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INVESTIGATION OF THE RELATIONSHIP BETWEEN CHRONIC METFORMIN USAGE AND DNA DAMAGE IN TYPE 2 DIABETES PATIENTS

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TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

Page

ABSTRACT

Metformin, the sole member of the biguanide class of antidiabetic medications, is one of the oldest antidiabetics in the market. Metformin is recommended to be used by the American Diabetic Association and European Association for the Study of Diabetes as a first line drug against Type II Diabetes. Metformin acts to reduce the blood glucose levels by inhibiting hepatic glucose production through gluconeogenesis. The most common adverse effects were gastrointestinal effects such as diarrhoea and nausea, with the possibility of vomiting, and abdominal pain. The use of metformin has recently been associated with a decreased risk of the occurrence of various types of cancers, especially of pancreas, colon and hepatocellular carcinoma. This observation was also confirmed by the results of numerous meta-analyses. In this study, Alkaline Comet Assay was validated using 6 persons in the 24-27 age range who were non-smokers, did not use any drugs or any other risk factors, both in freshly isolated lymphocytes and peroxide-exposed lypmhocytes. Diabetic patients using metformin (17 metformin using patients and 6 non-metformin using control patients residing in Northern Cyprus) were included in the study. Blood samples were obtained and their peripheral blood lymphocytes were analysed for DNA damage using Alkaline Comet Assay. Results indicate that control group and metformin group alike had varying levels of DNA damage. The low number of samples in the control group prevented the usage of statistical tests between the two groups. By increasing the number of samples, the patients will be able to be broken down into categories according to factors such as concominant drug use, lifestyle circumstances, smoking habits, drinking habits and age, the extent of this study will thus be enlarged.

Keywords:metformin, DNA damage, comet assay, diabetic patients

1– INTRODUCTION

1.1 – Diabetes Mellitus& Diagnoses

Diabetes Mellitus, commonly known simply as Diabetes, is a chronic ailment associated with a loss of native homeostatic control of blood glucose concentration. Therefore, its hallmark is an elevated blood glucose level. This elevation has multiple side effects, some of them relatively mild, and some of them life-threateningly severe. Such side effects include generally feeling unwell, frequent urination, insatiable thirst, rapid and unstoppable weight loss, slow healing of injuries etc. [3].

Diabetes is usually diagnosed using two main tests: OGTT and HbA1c. OGTT or Oral Glucose Tolerance Testing involves a measurement of the person's blood glucose level as part of a Serum Biochemistry test at the beginning of the test, immediately followed by the administration of 75g oral glucose in drinking water. Either a fasting glucose concentration above 110 mg/dl or a glucose concentration of 200 mg/dl two hours after oral glucose will indicate diabetes. However, the healthcare standard for diabetes diagnosis is the HbA1c test (Hjellestad et al., 2013).

HbA1c designates a variant of haemoglobin, called glycated haemoglobin. Its concentration in the blood is directly dependent on the concentration of glucose in the blood and gives a high accuracy indication of the glucose concentration in the blood over the last 2-3 months. As blood glucose concentration in a diabetic patient can fluctuate drastically between meals, HbA1c is generally thought to give a better indication of the overall glucose levels in the blood. HbA1c is measured as a percentage of the total haemoglobin in the blood and the cut-off value is established at 6.5%. A percentage lower than this would indicate a healthy person whereas values above this would strongly point towards diabetes [8].

1.2 – Types of Diabetes And Their Treatment

Diabetes Mellitus has two recognised types, designated as Type I and Type II. These two types have similar outset and generally similar symptoms, although the underlying pathology is fundamentally different. Both types are ultimately founded in malfunctions in the insulin cycle. Type I is associated with a loss of the body's ability to produce insulin. Type II is associated with the emergence of a resistance and insensitivity of the target cells toward insulin.

Type I Diabetes is thought to be an auto-immune disease, where an aberrant humoral (antibody-mediated) response is initiated against the β-Langerhans cells of the pancreas, which produce insulin (Roep and Parkman, 2012). This humoral immunoreaction is thought to induce in turn a T-cell mediated cytotoxic reaction where $CD8^+$ T lymphocytes attack and destroy the β-Langerhans cells, resulting in a complete loss of insulin production. The result is an inability to respond to naturally rising postprandial (after-meal) glucose concentrations, leading to very high blood glucose levels. Due to this drastic destruction of insulin-producing cells, the only viable treatment for Type I Diabetes is subcutaneous injection of insulin into the body. There are proposed therapies involving transplantation of β-Langerhans cells into the malfunctioning body (Guo et al., 2009)(Kumar et al., 2013) however, these are experimental at best and these have not yet been attempted in living human patients in a clinical setting.

Type II Diabetes is an easier disorder to treat, due to the problem lying not in a lack of insulin production, but a lack of response to it. As a result, there is a large plethora of medications aimed at either reducing blood glucose load or sensitising the target tissues to the effects of insulin, both factors facilitating the normalisation of blood glucose levels. Such medications include the sulphonylurea class, which induce the secretion of insulin from the β-Langerhans cells of the pancreas. The most commonly used members of this class are glimepiride (Amaryl), gliclazide (Diamicron) andglibenclamide (Gluconorm). Closely related to this class is the meglitinide class, the most commonly used member of which is repaglinide (NovoNorm). There are also adjuvant therapies such as α-glucosidase inhibitors (most commonly used member of which is acarbose (Glucobay)), which slow the liberation and subsequent absorption of glucose from the intestinal wall and the gliptin class of secretagogue proteins, which also serve to induce insulin secretion from β-Langerhans cells of the pancreas. Another class in the glitazone class, which bind the PPARγReceptor in the target cells, directly enhancing insulin sensitivity of the target tissues and the utilisation of glucose by such tissues. The sole marketed member of the glitazone class is pioglitazone, whereas its siblings rosiglitazone and troglitazone were both removed from the market due to severe cardiovascular side effects and hepatotoxicity, respectively.

Finally, there is the biguanide class, the sole member of which is metformin, which reduces blood glucose through a combination of hepatic gluconeogenesis inhibition and insulin sensitisation.

1.3–History Of Metformin

Metformin is an antidiabetic drug (Fig. 1.1), an analogue of the chemical galegine, isolated from the medicinal herb *Galegaofficinalis*(Fig. 1.2). It has a long, convoluted history, with its discovery being in 1922 (Janos, 2010), although the development of animal-extracted insulin soon overshadowed its discovery and delayed its market introduction for 3 decades.

Metformin was introduced into the European market in 1957[2]as the first marketed synthetic antidiabetic of the biguanide class (Fig. 1.3). Soon after, its analogues phenformin and buformin were also introduced. Phenformin and buformin were both more potent than metformin, which was used in milder cases of diabetes mellitus (DM) Type II.

