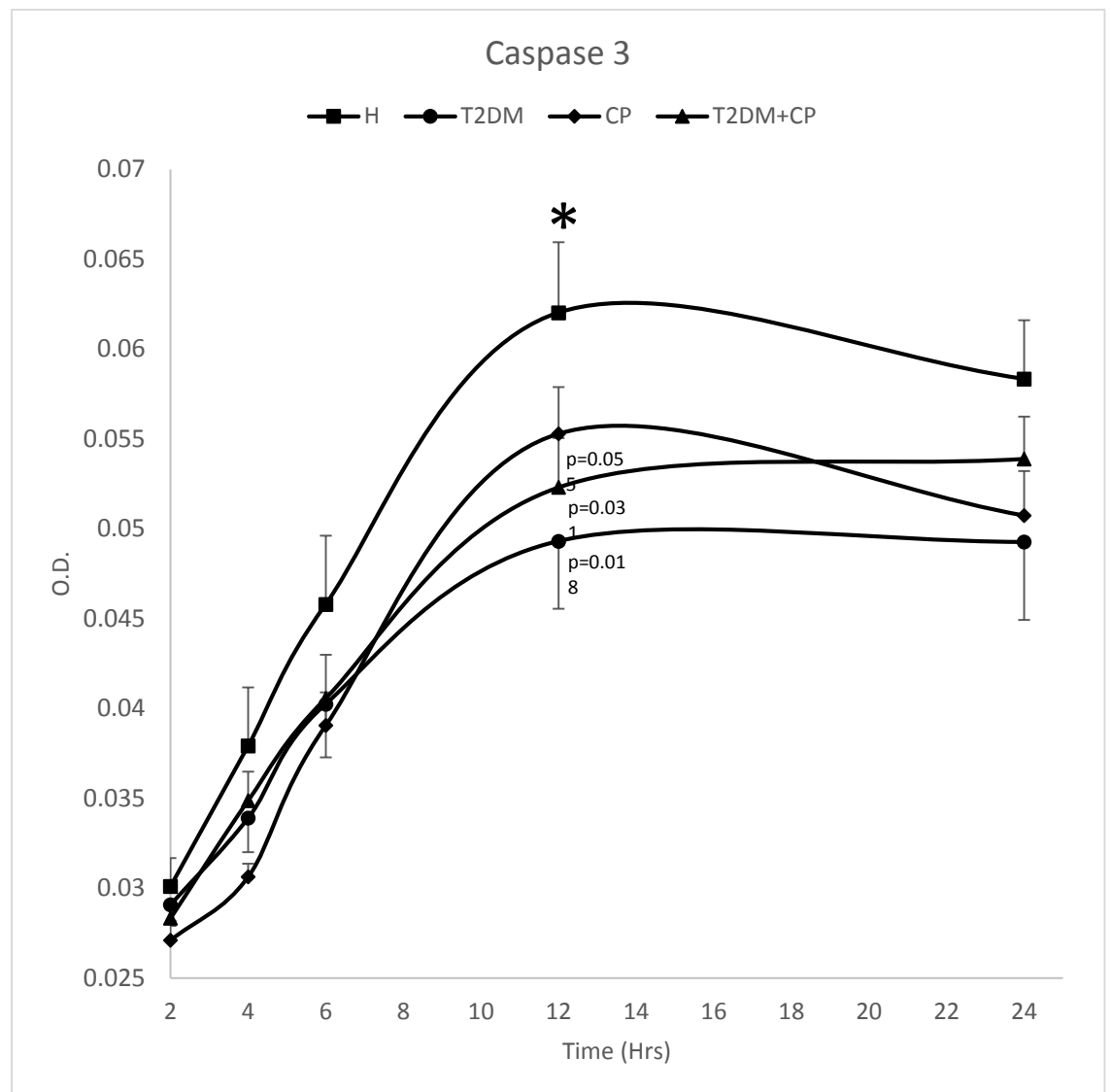
Our results have shown that spontaneous neutrophil apoptosis was delayed in patients with type 2 diabetes and chronic periodontitis. To further explore the mechanism of apoptosis, we measured the caspase activity, an essential downstream enzyme in regulating the apoptosis cascade, by using a caspase colorimetric assay. Neutrophils ( $1 \times 10^6$  cells/mL) were resuspended and cultured in RPMI-1640 supplemented with bovine fetal serum at 37°C in a 5% CO<sub>2</sub> atmosphere and were analyzed with caspase protease assay kit. Caspase 3 activity was measured after 2, 4, 6, 12 and 24 hrs of incubation (n=12). The healthy group showed a significantly higher activity at 12 hrs as compared to the T2DM (p= 0.018) and T2DM+CP groups (p= 0.031) **(Fig.5)**.

As there was a significant decrease in diseased subjects' caspase-3 activity compared to the healthy group, we investigated whether the inhibition of caspase-3 activation was due to upstream decreases in either caspase-9 activity, which is the initiator caspase enzyme representing the intrinsic (mitochondrial) pathway; caspase-8 activity, representing the extrinsic (death receptor) pathway; or both.



Neutrophils ( $1 \times 10^6$  cells/mL) were resuspended and cultured in RPMI-1640 supplemented with bovine fetal serum at 37°C in a 5% CO<sub>2</sub> atmosphere for 6 hrs and were analyzed with caspase protease assay kit (n=5). In figure 6A, neutrophil caspase-8 enzyme activity from T2DM+CP was significantly decreased as compared to healthy subjects (p= 0.046). A decrease in caspase-8 activity was also observed in the T2DM and CP groups; however, the result was not significant (p=0.059, p=0.055 respectively). Neutrophils from neither the T2DM nor T2DM+CP groups showed a significant change in caspase-9 activity compared to healthy group (**Fig. 6B**).

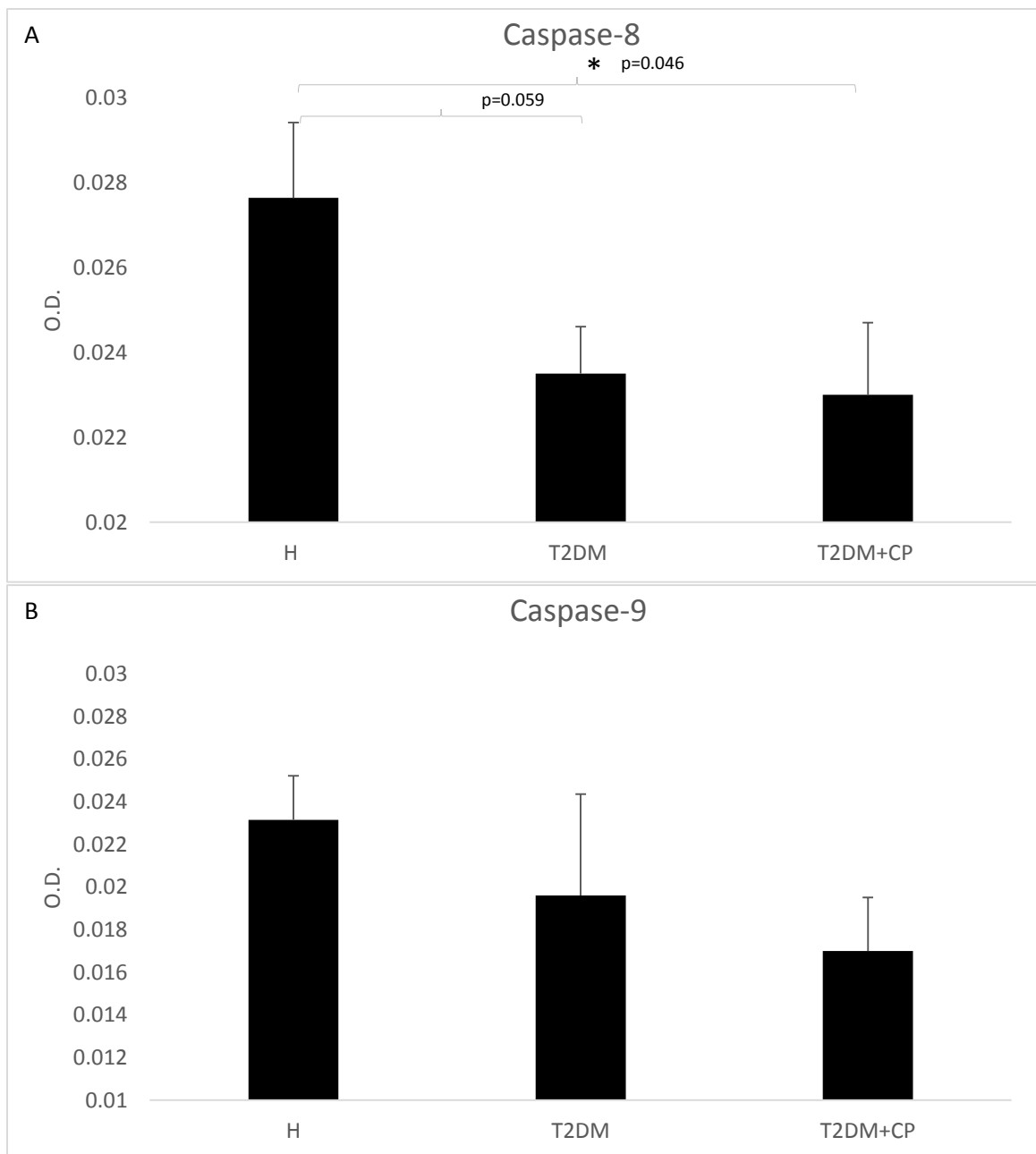
**Figure 5.** Comparison of caspase 3 activities between H, T2DM, and T2DM+CP subjects.



**Figure 5.** Neutrophil ( $1 \times 10^6$  cells/mL) were resuspended and cultured in RPMI-1640 supplemented with bovine fetal serum at 37°C in a 5% CO<sub>2</sub> atmosphere and were analyzed with caspase protease assay kit. Comparison of caspase-3 activity between groups after 12 hrs incubation (n=12) showed significantly higher levels of caspase-3 activity in healthy neutrophils than in both the T2DM and T2DM+CP groups (12 hrs). No significant differences between diseased groups.

\* indicates  $p < 0.05$  vs healthy neutrophil by One-way ANOVA.

**Figure 6.** Comparison of caspase 8, and 9 activities between H, T2DM, and T2DM+CP subjects.



**Figure 6.** Neutrophils ( $1 \times 10^6$  cells/mL) were resuspended and cultured in RPMI-1640 supplemented with bovine fetal serum at 37°C in a 5% CO<sub>2</sub> atmosphere and were analyzed with caspase protease assay kit. (6A) Caspase-8 activity between healthy and diseased groups after 6 hours (n=5) shows a significant decrease in caspase-8 activity in the T2DM+CP group as compared to the H group. A decrease was also observed in the T2DM group; however, the difference was not significant. (6B) Comparison of caspase-9 activity between groups after 6 hrs incubation (n=5) shows no significant differences between the healthy and diseased groups.

\* indicates  $p < 0.05$  vs healthy neutrophil by one-way ANOVA.

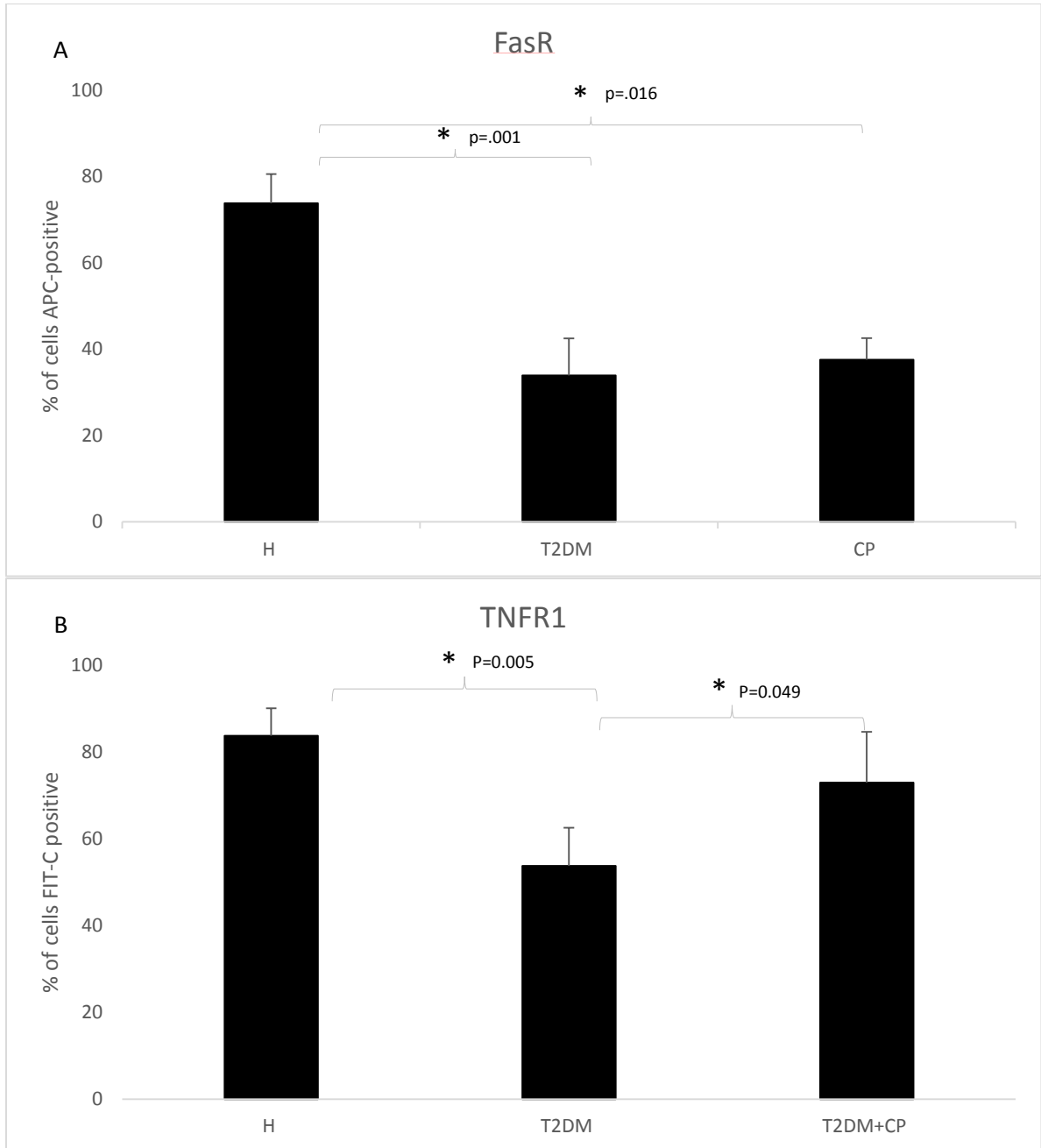
#### **4. Diminished cell surface death receptor expression and their ligands are found in neutrophil from subjects with type 2 diabetes and chronic periodontitis**

In the extrinsic pathway of apoptosis, signaling molecules such as FasL and TNF- $\alpha$ , which are released by other cells, bind to transmembrane death receptors on the neutrophil to induce apoptosis. The binding of the FasL to Fas receptors or TNF- $\alpha$  to TNF receptors (a death receptor) on the neutrophil cell surface triggers caspase 8 activation. Our results have shown a decrease in caspase 8 activation in neutrophil from T2DM and CP patients.

In order to further characterize the decrease in caspase 8 in subjects with type 2 diabetes and chronic periodontitis, the expression of cell surface death receptors including FAS receptor (CD95) and TNF receptor 1 (CD120a) which activated the caspase 8 enzyme through cooperation with their adaptor proteins was measured. Paraformaldehyde-fixed neutrophils ( $1 \times 10^6$ ) were incubated with FITC conjugated mouse anti-human CD120a antibody and APC conjugated mouse anti-human CD95 antibody (n=6). All samples were analyzed using a flow cytometer. The results have shown a drastic reduction of cell surface Fas receptor in neutrophils from T2DM (p=0.001) and T2DM+CP (p=0.016) subjects compared to healthy control (**Fig. 7A**). Cell surface TNF receptor results from T2DM also showed a significant decrease compared to healthy subjects (p=0.005). No significant decreases were observed in CP group or T2DM+CP group compared to healthy control (**Fig. 7B**).

In order to further characterize the decrease in caspase-8 in subjects with type 2 diabetes and chronic periodontitis, we measured the levels of FasL and TNF- $\alpha$  in serum. Ten ml of whole blood was collected for serum separation in a non-heparinized tube. Multiplexed sandwich immunoassays, based on flowmetric Luminex™ xMAP technology, were used to measure the amount of both cytokines in soluble in the serum (n=10). Results showed a decrease in FasL in all diseased subjects compared to healthy subjects, especially in T2DM+CP which presented a significantly lower level than in the healthy group (p= 0.035) **(Fig. 8A)**. However, no significant differences in TNF- $\alpha$  levels were observed compared to healthy subjects **(Fig. 8B)**.