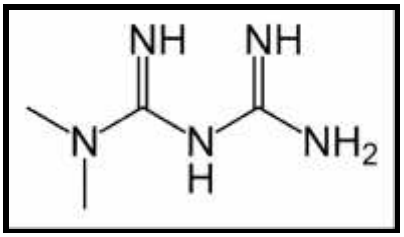

Figure1.1 – Chemical Structure of Dimethyl biguanide (Metformin)

Figure1.2 – *Galegaofficinalis*

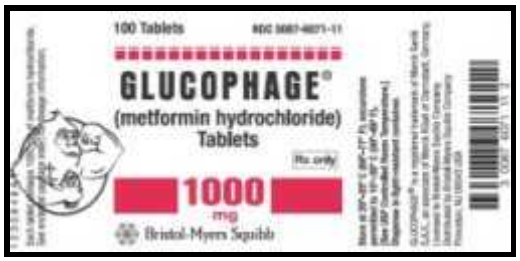

Figure1.3 – Glucophage Tab. from 1960s (http://www.evolutionary.org/metformin-HCl)

Although largely overshadowed by its more potent siblings, the use of metformin skyrocketed in the 1970s, when buformin and phenformin were both found to lead to significantly increased risk of systemic lactic acidosis (Berger et al., 1976). Similar reasearch conducted on metformin failed to yield any proof of increased tendency to go into lactic acidosis (Salpeter et al., 2003) , and with the market withdrawal of phenformin and buformin, metformin was left as the sole marketed member of the biguanide class, a distinction it still holds today.

After the withdrawal of its analogues, the use of metformin increased massively due to its very low toxicity and concurrent safety, as well as its ability to effectively handle mild cases of DM Type II. Metformin is still the most widely used antidiabetic of any type in the world, with 48 million prescriptions of it being filled in the USA in 2010 alone (techtimes.com, 2015).Itssome trade names include Glucophage, Glifor, Diaformin, Gluformin, Glukofenand also combination formulations such as Glifix Plus and Acort (with pioglitazone), Galvus Met (with vildagliptine) and Janumet (with sitagliptine) which are now marketed in TRNC, Turkey ,and many other countries.

1.4 - Mechanism Of Action And Potential Benefits

For a drug being used for almost 60 years, there is considerable uncertainty and disagreement concerning the mechanism of action of metformin. However, the almost universally held conviction, one supported by a vast body of scientific evidence, is that metformin functions by inhibiting the process of hepatic gluconeogenesis, the production of glucose from pyruvate in the hepatocytes (Kirpichnikov et al., 2002).The resulting fall in the net amount of glucose circulating in the blood is thought to be the mein medium of effect of metformin. The use of metformin has been shown to reduce blood glucose levels as evidenced by HbA1c level decreases of up to 1% (Hirst et al., 2012) as well as increased cellular responsivity towards other antidiabetic medications and reduced insulin resistance,

as evidenced by a requirement of lesser antidiabetic doses and improved dose responses to co-administered subcutaneous insulin.

1.5 - Side Effects Of Metformin Usage

The most commonly observed side effects of metformin use are general gastrointestinal upset, such as nausea, vomiting or diarrhoea, as well as abdominal pain. Another side effect associated with metformin intake is an elevated risk of Vitamin B_{12} deficiency, followed by a recommendation from the authors of the research paper that the levels of serum Vitamin B_{12} monitored during metformin therapy (Fig. 1.4) (de Jager et al., 2010).

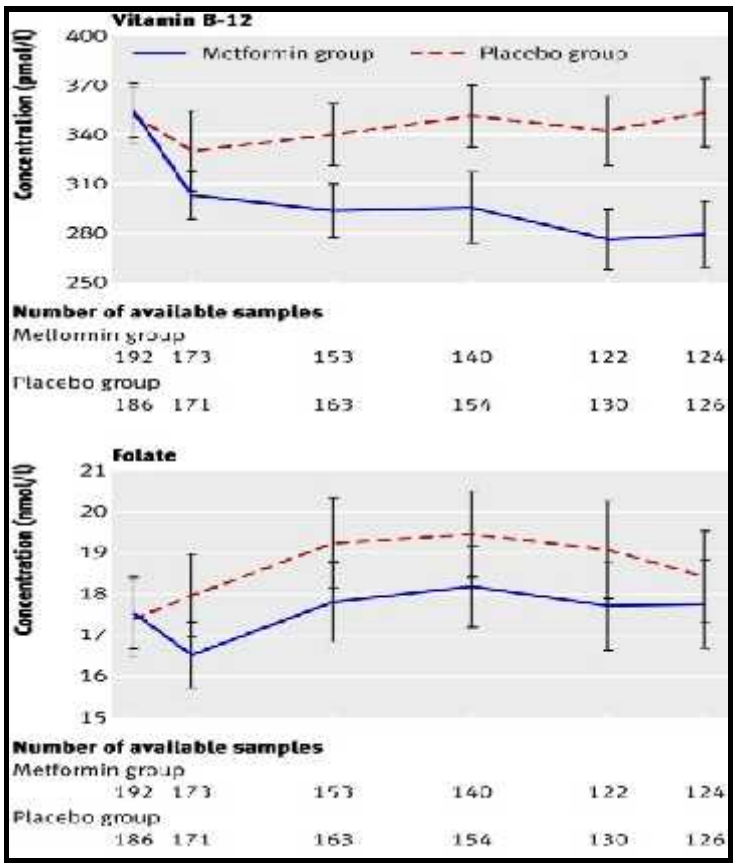

Figure1.4 – Correlation between metformin usage and Vit.B12 deficiency (de Jager et. al.)

Despite years of research, no definitive link between metformin intake and risk of lactic acidosis has been identified in diabetic patients. In the meta-analysis reported by Salpeter (2003), from which a graph is shown, metformin is shown not to induce lactic acidosis with

p=0.07.(Fig. 1.5).However, carestill must be taken in patients with hepatic or renal insufficiency as these patients are less capable of filtering out or metabolising the natural lactate production of the body and might therefore be more susceptible to metformin-associated lactic acidosis (MALA).