**Figure 7.** Cell surface death receptor expression in T2DM and CP.

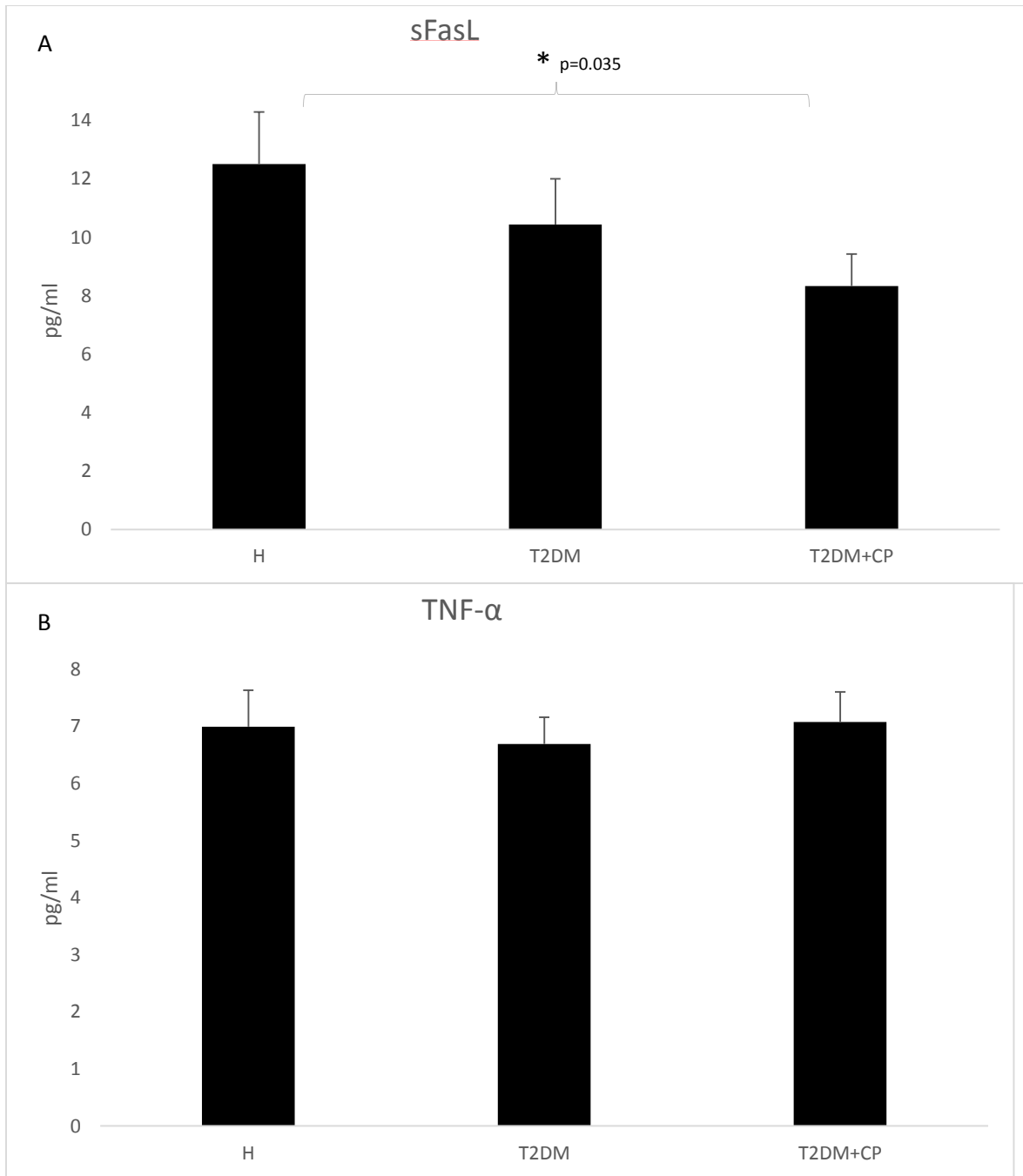




**Figure 7.** Neutrophils( $1 \times 10^6$  cells) were fixed with 1% paraformaldehyde at 4°C. Cells were incubated with PBS with 1% BSA and then stained with FITC conjugated anti-human CD120a (TNFR1) antibody and APC conjugated anti-human CD95 (FasR). All samples were analyzed using a flow cytometer. (7A) Cell surface Fas receptor expression in H, T2DM, and T2DM+CP was then examined, and a significant decrease in serum Fas receptor expression was observed in the T2DM and T2DM+CP groups as compared to healthy subjects (n=6). (7B) Cell surface TNF receptor expression was similarly evaluated in the H, T2DM, and T2DM+CP groups. A significant decrease in serum Fas receptor expression was observed in the T2DM group as compared to healthy subjects (n=6).

\* indicates  $p < 0.05$  vs healthy control by One-way ANOVA.

**Figure 8.** Death receptor ligand expression in T2DM and CP.



**Figure 8.** Whole blood was collected for serum separation and multiplexed sandwich immunoassays were conducted. (8A) Serum levels of sFasL in H, T2DM, and T2DM+CP was then examined, and a significant decrease in serum sFasL levels was seen in the T2DM+CP groups as compared to healthy serum. (n=10). (8B) Serum levels of sFasL in H, T2DM, and T2DM+CP was then examined, and no significant difference was seen between groups (n=10).

\* indicates  $p < 0.05$  vs healthy control by One-way ANOVA.

**Specific aim 3:** To mimic type 2 diabetes + chronic periodontitis neutrophil apoptosis in healthy donors in vitro.

## **5. Mimicking chronic periodontitis delayed neutrophil apoptosis in healthy donors**

To further investigate the mechanism of neutrophil apoptosis in chronic periodontal disease as a factor that modulates diabetic neutrophil response, we investigated the action of *P. gingivalis* on neutrophil apoptosis. *P. gingivalis* is a nonmotile, gram negative pleomorphic rod. It is most closely associated with chronic periodontitis and is a member of the red complex of bacteria. *P. gingivalis* is a major member of the pathogenic microbiota in severe periodontal diseases.

Neutrophils ( $1 \times 10^6$  cells/mL) (n=4) were resuspended and cultured in RPMI-1640 supplemented with bovine fetal serum at 37°C in a 5% CO<sub>2</sub> environment. The collected neutrophils were analyzed with Annexin V-FITC staining. Our results showed that neutrophils co-cultured with *P. gingivalis* resulted in delayed neutrophil apoptosis. Time course studies using a *P. gingivalis* A7436 multiplicity of infection (MOI) of 20 and neutrophil co-incubation for 6, 8, 12 and 24 hrs were performed. Significant differences were found at all-time points, except baseline (p<0.001) (**Fig. 9**). **Figure 10** demonstrates that this effect was dose dependent. *P. gingivalis* A7436 MOI of 5, 10, and 20 resulted in significant delays in neutrophil apoptosis after 8 hrs of co-incubation (p=0.004, p=0.003 and p=0.001, respectively). Interestingly, at an MOI of 50, the effect was slightly reversed but still significantly

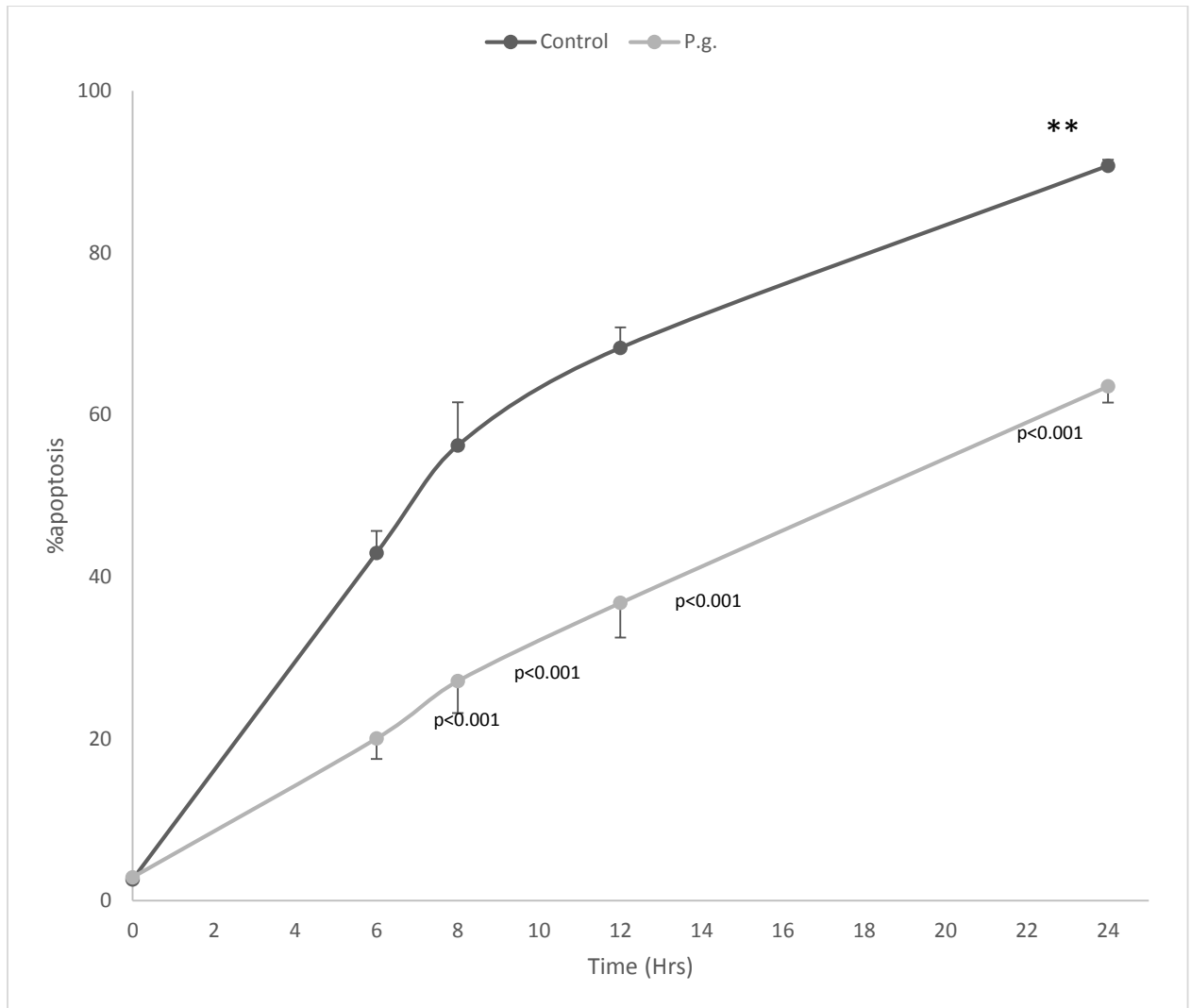
different compared to PBS control ( $p=0.009$ ). We evaluated the ability of *P. gingivalis* to inhibit neutrophil apoptosis from 2 strains, A7436 and W50, both used at an MOI of 20. **Figure 11** shows similar results after 8 hrs of co-incubation between the 2 strains, both of which demonstrate significant decrease in neutrophil apoptosis as compared to the controls ( $p<0.001$  and  $p=0.001$ , respectively). To evaluate which component or factor from *P. gingivalis* plays a role in delaying apoptosis; live, heat killed (HK) *P. gingivalis* A7436 (MOI 20) and *P. gingivalis* A7436 LPS at 200 ng/ml (which corresponds to  $2 \times 10^7$  bacteria per ml) were co-incubated with neutrophils for 8 hrs. All groups exhibited a reduction in neutrophil apoptosis. Live, heat-killed and *P. gingivalis* LPS significantly delayed neutrophil apoptosis compared to PBS control ( $p=0.001$   $p=0.002$  and  $p=0.011$  respectively) (**Fig. 12**). *E. coli* was used as a positive control which has previously shown an increase in neutrophil apoptosis as reported by Watson et al (288).

Our results showed that neutrophil apoptosis was delayed by co-culture of neutrophils with *P. gingivalis*. To confirm these results, we measured the caspase activity, which is an essential downstream enzyme in regulating the apoptosis cascade, by using a caspase colorimetric assay. The results demonstrated a significant decrease in caspase-3 activity compared to the control ( $p< 0.001$ ) (**Fig 13A**).

As there was a significant decrease in caspase-3 activity compared to control, we then investigated whether the inhibition of caspase-3 activation was due to an upstream decrease in caspase-9 activity. Caspase-9 is the initiator caspase enzyme

representing the intrinsic pathway. We also examined the caspase-8 activity, which represents the extrinsic pathway. As shown in **Figure 13B**, no significant change in caspase-8 activity was observed; however, caspase-9 activity decreased significantly compared to control neutrophils ( $p=0.003$ ) (**Fig 13C**).

**Figure 9.** *P. gingivalis* delays neutrophil apoptosis by time point

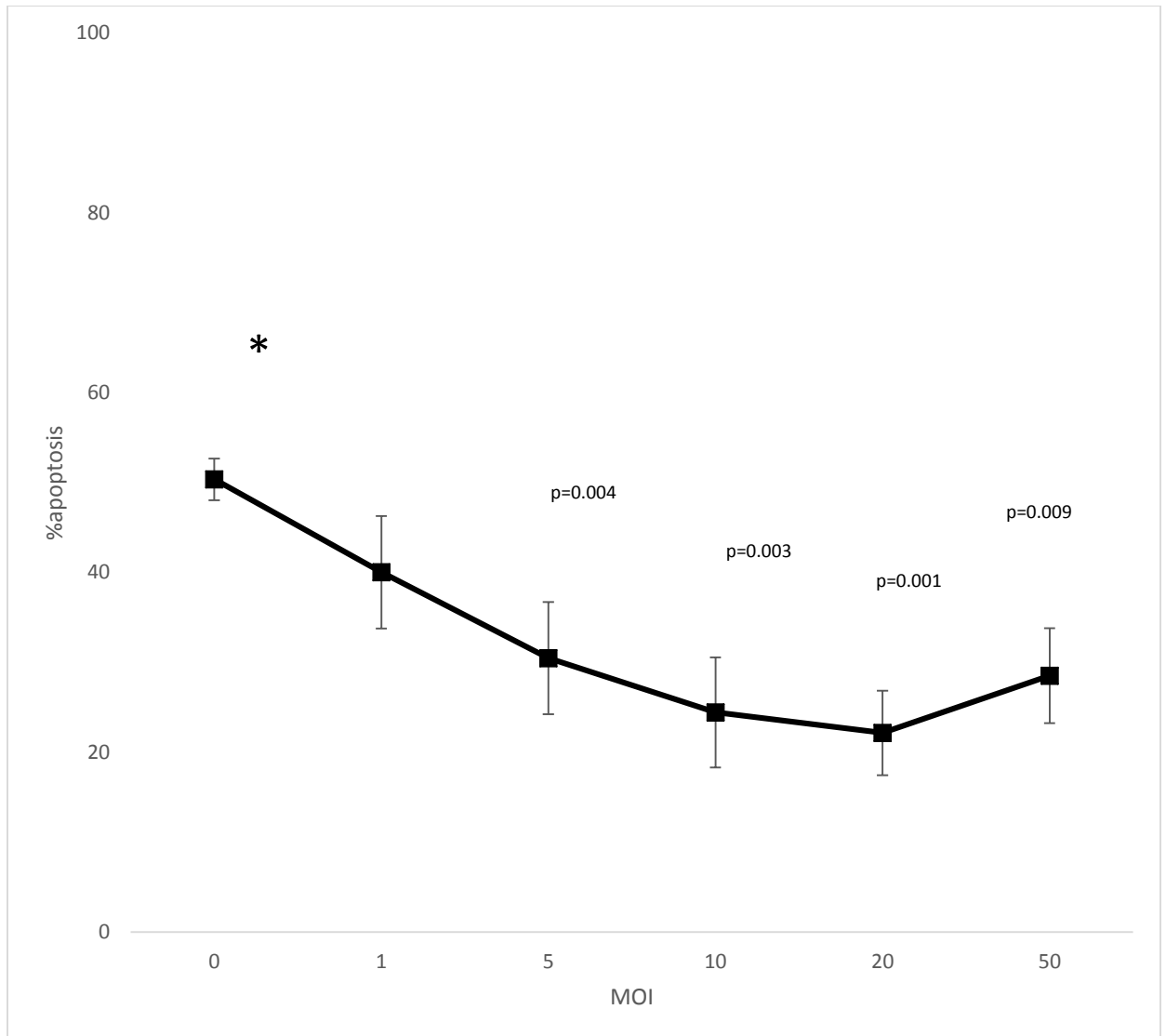


**Figure 9.** Neutrophils ( $1 \times 10^6$  cells/mL) (n=4) were resuspended and cultured in RPMI-1640 supplemented with bovine fetal serum at 37°C in a 5% CO<sub>2</sub> environment. The collected neutrophils were analyzed with Annexin V-FITC staining. Comparison of spontaneous apoptosis of neutrophil-*P. gingivalis* A7436 (MOI 20) co-incubation vs control at time points 0, 6, 8, 12 and 24 hrs showed a significant decrease in apoptosis of co-incubated neutrophils.

\*\* indicates  $p < 0.05$  vs control by Student's t test.



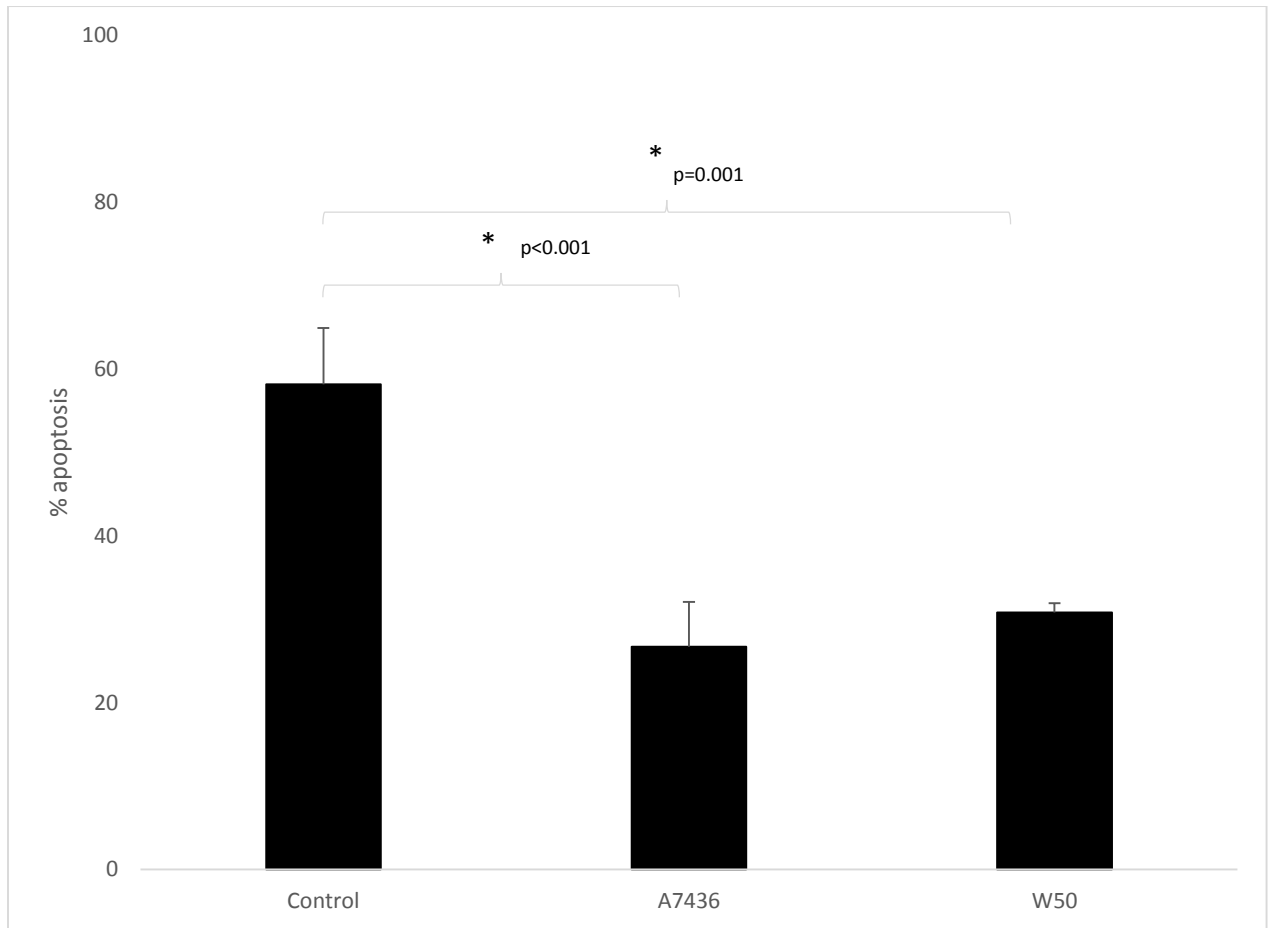
**Figure 10.** *P. gingivalis* delays neutrophil apoptosis by MOI



**Figure 10.** Percentage of neutrophil apoptosis seen at various concentrations of *P. gingivalis* A7436 after 8 hrs co-incubation. Neutrophils ( $1 \times 10^6$  cells/mL) (n=4) were resuspended and cultured in RPMI-1640 supplemented with bovine fetal serum at 37°C in a 5% CO<sub>2</sub> environment. The collected neutrophils were analyzed with Annexin V-FITC staining. The most significant decrease in apoptosis was at an MOI of 20.

\* indicates  $p < 0.05$  vs control by one-way ANOVA.