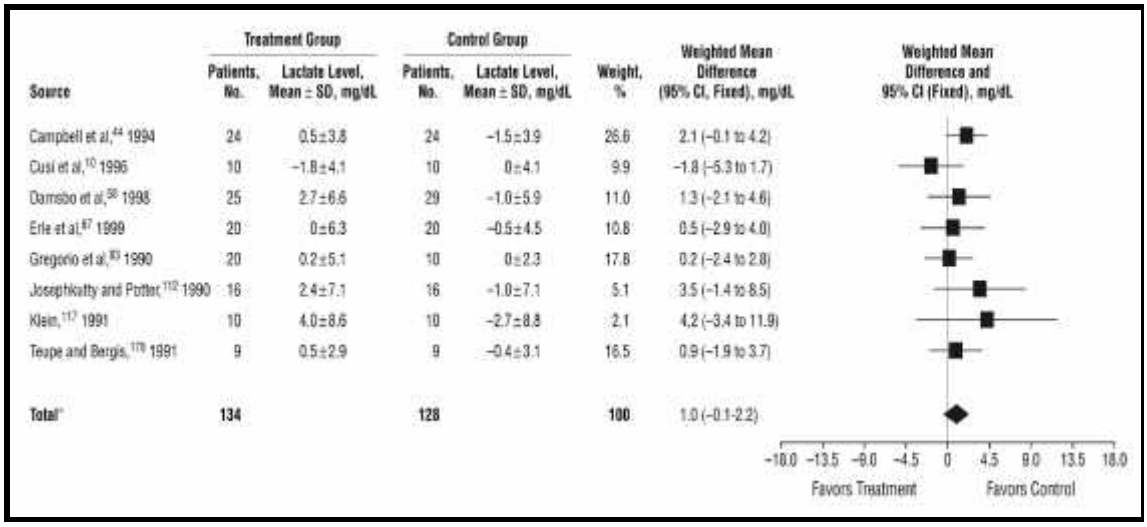

Figure1.5 – Correlation between metformin use and risk of lactic acidosis (Salpeter et al., 2003)

2 - METFORMIN AND CANCER

There is a significant (and continuously growing) body of evidence that chronic long-term use of metformin leads to reduced occurrences of a multitude of different cancer forms (Kasnicki et al. 2014), including those of the colon and the pancreas, as well a marked reduction in the frequency of hepatocellular carcinoma. These findings were also confirmed through data obtained by numerous meta-analyses of such research.

2.1 – Direct Hypoglycaemic Effect

Due to its method of action, there is considerable curiosity surrounding how metformin acts to hinder the development of these cancerous cells. Cancerous cells are known to possess glycolysis rates of up to 200 times faster than ordinary cells (Alfarouk et al., 2014) (Oncological Warburg Effect), and some scientist propose that by reducing hepatic gluconeogenesis and thus free blood glucose, metformin effectively starves these cells to apoptotic or necrotic death. In this view, the drug itself has no anti-cancer effects, but its intended effect of blood glucose reduction merely serves to facilitate cancerous cell death.

2.2 – Insulin-Like Growth Factor (IGF) Signalling

A related idea links this with Insulin-like growth factor (IGF) signalling. IGFs are a family of two proteins, IGF-1 and IGF-2, binding to 3 receptors, IGF-1R, IGF-2R and Insulin receptor. IGF-1 and IGF-2 show high structural similarity with insulin (hence their name) and all three proteins act as anti-apoptotic cell survival signals to a vast variety of cells. The idea suggests that the metformin-induced fall in blood glucose levels result in a fall in blood insulin levels, which is directly dependent on blood glucose concentration, and this fall in insulin concentration results in reduced pro-survival signalling through the IGF signalling axis, resulting in easier apoptotic death of the cancer cells (Fig. 2.1)

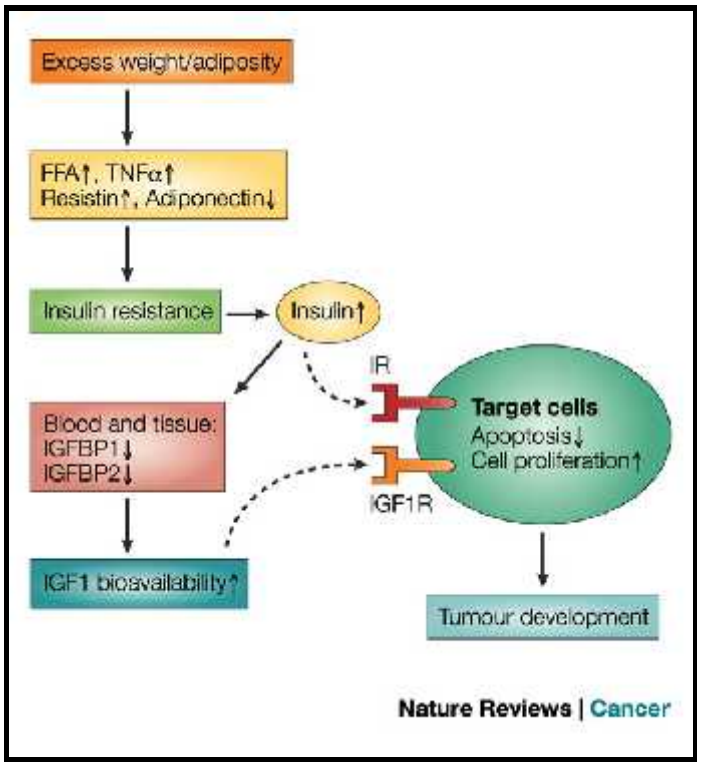

Figure2.1 – Insulin and Tumorigenesis
(http://www.nature.com/nrc/journal/v4/n8/fig_tab/nrc1408_F2.html)

2.3 – AMP-Activated Kinase Enzyme (AMPK) – Mammalian Target OfRapamycin (mTOR) Axis

Another set of hypotheses employ the primary known target of metformin, AMP-activated Kinase enzyme (AMPK). This enzyme is in the centre of two different proposed mechanisms in which metformin could lead to cancer cell death (Martin-Montalvo et al., 2013).

One of these hypotheses is that metformin-activated AMPK phosphorylatively inactivates mammalian Target of Rapamycin protein (mTOR). mTOR is a known upregulator of cellular survival signalling (Xu et al., 2012). Its inhibition has been associated with reduced cancer cell survival. Thus, this idea suggests that metformin, through the activities of AMPK, could indirectly inhibit mTOR and result in death of cancer cells (Fig. 2.2).

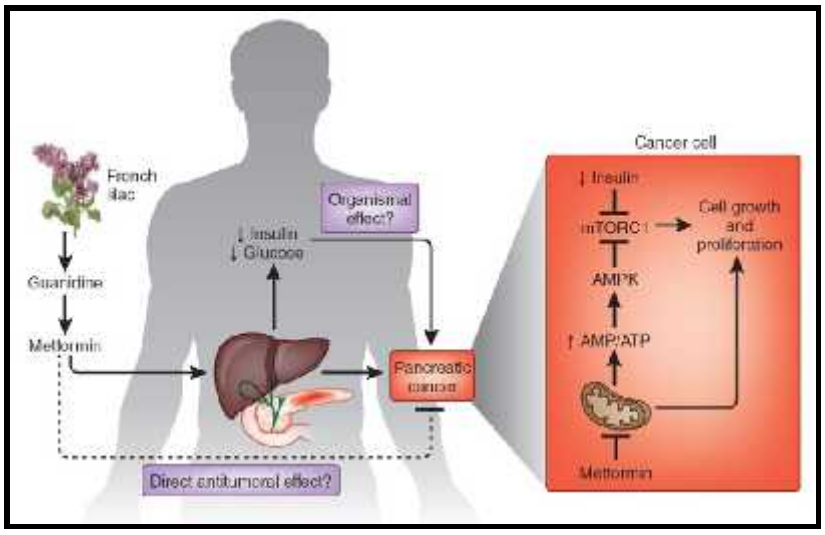

Figure2.2 – Potential Direct and Indirect pathways of metformin antitumor activity (http://www.nature.com/nm/journal/v18/n7/fig_tab/nm.2870_F1.html)

2.4 – AMPK – cAMP Activated Protein Kinase A (PKA) – p53 Axis

The other hypothesis suggests that metformin-activated AMPK could activate cAMP activated Protein Kinase A (PKA) an enzyme with far-reaching function in the cell. It is activated by the presence of cyclic AMP (cAMP) in the cell, which is a product of many signalling cascades, including cell survival or apoptotic signalling. PKA activation is known to have epigenetic consequences, with PKA activation being intricately linked with changes in the regulation of many genes, one of them being p53 (Surget et al, 2013).

Tumor suppressor protein p53 is one of the most important, if not the most important, protein to maintain the genetic and genomic integrity of a cell. It is a vastly multifunctional protein, as it can detect mismatched DNA base and single-stranded DNA (ssDNA), as well as receive input from other proteins that detect other forms of DNA abnormalities. In a human cell,, it has 3 major functions: It arrests cellular growth cycle (through p21), it induces DNA repair damage pathways and it forces the cell into apoptosis (Rahimi et al., 2013). The International Cancer Genome Consortium estimates that a mutation or otherwise inactivation of p53 is found in more than 50% of all human cancers. Thus, it is thought that metformin-induced p53 activation would upregulate DNA damage repair or apoptosis, protecting against carcinogenesis (Fig. 2.3).

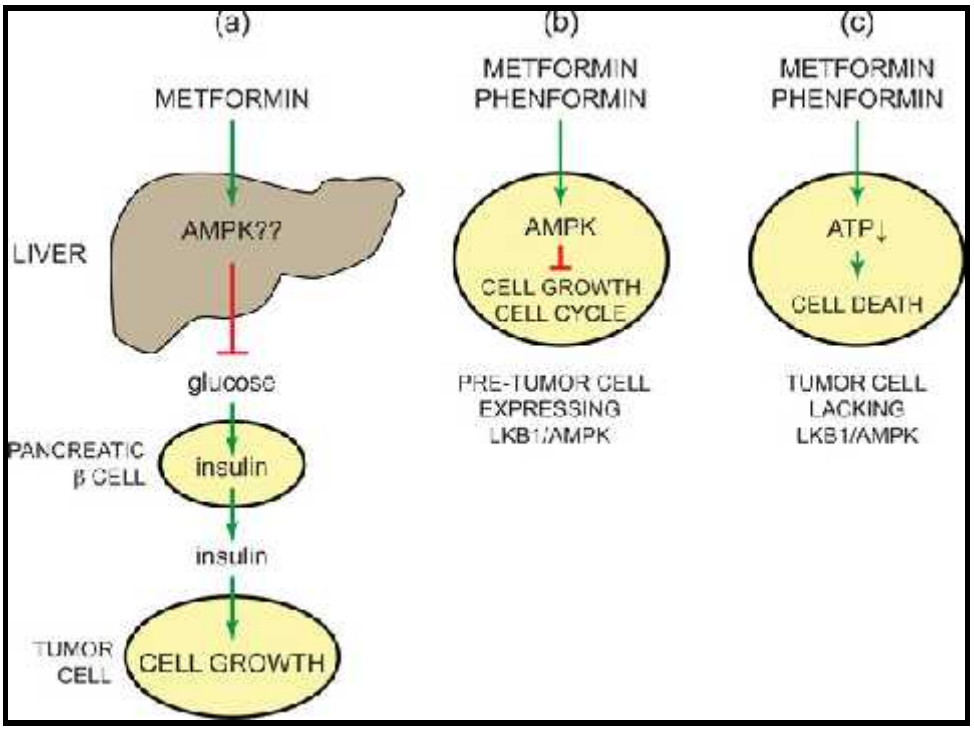

Figure2.3 – Metformin antitumor effects (http://pharmaceuticalintelligence.com/2014/09/28/metformin-thyroid-pituitary-axis-diabetes-mellitus-and-metabolism/)

2.5 – Non-AMPK Dependent r-ETC Inhibition

An alternative view states that metformin function is only partially dependent on AMPK, but it also targets Complex I of the respiratory electron transport chain (r-ETC), thus effectively short-circuiting the respiratory power production and reducing the level of ATP within the mitochondrion, starving rapidly dividing cells such as cancer cells from the energy supply they critically depend on and resulting in their necrosis or apoptosis (Birsoy et al., 2012).

3 – GENOTOXICOLOGICAL TESTING

Genotoxicology testing is the umbrella term used for any and all tests that aim to establish and investigate the genetic and genomic integrity of an individual's cells and to determine DNA damage and lack thereof. These cells are an important factor in determining the toxicity of a chemical due to the particular details of genotoxicity. Unlike other forms of toxicity, which manifest in a matter of minutes to months, genotoxicity is a form of cumulative damage that builds up in the DNA of an individual and can manifest its effects in many years, even across generations. Therefore, its overt symptoms can appear even after the exposure that caused the DNA damage in the first place has already ended. However, this latency also allows a length of time between the infliction of the damage and the appearance of the symptoms, a length of time during which DNA testing procedures, also known as genotoxicological testing procedures can be used to assess and evaluate DNA damage, take precautions to avoid the risks associated with such DNA damage, and even mitigate and repair DNA damage.

3.1 – Cell Cycle and DNA Damage

In all living organisms, there exist mechanisms that evolved for the express purpose of preventing or repairing DNA damage. These mechanisms are tightly coupled with the biochemical pathways and cycles, the most prominent being the Cyclin Cycle, that govern and control the cell cycle. As a result of this entanglement, DNA damage detection and repair mechanisms have the ability to arrest the cell cycle at 3 primary checkpoints, the G_1-S Checkpoint, the G_2-M Checkpoint and the Spindle Checkpoint. Detection of DNA damage in either of these checkpoints will automatically induce the arrest of the cell cycle through their effects on Cyclins and Cyclin-dependent Kinases (CDKs). Cyclins and CDKs are the effector proteins of the cell cycle. Cyclin levels fluctuate in the cell depending on the phase the cell is in, and the CDKs are dependent on their specific cyclins for activation. Upon binding with their respective Cyclin, the activated CDKs phosphorylate key proteins, which allow progression through the cell cycle through the activation of key biochemical pathways[4].