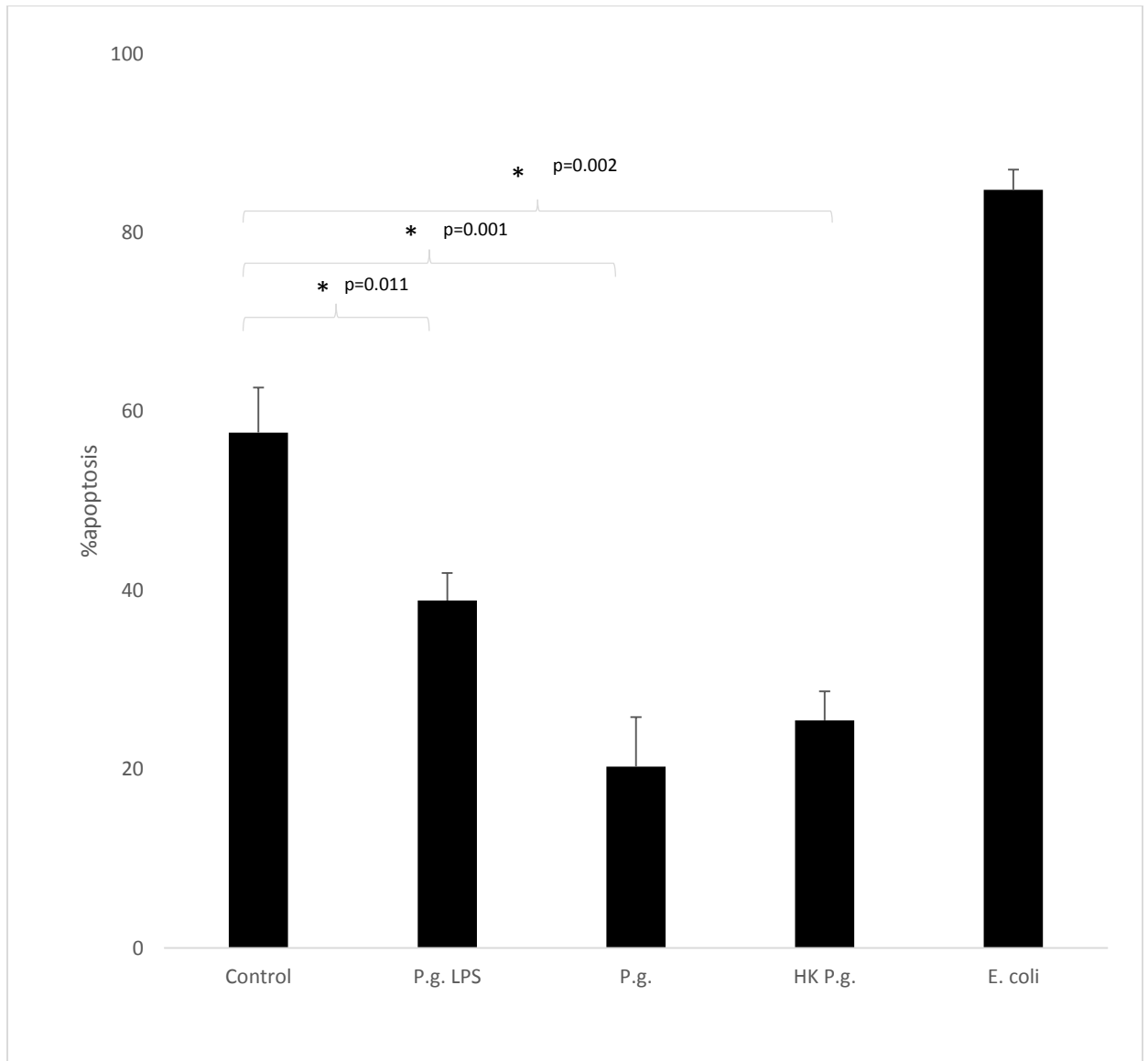
**Figure 11.** *P. gingivalis* delays neutrophil apoptosis by strains



**Figure 11.** Comparison between *P. gingivalis* strains A7436 and W50, both at MOI 20. Neutrophil ( $1 \times 10^6$  cells/mL) (n=4) were resuspended and cultured in RPMI-1640 supplemented with bovine fetal serum at 37°C in a 5% CO<sub>2</sub> environment. The collected neutrophils were analyzed with Annexin V-FITC staining. *P. gingivalis* strains A7436 and W50 both produced significant decreases in the amount of apoptosis after 8 hrs co-incubation.

\*indicates  $p < 0.05$  vs control by one-way ANOVA.

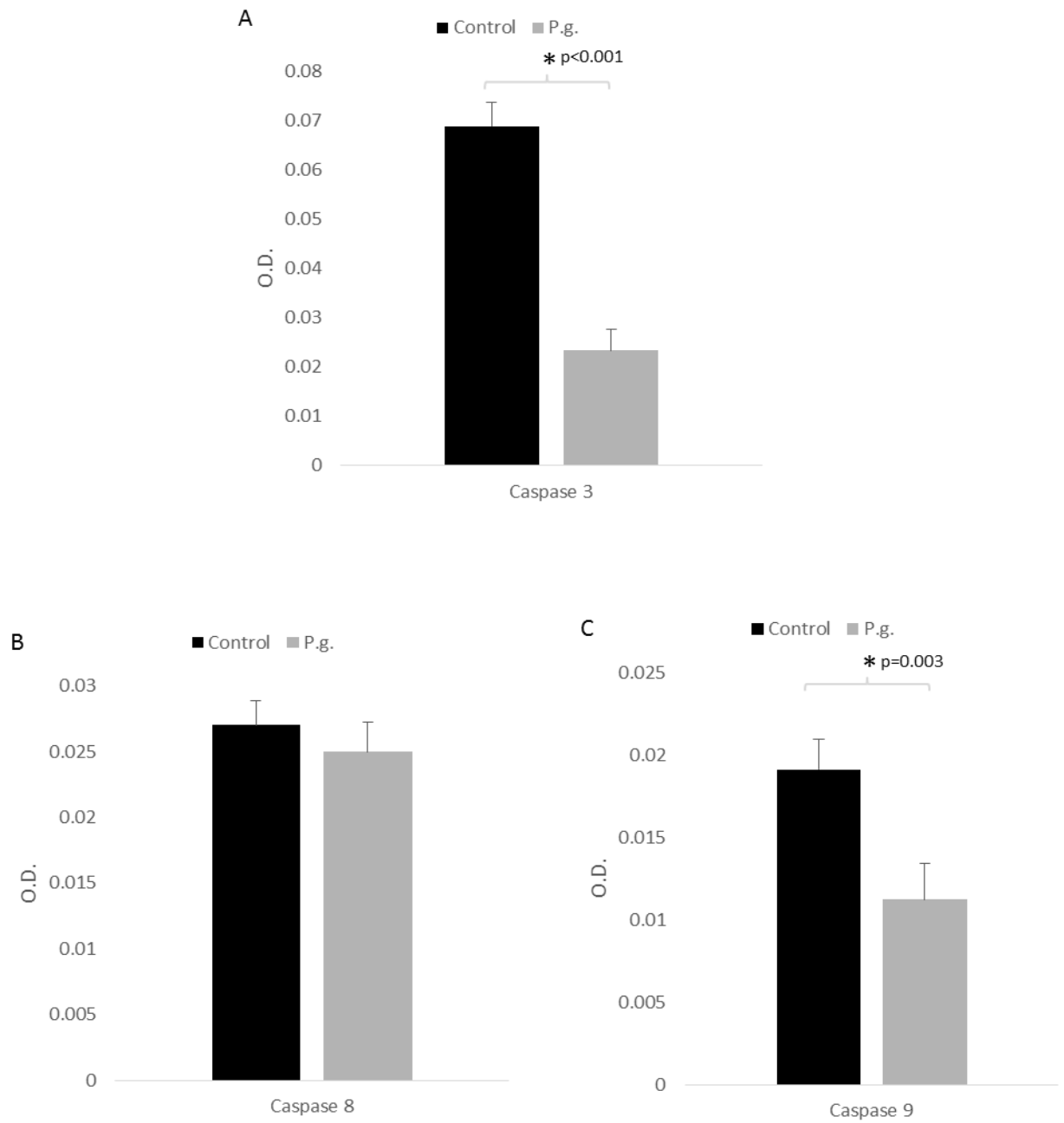
**Figure 12.** *P. gingivalis* delays neutrophil apoptosis



**Figure 12.** Comparison between co-incubation for 8 hours with live and heat-killed *P. gingivalis*, as well as LPS of *P.g.* A7436 and *E. coli* as a positive control. Neutrophils ( $1 \times 10^6$  cells/mL) (n=4) were resuspended and cultured in RPMI-1640 supplemented with bovine fetal serum at 37°C in a 5% CO<sub>2</sub> environment. The collected neutrophils were analyzed with Annexin V-FITC staining. All three forms of *P. gingivalis* produced significant decreases in apoptosis. *E. coli*, as expected produced a substantial increase in apoptosis.

\* indicates  $p < 0.05$  vs control by One-way ANOVA.

**Figure 13.** Mechanism of delayed neutrophil apoptosis by *P. gingivalis*:



**Figure 13.** Neutrophils ( $1 \times 10^6$  cells/mL) ( $n=6$ ) were resuspended and cultured in RPMI-1640 supplemented with bovine fetal serum at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  atmosphere with or without *P. gingivalis* A7436 (MOI 20) and were analyzed with caspase protease assay kit. (2A) Caspase-3 activity after 12 hrs of co-incubation shows a significant decrease in activity compared to control ( $p < 0.001$ ). (2B) Caspase-8 activity after 6 hrs of co-incubation demonstrated no significant differences. (2C) After 6 hrs of co-incubation, caspase-9 activity demonstrated a significant reduction in caspase-9 activity compared to control ( $p < 0.05$ )

\* indicates  $p < 0.05$  vs control by Student's t test.



## 6. Mimicking type 2 diabetes with chronic periodontitis delayed neutrophil apoptosis in healthy donors

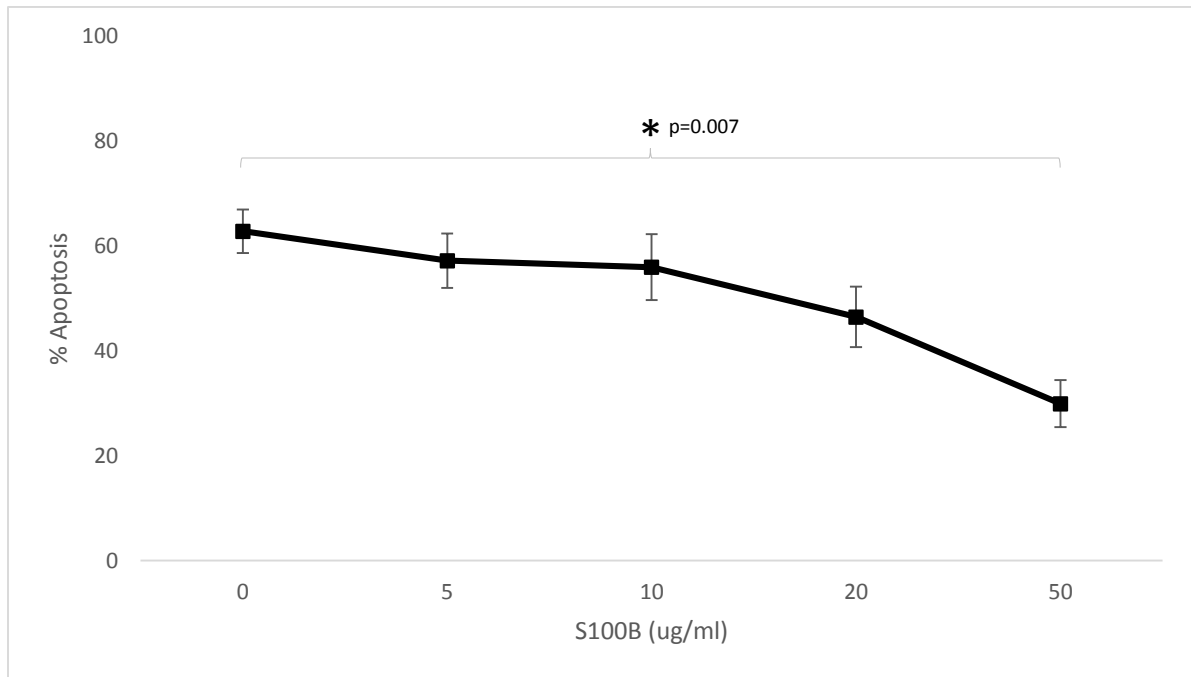
To further investigate the mechanism of neutrophil apoptosis in T2DM, we used a model mimicking diabetes adapted from Omori et al. (284) by culturing healthy subject neutrophil in diabetic conditions consisting of high glucose, with or without S100B, a ligand for Receptor for Advanced Glycation Endproducts (RAGE). Neutrophils ( $1 \times 10^6/\text{ml}$ ) (n=3) were cultured for 8 hrs in RPMI supplemented with 5.5 mM glucose (normoglycemic, NG) or 25 mM glucose (hyperglycemic, HG), with or without RAGE ligand, S100B. The results reveal that high glucose alone did not affect neutrophil apoptosis, but when incubated with high glucose and S100B, neutrophil apoptosis was delayed in a concentration dependent manner. Significant differences were found when the concentration of S100B increased to 50  $\mu\text{g}/\text{ml}$  (p=0.007) (**Fig. 14**).

To represent the T2DM+CP condition, *P. gingivalis* (MOI 20), a known pathogen in periodontal disease, was co-incubated with neutrophils ( $1 \times 10^6/\text{ml}$ ) (n=3) for 8 hrs in RPMI supplemented with 25 mM glucose (hyperglycemic, HG) and RAGE ligand, S100B (50  $\mu\text{g}/\text{ml}$ ) (**Fig. 15**). Results from Annexin V staining indicate a significant delay in apoptosis compared to normal glucose control (p=0.002).

Our previous results have shown a decrease in cell surface Fas and TNF receptor in neutrophils from T2DM. In order to further characterize the impact of high glucose and S100B on the expression of cell surface death receptors, FAS receptor (CD95) and TNF receptor 1 (CD120a) which activated the caspase 8

enzyme through cooperation with their adaptor proteins were measured. Neutrophils from healthy donors ( $1 \times 10^6$ ) (n=3) were cultured for 8 hrs in RPMI supplemented with 5.5 mM glucose (normoglycemic, NG) or 25 mM glucose (hyperglycemic, HG), with or without RAGE ligand, S100B. Neutrophils were paraformaldehyde fixed and were incubated with FITC conjugated mouse anti-human CD120a antibody and APC conjugated mouse anti-human CD95 antibody. All samples were analyzed using a flow cytometer. The results showed a significant reduction of cell surface Fas receptor in neutrophil incubated under HG with S100B compared to NG (p=0.004) and HG only (p=0.04) controls (**Fig. 16A**). Cell surface TNF receptors in neutrophils incubated under NG, HG, and HG with S100B showed no significant difference (**Fig. 16B**).

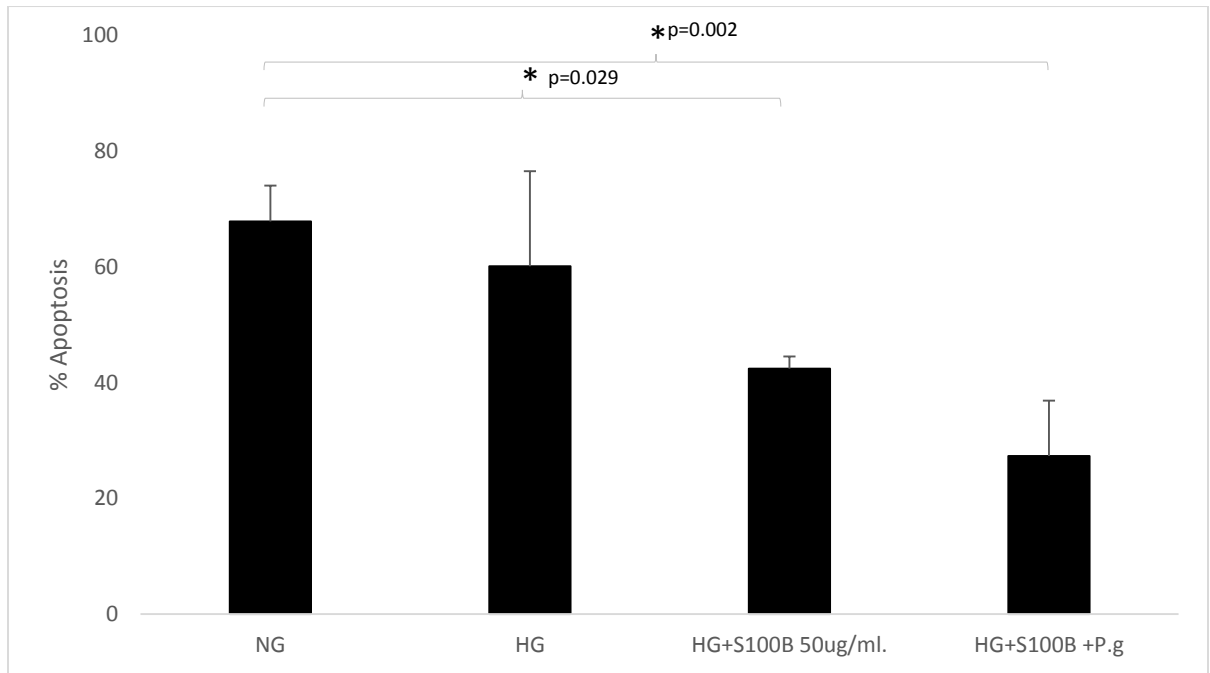
**Figure 14.** *Mimicking T2DM delays neutrophil apoptosis*



**Figure 14.** Neutrophils ( $1 \times 10^6/\text{ml}$ ) from healthy subjects ( $n=3$ ) were cultured for 8 hrs in RPMI with 25 mM glucose (hyperglycemic, HG), with different concentrations of S100B. Neutrophil apoptosis was analyzed by Annexin V staining kit. A concentration of 50  $\mu\text{g}/\text{ml}$  produced a significant decrease in the percentage of apoptosis.

\* indicates  $p < 0.05$  vs control by One-way ANOVA.

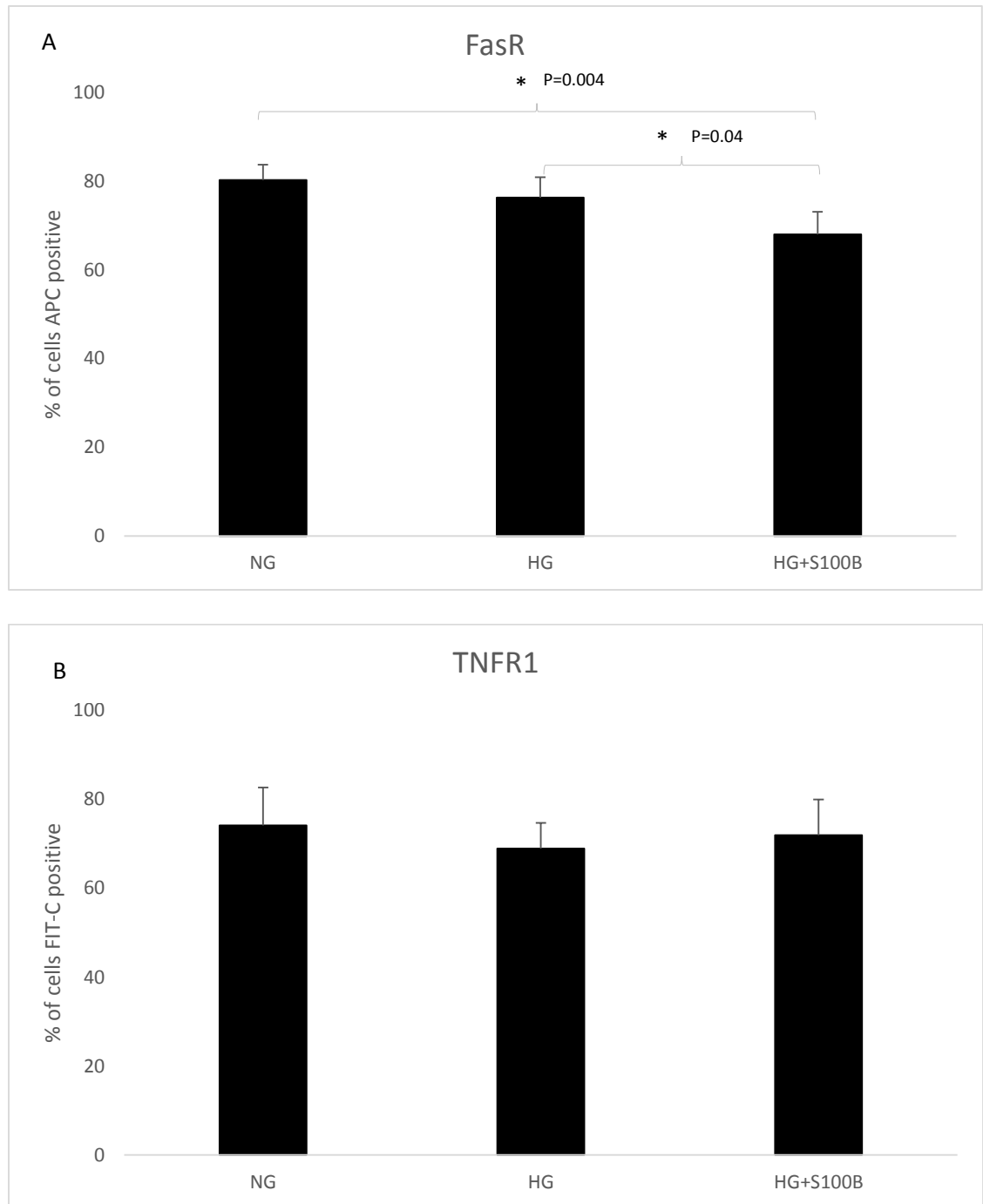
**Figure 15.** *Mimicking T2DM+CP delays neutrophil apoptosis*



**Figure 15.** Neutrophils ( $1 \times 10^6$ /ml) from healthy subjects (n=3) were cultured for 8 hrs in RPMI with 5 mM glucose (normoglycemic, NG), 25 mM glucose (hyperglycemic, HG), with or without S100B (50  $\mu$ g/ml), with or without *P. gingivalis* (MOI 20). Neutrophil apoptosis was analyzed by Annexin V staining kit. The combination of HG+S100B produced a significant decrease in spontaneous neutrophil apoptosis compared to the normal glucose (NG) control. Addition of *P. gingivalis* to the HG+S100B condition produced a more dramatic decrease in neutrophil apoptosis compared to NG control.