Two prominent DNA damage detector proteins areAtaxia telangiectasia mutated (ATM) Kinase and Ataxia telangiectasia and Rad3 related protein (ATR), which recognise double strand DNA breaks or single strand DNA breaks, respectively. Upon recognition of such double strand breaks, ATM Kinase phosphorylates many proteins, including Chk2, whereas ATR will phosphorilatively activate Chk1. Chk1/2 will in turn phosphorylate and mark Cdc25A for degradation. Under normal circumstances, Cdc25A is necessary for the activation of the Cyclin E – CDK2 complex, which mediates progression through the G_1 – S Checkpoint. Therefore, this activation through ATM/ATR halts the progression through this checkpoint temporarily. Permanent suppression of the cell cycle progression is achieved through the phosphorylation of p53 through Chk1/2, which both activates and protects it against degradation by the ubiquitin ligase Mdm2 (Canman et al., 1998). The activation of p53 itself is associated with a large number of cellular responses, such as the arrest of cell cycle through its activation of p21 which in turn inhibits Cyclin E – CDK21 Complex, the induction of DNA repair pathways and in the presence of excessive DNA repair despite the activation of the repair pathways, the induction of the intrinsic pathway of apoptosis (Rahimi et al., 2013).

3.2 – DNA Damage Tests

Should the DNA damage detection and repair or apoptosis pathways detailed above fail, the cell becomes prone to losing all control over its own cell cycle and entering a vicious cycle of unregulated cell divisions in quick progression, with minimal or no opportunity in between to repair DNA damage. Such cells are said to be “transformed”, that is become cancerous. This loss of control over the progression of the cell cycle, in particular the loss of pathways that could lead to apoptosis, means that cancer cells divide very rapidly without DNA damage being mitigated, which sets them apart from normal cells, where DNA integrity is tightly controlled. This permanence of damage is why Genotoxicology tests are very useful tests in probing the DNA damage in cells.

As DNA damage, unlike most other forms of cellular damage, cannot be repaired by the induction of mitosis and the replacement of the damaged cells, it is particularly detectable by a multitude of tests designed to detect this specific type of damage. This DNA damage need

not be damage inflicted directly on the DNA, but rather encompasses any and all forms of abnormalities that ultimately result in an aberrant distribution and function of DNA.

The simplest forms of DNA damage are alkylation, intercalation or chemical attack, in which chemical groups are added onto the nucleic bases, between the nucleic bases or replaced in the bases, respectively. These forms of damage are unfortunately usually the hardest to detect, as they generally result in no strand breakage, instead manifesting as abnormalities in gene expression. Other forms of damage include Single Strand DNA Breakage or Double Strand DNA Breakage, in which a single strand or both strands are cleaved, respectively. These types of DNA damage can be detected using the methodology known as the Alkaline Comet Assay. The original, unmodified assay can only detect Single Strand and Double Strand DNA breaks (SSBs and DSBs), however with the addition of the appropriate endonucleases, Alkaline Comet Assay would also acquire the capability to detect chemical insults and alkylation.

Another form of DNA damage would be base mismatch, where mismatching bases would be found in pairs in the DNA helix. Such mismatching bases would result in the formation of SSBs during DNA repair and a lack of repair of these errors would result in an increased frequency of SSBs that can be visualised in the Alkaline Comet Assay.

Aberrant repair could also be detected through Genotoxicology testing. One such scenario involves the DSB of a large DNA fragment of a chromosome. Due to both strands breaking, this DNA fragment would then be free to move within the nucleus, under normal circumstances, all chromosome ends contain about 2,500 repeats of the sequence TTAGGG, which is called the telomere. These ends protect the chromosome ends from damage during mitosis and sacrificially protect the coding DNA [6]. As all normal ends of the DNA sequence contain this sequence of nucleotides, any end that does not contain the telomere is recognised as an aberrant end. Under normal circumstances, due to the two ends of a break being in close proximity, the two ends can be ligated back together, restoring DNA stability. However, in the presence of genotoxic compounds, this process may be hampered and the fragment of DNA may become free floating, or may erroneously attach itself to another DSB site, resulting in abnormal fusion chromosomes with abnormal morphology. Such abnormalities may be detected using the Chromosomal Aberration Assay (Rieger, 1968).

Yet another assay is the Cytokinesis Blocked Micronucleus Assay, which exploits the development of erythrocytes to check for genotoxic exposure. Under normal circumstances, an erythrocyte, during the reticulocyte stage of its development into an erythrocyte, will lose

its nucleus, becoming an enucleated cell. During this nuclear elimination, genotoxin exposure may result in increased unusual segregation of DNA material apart from the main nucleus, creating a micronucleus. This is a normal occurrence and these micronucleated cells are then eliminated in a short timeframe (Fenech et al., 2011). Under genotoxin exposure, the rate of micronucleus formation will increase and a significantly higher count will be observed. Erythrocytes are ideal cells to run CBMN Assay as they lack a proper nucleus and the observation of micronuclei is much easier in erythrocytes compared to other cells[7].

3.3 – Alkaline Comet Assay

The Alkaline Comet Assay is a powerful tool that can be used to search for and quantitatively detect DNA damage. It is fast, sensitive, cheap and easy to conduct and can give results with high precision (Nandhakumar et al., 2011).Comet Assay in its modern form was first proposed in 1984 (Östling and Johannson, 1984). While the idea itself was revolutionary, it suffered from relatively weak detection and quantisation performance. It was in 1988 (Singh et al., 1988) that the Comet Assay was run at very alkaline conditions (pH = ~13) that a truly viable test was born. Since then, the Alkaline Comet Assay has found uses in many fields investigating the damage or repair of DNA, such as fertility studies, chemotherapy response and genotoxicology.

The power of the Comet Assay is in its simplicity: It rests on the assumption that in such a severely alkaline condition, all DNA will remain charged and, if put in an electrical field, will travel away from the negatively charged end and towards the positively charged end. The alkalinity will also dissociate the two DNA strands from each other, allowing for independent migration of the two strands and the detection of single-strand breaks.

For purposes of DNA damage and genotoxicity testing, CometAssay is one of the most common and widespread assays. It is recommended since 2011 by European Food Safety Association (EFSA) as a follow-up test using cultured human cells if other in-vitro tests show potential genotoxicity [1].

This study has two major aims: The first is the development and establishment of the Alkaline Comet Assay methodology as a viable genotoxicity and DNA damage testing method in the Near East University in North Cyprus. The other aim is to investigate the potential links between chronic metformin usage and potential DNA damaging effects of such chronic usage.

3.4 – Visualisation of DNA Damage

The visualisation of DNA damage, being the last step in any Genotoxicology assay, is done through the exposure of the samples to DNA binding dyes such as Ethidium Bromide. These dyes amplify by a large factor when they are bound to DNA and are useful in such assays as they lead to low background glow and high-accuracy representations of DNA locality.

Historically, the method of visualising DNA damage has been visual examination, where a cell counter observed randomly selected cells on a slide and categorises them in accordance with methods similar to the one described in this Thesis' Materials & Methods section. In this form of evaluation, a cell counter randomly selects a total of at least 100 cells per patient and takes pictures of these cells under a suitable fluorescence microscope. These pictures are then evaluated by another person (to reduce observer bias) and sorted into categories according to the level of DNA damage they bear.

An emerging technology in Alkaline Comet Assay research is the use of computer technology to perform the sorting and computation step, completely eliminating the observer bias. These software, collectively called Image Analysis Software, measure the percentage of DNA of the cell that has spread into the comet tail ($\%DNA_{tail}$) and compares these values between different samples.

For all means and purposes, Image Analysis Software is decidedly better than visual evaluation in suitably prepared samples with ideal conditions. The method of visual examination functions with a limited number of classes into which all cells must be classified and there will likely be significant DNA damage level differences between

cells in a class. Image Analysis, on the other hand, in computing $\%DNA_{tail}$, will sort the cells into 101 classes (0% to 100% DNA_{tail}), therefore is much more accurate in sorting cells.

Image Analysis does have its own problems. One problem is that unusually shaped comets are not easy to calculate using Image Analysis, whose computation algorithm assumes a distinct head and a distinct tail. Where there is DNA scatter to the sides as well as towards the tail end, Image Analysis software is likely to give inaccurate results. The other problem is cost. Image Analysis Systems require the same equipment as visual evaluation (Flurorescence Microscope, Camera and Computer) but also require specialised software in order to perform the $\%DNA_{tail}$calculation. These software, being custom-built, are usually very expensive. BABPro Image Analysis Software, one of the cheapest on the market, costs 25,000 TRY.

4 – MATERIALS AND METHODS

4.1 - Materials

4.1.1 - Chemicals

The chemicals below have been used in the experimental procedure. They are; Sodium hydroxide (Sigma Aldrich, 06203), Di-sodium EDTA (Merck Millipore, 108421), Triton X-100 (Merck Millipore, 108603), Hydrochloric Acid 37% (Sigma-Aldrich, 30721), Di-methyl Sulphoxide (Merck Millipore, 102952), Sodium Chloride (Merck Millipore, 106404), Ethidium Bromide (Sigma-Aldrich, E8751), High-melting Agar (Sigma-Aldrich, A7174), Low-melting Agar (Sigma-Aldrich, A4018), Phosphate-buffered Saline Solution (Lonza, BE17-516F), Histopaque 1077 (Sigma, 10771), Ethanol, Absolute (Merck Millipore, 100983).

4.1.2 - Laboratory Equipment and Instruments

The laboratory equipment used in the study were: refrigerated centrifuge (HettichMikro 220R), Laboratory Balance (Mettler Toledo AB204-S/FACT), pH meter (Mettler Toledo SevenEasy), Electrophoresis Assembly (Wealtec Elite 300 Plus + GES), Microscope Slides (Isotherm Superior Quality Coloured Line), Microscope Slide Covers (Honka, 24 x 60 mm), Magnetic Stirrer Hotplate (HeidolphHei-Standard), Vortex (HeidolphReax Top), Li-heparin blood collection tubes (BD Vacutainer #368494), Centrifuge Tubes 15 ml (ISOLAB, 078.02.007), Centrifuge Tubes 50 ml (ISOLAB, 078.02.008), Microcentifuge Tubes 1.5 ml (ISOLAB 078.03.002), Micropipettes 20-200 µl and 100-1000 µl (Eppendorf Research), Micropipette Tips 200 µl and 1000 µl, Graduated Pipettes 10 ml (ISOLAB, 021.01.010), Staining Jar, Vertical (ISOLAB, 073.01.001) Fluorescence Microscope (Carl Zeiss Axio Imager M2 with fluorescence attachment).

4.1.3 - Blood Samples

Blood samples were obtained from the patients at the Endocrinology and Metabolic Diseases Service of the Dr.BurhanNalbantoğlu State Hospital, Nicosia,TRNC.

4.2 – Methods

4.2.1 – Patient Selection and Sample Acquisition

The collection of patient blood samples was conducted between June 2015 and January 2016. The patients were informed on the aim and the details of the study and were asked whether they would like to participate in the study. Those who accepted to participate were asked to sign Informed Consent Forms and fill out questionnaires designed to acquire general demographic information of the participants, as well as the drugs they have been using for the last year, their radiation exposure, the presence of malignancies or other genetically transmitted maladies in their families, their occupational exposure and other factors. (A full copy of the questionnaire form can be found in Appendix A). During this period, 43 metformin-using patients and 22 control group patients agreed to participate in the study. Ethics approval for the study was obtained from The NEU Joint-Committee of the Research and Ethics Committee.

The main determinant of patient selection was whether they take metformin or not. Most of the patients were also taking other medications along with metformin, therefore metformin group patients taking a combination of metformin and other medications and the control group taking only other medications. Of these patients, only those with no close familial malignancies, no X-ray or radiation exposure within the last 3 months and no genetically inherited diseases in the family were selected to participate in the study.

For the participants that filled out the Informed Consent Forms and the questionnaire, 2 ml of blood was withdrawn into heparinised blood collection tubes (BD Vacutainer 2ml green top Li heparin, ref #368494). Blood samples were processed the same day. Trypan Blue Viability testing was not conducted on the samples (see Appendix B). Lymphocytes were isolated from the blood samples with density-gradient centrifugation. For each patient, two lymphocyte samples were obtained. Both samples were frozen at -20°C. The day before the visualisation, the samples were thawed at 4°C and the procedure as detailed in Materials & Methods section was carried out.

4.2.2 – Analysis Methodology and Evaluation

Alkaline Comet Assay was used as the genotoxicological assay methodology in the study. To quantitate the results, Total Comet Scoring System, where 100 cells are counted and scored according to the damage they possess, was used. Non-damaged cells were given a score of 0, lightly damaged cells were given 1 and heavily damaged cells were given 2 on the TCS scale. For each patient, two sample slides were produced, 100 cells on each slide were counted and their average was taken. The TCS for each slide was the sum of the scores of the 100 randomly selected cells counted on them.

4.2.3 – Alkaline Comet Assay Methodology

In this study, the Alkaline Comet Assay methodology as used by the Pharmaceutical Toxicology Department of the Faculty of Pharmacy at Marmara University was used with adaptations (Sardas et al, 2010). A week before the acquisition of the blood samples, 2 slides for each patient were coated with High-melting Agar (HMA) by rapidly dipping the slides into hot, molten 0.65% agar solution and quickly wiping the non-frosted back side of the slides with a piece of drying paper, followed by the pipettage of 0.5ml of agar solution onto the slide. The slides were then left under vacuum extraction overnight to dry and dehydrate. After this, they were collected and stored in their original box at 4°C.

A day before the acquisition of blood samples, stable solutions to be used in the experiment were prepared. The formulation and the preparation of these solutions is given below.