\* indicates  $p < 0.05$  vs control by One-way ANOVA

**Figure 16.** Cell surface death receptor expression in neutrophil after incubated in HG and S100B



**Figure 16.** Neutrophils ( $1 \times 10^6$ /ml) from healthy subjects (n=3) were cultured for 8 hrs in RPMI with 5 mM glucose (normoglycemic, NG), 25 mM glucose (hyperglycemic, HG), with or without S100B (50  $\mu$ g/ml), and fixed with 1% paraformaldehyde at 4°C. Cells were incubated with PBS with 1% BSA. The cells were then stained with FITC conjugated anti-human CD120a (TNFR1) antibody and APC conjugated anti-human CD95 (FasR). All samples were analyzed using a flow cytometer.

(15A) Cell surface Fas receptor expression: The combination of HG+S100B produced a significant decrease in cell surface Fas receptor expression compared to NG and HG groups.

(15B) Cell surface TNF receptor expression: No significant difference was seen between groups. \* indicates  $p < 0.05$  vs healthy control by One-way ANOVA.



### **XIII. DISCUSSION**

Our study objective was to explore the impact of T2DM and CP on peripheral blood spontaneous neutrophil apoptosis. Although considerable research has focused on neutrophil apoptosis and resolution of inflammation in subjects with T2DM and CP, it remained unclear whether peripheral blood neutrophil from these patients demonstrated any defects in apoptosis. Our results demonstrated that peripheral blood spontaneous neutrophil apoptosis was delayed in subjects with T2DM and T2DM+CP by using quantitative TUNEL assay. This was confirmed by diminished activity of essential extrinsic apoptosis pathway enzymes including caspase 3 and 8 in both subjects. The lack of caspase activity was possibly regulated by reduction of cell surface Fas receptor, TNF receptor, and serum Fas ligand which were found in T2DM and T2DM+CP subjects. To further investigate the mechanism, we mimicked T2DM+CP delayed neutrophil apoptosis in healthy donors. Our results showed that high glucose alone did not affect neutrophil apoptosis. When hyperglycemia was combined with S100b and *P.gingivalis*, neutrophil apoptosis was delayed.

Diabetes mellitus is a group of diseases characterized by chronic hyperglycemia and other metabolic abnormalities. The effects of prolonged exposure to hyperglycemia are now recognized as the primary causal factor in the majority of diabetic complications including coronary artery disease, cerebrovascular disease and peripheral vascular disease, inhibited wound healing, and periodontitis (2, 13). In type 2 diabetes, neutrophil dysfunction was noted in

assays of neutrophil chemotaxis, adherence, and phagocytosis, (128) and this dysfunction could lead to impaired host resistance to infection. Functional abnormalities within the neutrophil apoptotic mechanisms would allow it to continue circulation at the site of infection, continuing their pro-inflammatory potential and host tissue destruction, ultimately delaying wound healing (289). To date, research on neutrophil apoptosis as a crucial role in resolution of inflammation in patients suffering with diabetes is still controversial. In this study, we evaluated the defects in spontaneous neutrophil apoptotic responses in type 2 diabetes and found a significant delay in apoptosis compared to the healthy controls. The results agreed with Sudo et al. (274) who found delayed neutrophil apoptosis in diabetic subjects compared to healthy controls by using the active caspase-3 positive rate as the only measurement tool. This was reinforced by another study which indicated that higher amounts of anti-apoptosis cytokines were produced from neutrophils in diabetic patients (275). Studies in mice and rats, on the other hand, suggested that there was no significant difference between spontaneous neutrophil apoptosis in diabetic and non-diabetic groups (276-278) suggesting that murine models of diabetes may not be fully relevant to human neutrophil for studying the apoptosis.

Many studies have concluded that neutrophil apoptosis is delayed in chronic periodontal disease. Gamonal et al. (280) demonstrated that granulocyte monocyte-colony stimulating factor (GM-CSF) and TNF- $\alpha$ , responsible for inhibition of neutrophil apoptosis, were present within the gingival crevicular fluid collected

from chronic periodontitis patients. Similarly, other studies also observed a decrease in neutrophil apoptosis in subjects with periodontitis via a variety of methods used including *in situ* DNA breaks, electron microscopy, caspase-3 measurement and *in vitro* co-culture of HL60-derived neutrophil with *P. gingivalis*, a well-known periodontal pathogen (255, 268, 279). Since chronic periodontitis increased the anti-apoptotic cytokine and delayed neutrophil apoptosis at a local tissue level, we wanted to further investigate the impact of chronic periodontitis on spontaneous neutrophil apoptosis at a systemic level, also complicated by chronic hyperglycemia. Our results demonstrated a significant delay in spontaneous neutrophil apoptosis in the T2DM+CP group compared to healthy subjects, where the effect was additive but not synergistic. This finding suggests that periodontal disease not only affected neutrophil apoptosis at the local site of periodontal infection but also systemically influenced the resolution of inflammation and neutrophil clearance, which may result in intensifying other systemic inflammatory conditions.

There are two major signaling pathways that initiated the apoptosis, the death receptor pathway (extrinsic pathway) and the mitochondrial pathway (intrinsic pathway) (290). These signals activate cytoplasmic protease enzymes called caspases. Caspases are activated by precursor caspases in a protease cascade mechanism. Each precursor caspase is activated by cleavage of a peptide bond by an upstream caspase. Cleavage of the enzyme DNA fragmentation factor 45 (DFF45) to the active 40-kDa form by active caspase 3 causes endonucleolytic breakage of

chromatin and eventual chromatin condensation, one of the classic morphological features of cell apoptosis. Understanding the molecular events of apoptosis has allowed the development of methods to measure various markers of apoptosis, for example, the degree of caspase 3 activation, cell membrane asymmetry of phosphatidylserine, shifts in mitochondrial membrane potential, or detection of intact and cleaved poly-ADP-ribose-polymerase that binds at DNA strand breaks. However, methods to assess apoptotic markers can have numerous limitations; examples include a qualitative or comparative ability to measure, a lack of sensitivity especially to measure changes at low levels, a requirement for live cells at the time of measurement, a restricted and non-linear dynamic range and low throughput. In order to overcome the limitation and weakness of each method, we utilized multiple techniques including detection of DNA strand breaks by TUNEL assay, cell membrane exposure of phosphatidylserine by Annexin V and caspase enzyme activity, which each efficiently measured apoptosis inside the cell at a different time point and location. By utilizing most of the measurement with flow cytometer, we were able to obtain a more accurate data compared to traditional method of microscope counting.

Our results demonstrated that spontaneous neutrophil apoptosis was delayed in patients with type 2 diabetes and chronic periodontitis. We confirmed these results by measuring the caspase-3 enzyme, which is a well-known and reliable method used to measure the amount of apoptosis (291). Caspase-3 is a member of the cysteine-aspartic acid protease family produced in the cascade

pathway which acts on protein substrates and plays a main role in the execution-phase of cell death by apoptosis. This process occurs before DNA fragmentation, a characteristic of apoptosis that can be detected by the TUNEL assay as used in our experiments. As expected, our results have shown that healthy subjects exhibited the highest caspase-3 activity in comparison to other diseased subjects. This experiment revealed that delays in spontaneous neutrophil apoptosis in type 2 diabetes and chronic periodontitis occurs by a caspase-dependent pathway.

Since there was a significant decrease in neutrophil caspase-3 activity in diseased subjects' as compared to a healthy group, we investigated whether the process of caspase-3 activation was due to an upstream increase in initiator caspase-9 activity, representing the intrinsic pathway; or caspase-8 activity, representing the extrinsic pathway; or both. Our results have shown a significant increase in caspase-8 activity in the healthy group compared to diseased groups. Caspase-9 activity similarly exhibited the same trend but was not statistically significant. This demonstrated that the delayed spontaneous neutrophil apoptosis in T2DM+CP was triggered by inhibition of the caspase-dependent pathway via down-regulation of caspase activation by the death receptor pathway. The extracellular signals can be triggered by engagement of cell surface death receptor with their specific death ligands, which play a central role in apoptosis. Cell surface death receptors (DR) are a family of transmembrane proteins that belong to the TNF receptor superfamily. Mammalian death receptors include Fas (also called CD95) and TNFR1 (Tumor necrosis factor receptor 1 or CD120a) (292), (209). Additional

death receptors such as DR3 (death receptor 3), DR4, and DR5 are also structurally related to the TNF receptor superfamily (211). These receptors share a conserved cysteine-rich repeat at their extracellular domains. Although the regions of greatest sequence homology between superfamily members are extracellular, Fas and TNFR1 share a region of homology at the cytoplasmic face termed the 'death domain'. This domain is required for apoptotic signaling by both Fas and TNFR1. The activating ligands for these DR are structurally related molecules that belong to the TNF gene superfamily (195, 293). For example, Fas/CD95 ligand (FasL) binds to Fas, TNF binds to TNFR1, Apo3 ligand (Apo3L) binds to DR3, and Apo2 ligand (Apo2L, or TRAIL) binds to DR4 and DR5 (211). In this study, we measured the cell surface death receptor expression. Results have concluded that both TNF receptor and Fas receptors are decreased in type 2 diabetes, but with the addition of chronic periodontitis, TNF receptor levels were restored. The levels of soluble Fas ligand and TNF- $\alpha$  in serum separated from the subjects' whole blood were assessed. Our results have shown no significant difference in TNF- $\alpha$  level in all groups, agreeing with Makino et al. who found no significant difference in serum TNF- $\alpha$  levels between uncomplicated diabetic patients vs healthy normal participants (294). A significant decrease in sFas ligand was observed, specifically in T2DM+CP compared to the healthy controls. This result agreed with previous literature which concluded that sFas ligand levels are decreased or similar in type 2 diabetes subjects (295). On the other hand, Guillot et al. reported an increase in sFas ligand in diabetes patients especially in patients with diabetic neuropathy (295).