- **10M NaOH:** 200 grams of pelletisedNaOH were slowly added to 450ml of water in a volumetric flask in a 4°C water bath. The solution was gently swirled until the NaOH had completely dissolved and the solution was made up to 500ml, then transferred to a 500ml ISO Bottle and kept at 4°C.
- **Stock Lysis:** 73.05 g of NaCl and 18.6 g of EDTA were added to 400 ml of distilled water and left to mix on a magnetic stirrer. After 10 minutes of

mixing, the pH was adjusted to 12 with 10M NaOH and 0.6g of Tris base was added. After the Tris base dissolved, the solution was made to 500ml. If the pH was different from 10, it was adjusted with NaOH (10M) or HCl (37%). The solution was stored at 4°C.

- **Neutralisation Buffer:** 25.25g of Tris base was dissolved in 450ml of distilled water and made up to 500ml. The pH was reduced to 7.5 using HCl (37%). It was stored at 4°C.
- **EDTA Solution:** 7.44 g EDTA was dissolved in 90 ml of distilled water and made up to 100 ml. It was stored at 4°C.

On the day of the experiment, 0.65% Low-melting Agar (LMA) solution was prepared by dissolving 0.065 g of agar powder in 10 ml of PBS. The solution was heated up to 60°C to dissolve the agar. To prevent it from solidifying, it was held in a 37°C incubator or firmly held in naked hand.To isolate the lymphocytes from the blood, whole heparinised blood was vortexed and 100µlof this blood was mixed with 1 ml of PBS at 4°C in a microcentrifuge tube. The diluted sample was kept at 4°C for 10 minutes. To the bottom cone of the tube, 100µlof Histopaque 1077 at 4°C was pipetted, care being taken not to mix the blood with Histopaque. These samples were then centrifuged in a refrigerated centrifuge at 250g reactive force to isolate the lymphocytes. At the end of the centrifugation, the lymphocytes were left as a cloudy layer on top of the erythrocytes and 100µlof these lymphocytes were pipetted out to a fresh, sterile microcentrifuge tube. At this point, the lymphocytes can be frozen at -20°C or ideally at -80°C or can be used immediately in the assay. If they were to be immediately used, 100µlof lymphocyte isolate was mixed with an equal volume of LMA solution, the sample was spread on slides pre-coated with HMA and a slide cover was applied, immediately followed by transfer to 4°C for 30-60 minutes for agar solidification. As the LMA set, the lysis solution was prepared by mixing 10 ml of Di-methyl sulphoxide (DMSO) with 1 ml of Triton X-100 and making up the solution to 100 ml with stock lysis solution. This solution was also stored at 4°C. When the agar had solidified, the slide covers were removed by gently blowing on the slide covers and sliding them across the agar towards the non-frosted end of the slide. The slides were then immersed in the lysis solution in a staining jar under dark for 2 hours. As the cellular lysis progressed, fresh electrophoresis buffer was

prepared by mixing 8.1 ml of EDTA solution and 45 ml of NaOH solution in 1L of distilled water and making up to 1.5L (1500 ml). This solution was also stored at 4°C. After the lysis was over, a fresh staining jar filled with electrophoresis buffer was prepared and the slides were transferred into this staining jar, where they resided at 4°C for 40 minutes under dark conditions. Once this alkalinisation treatment was over, the slides were transferred to an electrophoresis tank filled with fresh electrolysis buffer and were electrophoresed at 4°C, 25 volts and 300 mA for 30 minutes.At the end of the electrophoresis, the slides were then neutralised 3 times in staining jars filled with neutralisation buffer for 5 minutes each. They were then dehydrated and fixated in 50% ethanol, 75% ethanol and 100% ethanol for 5 minutes each. After the end of the 100% ethanol fixation, they were laid on drying paper in a cool, dark room and allowed to dry. Within 3 days, they were then dyed using 50µlof 100 ug/ml Ethidium Bromide solution and visualised under a fluorescence microscope.

4.2.4 – Initial Learning of the Assay and Establishment in Cyprus

After an initial Comet Assay learning period at the Pharmaceutical Toxicology Department of the Faculty of Pharmacy of Marmara University in Istanbul, Turkey between the dates of 22-26 December 2014, the initial equipment and chemicals for the Comet Assay were obtained and the “Genotoxicology Laboratory” in our department was set up. As part of these preparations, the laboratory was prepared to work with the samples using the equipment already present in the laboratory.

4.2.5 – Assay Validation Group Methodology

From each individual, 2x100 µl samples were taken. In order to see the infliction of damage on the lymphocytes, H_2O_2 solution (Merck, 30%) was used. One sample was mixed with 10 µl of PBS (control) and the other was mixed with 10 µl of 30% H_2O_2.Both samples were incubated on ice for 30 minutes and the assay methodology was continued.

5 - RESULTS

5.1 – Assay Validation Group

The establishment of the Comet Assay in North Cyprus, being the first objective of this study, was the first step in the development of the assay. For this, 6 individuals from 6 different families were chosen for blood donation. They had no history of cancer or malignant diseases for at least 3 generations. They were chosen among the ages of 24-26 and none of them were smokers.None used prescription medication or any other regular medication.The results obtained as a result of these assays is given below in Table 5.1, where the age, gender, smoking habits and the total comet score before and after H_2O_2 treatment are given. Figure 5.1 shows one of the undamaged cells as it was visualised under the microscope and Figure 5.2 shows cells from the same individual after H_2O_2 treatment. Of the participants 4 were male and 2 were female. Due to the limited sampling size, statistical comparison between males and females was not conducted.

Table 5.1 – Breakdown of assay validation patients

Individual	Age	Gender	Smoker/Non-smoker	TCS (control)	TCS (test)
1 (myself)	24	M	Non-smoker	4	116
2	24	M	Non-smoker	2	144
3	24	F	Non-smoker	6	151
4	24	M	Non-smoker	5	137
5	25	F	Non-smoker	9	100
6	26	M	Non-smoker	6	121

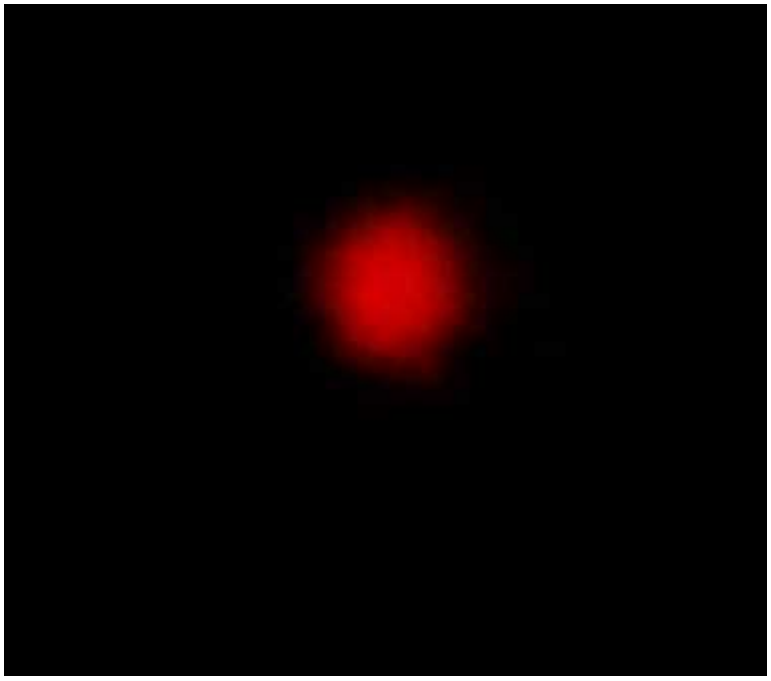

Figure5.1 – Undamaged DNA as seen in a fluorescence microscope (Assay Validation Group Patient 1 Control)