To further investigate the mechanism of neutrophil apoptosis, we used a model mimicking type 2 diabetes by culturing healthy subject neutrophil in diabetic conditions, modified from Omori et al. (284) who used human promyelocytic leukemia cells (HL-60). Our results demonstrated that high glucose did not affect the spontaneous neutrophil apoptosis, similar to the previous literature (296), signifying that hyperglycemia may not be the main cause of delayed spontaneous apoptosis of neutrophil in type 2 diabetes patients. However, our results also demonstrated a significant delay in spontaneous neutrophil apoptosis in poor glycemic control T2DM patients (HbA1c >7.5), in addition to the fact that poor glycemic control can increase RAGE activation (297). Therefore, we suspected that RAGE might play a role in delaying spontaneous neutrophil apoptosis in T2DM individuals. There is strong evidence of RAGE involvement in delayed apoptosis in monocytes, aggravating inflammation and altered neutrophil functions in diabetes patients (22, 298). Therefore, we assessed whether RAGE could be a potential factor in delaying neutrophil apoptosis by incubating healthy neutrophils with high glucose and S100B. These results showed a delay in spontaneous neutrophil apoptosis in a concentration-dependent manner. Our results also showed that by incubating healthy neutrophils with high glucose and S100B, cell surface Fas receptor expression was decreased as compared to normal glucose control. This outcome may explain our results which showed a decrease in Fas receptor on neutrophils from T2DM patients. Next, we wished to mimic type 2 diabetes with chronic periodontitis delayed neutrophil apoptosis in healthy donors. By combining

*P. gingivalis* and neutrophils with high glucose and S100B, apoptosis was significantly delayed as compared to control and its effect was similar to the result obtained from type 2 diabetes and chronic periodontitis patient.

Progression of periodontal disease results from a failure of the immune system to clear infectious agents and to restore periodontal homeostasis. Socransky et al. (48) grouped the microorganisms that colonize the subgingival plaque into five main color-coded complexes. Several associations were also characterized among species inside microbial complexes, among different complexes, and based on severity of periodontal disease. Specifically, the red complex (*Bacteroides forsythus*, *P. gingivalis*, and *Treponema denticola*) are associated with severe forms of periodontal disease. Several periodontal pathogens have been shown to be associated with cell death in leukocytes including *Fusobacterium nucleatum*, a gram-negative oral bacterium which has the ability to induce apoptotic cell death in neutrophils (299), and *Aggregatibacter actinomycetemcomitans can*, which can lyse human neutrophil cytoplasm and cause necrosis by the pore-forming and membranolytic properties of its leukotoxin (300). In our study, we used *P. gingivalis*, a Gram-negative black-pigmented microorganism, which has been implicated as the major pathogen in the development of adult periodontitis. The intimate interaction of this periodontal pathogen with the host has become a subject of intense investigation. Our results have shown that neutrophils and *P. gingivalis* (strain A7436) co-incubated significantly reduced neutrophil apoptosis. *P. gingivalis* inhibited apoptosis of neutrophil in a dose dependent manner and even when



different strain is used (W50), the inhibition persisted in a similar style. Heat killed *P. gingivalis* was also tested and a similar result was observed. This suggests that the mechanism of neutrophil survival may be due to direct inhibition of apoptosis by *P. gingivalis* virulence factors or components, or by indirect inhibition of neutrophil apoptosis by its own response. One of the components from *P. gingivalis* which is well known to inhibit neutrophil apoptosis is bacterial lipopolysaccharide (LPS). (255) Our results have confirmed that neutrophil apoptosis was delayed when incubated with LPS. In contrast, several publications report that *E. coli*, another gram negative anaerobic bacteria, significantly induced neutrophil apoptosis but its LPS intensely delayed neutrophil apoptosis. (288, 301)

Our results have shown that caspase-3 activity after neutrophil-*P. gingivalis* co-incubation was dramatically decreased. Consequently, we investigated whether the process of caspase-3 activation was due to an upstream increase in initiator caspase-9 activity, representing the intrinsic pathway, or caspase-8 activity, representing the extrinsic pathway, or both. The extrinsic pathway involved in caspase-8 enzyme activation was not interrupted. However, active caspase-9 enzyme, representing the end point of the intrinsic pathway, was greatly reduced, leading to a significant inhibition of caspase-3 enzyme activation and delayed neutrophil apoptosis. This result did not explain the mechanism of neutrophil apoptosis in chronic periodontitis patients who have shown a decrease in caspase 8 activity. We suggested that the effects of *P. gingivalis* in delaying neutrophil apoptosis is due to its LPS which signaled through the Toll-like receptors (TLRs).

François et al. demonstrated that TLR2/4 agonists delayed apoptosis by activation of NF- $\kappa$ B and PI3K. Consequently, increased levels of Mcl-1 and A1, which are anti-apoptotic members of the Bcl-2 family, involved in controlling the intrinsic pathway of apoptosis. (302) LPS from *P. gingivalis* was recently shown to bind to TLR2, not the common LPS receptor TLR4. (303, 304) LPS from *P. gingivalis* has also been reported as antagonistic for TLR4. (305) However, studies with fibroblasts have shown *P. gingivalis* LPS to signal through TLR4. (306, 307) This finding may indicate a difference in signaling in this particular cell type. Thus, in order to have a better understanding into the mechanism of *P.gingivalis* delayed neutrophil apoptosis, further study is needed to explore into *P.gingivalis* LPS-TLR activation in this cell.

This study gave us a better understanding of how neutrophils play a role in causing persistent inflammation and compromised wound healing in type 2 diabetes and chronic periodontitis. However, more detailed research is necessary to fully comprehend the mechanism of delayed spontaneous neutrophil apoptosis in type 2 diabetes and chronic periodontitis including determining which factors affect Fas and TNF receptors production and localization, the level of mitochondrial reactive oxygen species which involve in caspase-independent apoptosis pathway, and intracellular regulation of neutrophil survival protein molecules and pathways. One limitation of this study that needs to be addressed is the use of an *in vitro* neutrophil model, which would yield less definitive results, due to the inability to fully imitate the environment in the inflamed host tissue. Thus, an *in vivo* model might be the solution to this problem. Another limitation is the sample size and demographic

distribution such as, gender, race, age, and BMI, which might play a role in controlling neutrophil apoptosis. Therefore, in the future study, increasing the sample size will definitely help to produce a more solid result by using this study to serve as a pilot.

## **CONCLUSION**

We demonstrated that spontaneous neutrophil apoptosis in peripheral blood is impaired in subjects with type 2 diabetes and chronic periodontitis, giving us a better understanding of the systemically impeded resolution of inflammation in both of these chronic inflammatory conditions. Possible explanations for this occurrence stem from a lack of sFas ligand and its receptor expression on neutrophil cell surface, and in patients with type 2 diabetes, RAGE may also play a crucial role in diminishing spontaneous neutrophil apoptosis.

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**XV. CURRICULUM VITAE**

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***EDUCATION***

2002-2008 Dental School attended

Khonkaen University, Khonkaen, Thailand

2010-pres. Doctor of Science in Orthodontics

Boston University Henry M. Goldman School of Dental  
Medicine, Boston, Massachusetts, USA



## **XVI. AWARDS AND HONORS**

May 2008 First honor class rank

Khonkaen University, Khonkaen, Thailand

August 2010 Scholarship from Ananda Mahidol foundation

Under the Royal Patronage of H.M. the King. Bangkok, Thailand

### ***PROFESSIONAL EXPERIENCE***

2008–2010 General dental practitioner at Srinakarin Hospital, Khonkaen, Thailand.

2008-2010 Clinical instructor at the pre-doctoral dental clinic and laboratory of the department of Operative Dentistry at Khonkaen University Faculty of Dentistry, Khonkaen, Thailand.

### ***ORAL PRESENTATIONS***

March 2013 Delayed Neutrophil Apoptosis in Diabetes and Chronic Periodontal Disease. Presented at IADR conference 2013, 2013, Seattle, WA, USA

June 2013 Delayed Neutrophil Apoptosis in Diabetes and Chronic Periodontal Disease. Presented at Penn Perio Conference 2013, 2013, Philadelphia, PA, USA

March 2015 Porphyromonas gingivalis Delays Peripheral Blood Neutrophil Apoptosis. Presented at IADR conference 2015, 2015, Boston, MA, USA

### ***RESEARCH PROJECTS***

Aggasit Manosudprasit, et al.: A Comparison assessment of perceptions of Thai facial profile esthetic by 3 groups of clinicians dental students and general public; Faculty of dentistry, Khonkaen University, 2007

Doctor of Science in Orthodontics research project; “The impact of type 2 diabetes and chronic periodontal disease on peripheral blood neutrophil apoptosis” Boston University Henry M. Goldman School of Dental Medicine, Boston, Massachusetts, USA

### ***SCHOOL-RELATED EXTRACURRICULAR ACTIVITIES***

2003 Participate as an on-stage Dancer in Freshy-Dent activity at Chulalongkorn University

2004-2007 Member of the KKU Dent music band