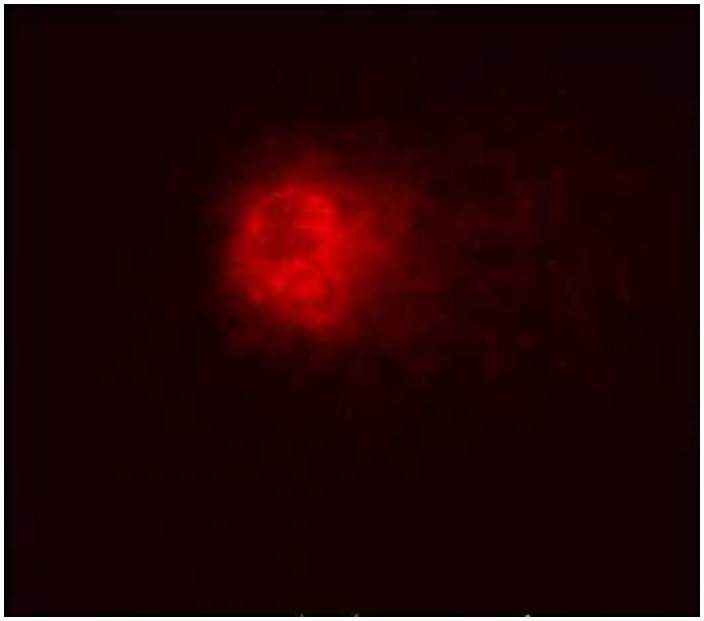

Figure5.2 – High-level DNA damage as seen in a fluorescence microscope (Assay Validation Group Patient 1 Test)

5.2 – Study Groups

A total of 67 patients agreed to participate in the study. The metformin group numbered 43 (29 females, 14 males), whereas control group numbered 22 (14 females, 8 males).Of these participants, 17 metformin group and 6 control group samples were deemed under fluorescence microscope to be in usably good condition.

Within the metformin group, the smokers' average (n=4) TCS was 153.5, the quitters' average (n=8) TCS was 147.5 and the non-smokers' average (n= 5) TCS was 151.6. The age averages were 60.0 for smokers, 61.63 for quitters and 52.8 for non-smokers. All respondents replied that they were social drinkers, with all respondents giving out replies of drinking once or twice per week. Within the control group, the average TCS for all patients (n=6) was 161.5, whereas the average TCS for non-smokers (n=5) was 153.8. The average age for all control group patients was 56.3 and for non-smokers, 55.2 years. The average TCS for females in the metformin group was 140.1, compared to 154.7 for females in the control group. Similarly, the males in metformin group had an average TCS of 168.5, compared to 168.3 for control group males.

All patients except one used at least one medication with metformin. Almost all of these other medications were cardiovascular medications, with 2 thyroid and 3 asthma medication using patients.

The visualisation of the Study Group results was conducted using the Genetics Laboratory Fluorescence Microscope with an excitation wavelength of 290nm and an observation wavelength of 550nm. A sample cell possessing low levels of DNA damage is shown below in Figure 5.3.

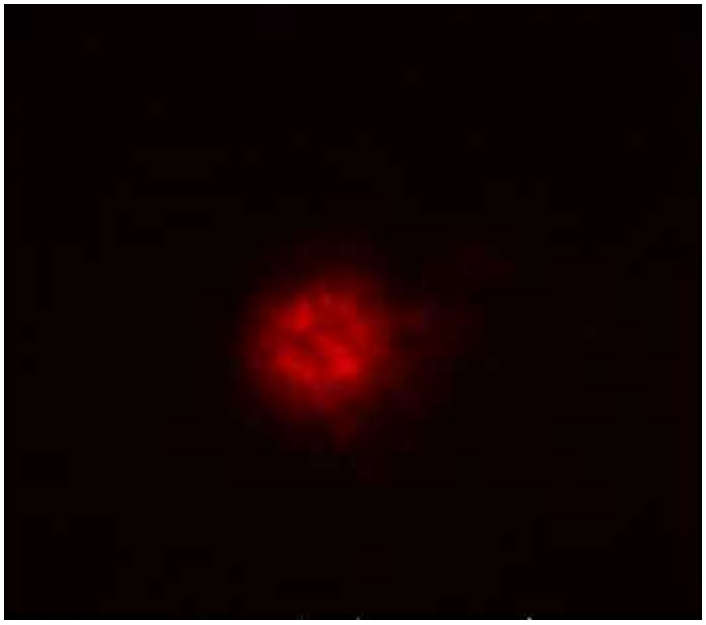

Figure5.3 – Low-level DNA damage as seen in a fluorescence microscope

(Study Group Patient 000)

A full breakdown of metformin-taking group and control group patients is given belowin Table 5.2 and 5.3., along with demographic values (i.e. age,gender, smoking habit), andtotal comet scores.

Table 5.2 – Breakdown of Metformin Group Patients

Metformin Patient	**Patient Code**	**Sex**	**Age**	**Smoking Status**	**TCS**	**ND**	**LD**	**HD**
1	H000	F	31	Non-smoker	81	39	41	20
2	H034	F	67	Quit 7 years ago	139	1	59	40
3	H032	F	62	Quit 10 years ago	200	0	0	100
4	H033	F	41	1-2 a day	195	0	5	95
5	H029	F	49	Quit 10 years ago	28	72	28	0
6	H017	M	64	1-2 a day	133	12	43	45
7	H032	M	61	Non-smoker	200	0	0	100
8	H018	F	64	Quit 13 years ago	56	56	30	13
9	H015	M	72	20 a day	106	35	24	41
10	H019	F	61	Non-smoker	89	32	47	21
11	H020	F	65	Quit 13 years ago	173	10	7	83
12	H022	M	52	Quit 19 years ago	184	7	2	91
13	H005	M	68	Quit 9 years ago	200	0	0	100
14	H002	M	62	Non-smoker	188	6	0	94
15	H004	F	49	Non-smoker	200	0	0	100
16	H003	F	63	5-6 a day	180	0	20	80
17	H010	F	66	Quit 20 years ago	200	0	0	100

Table 5.3 – Breakdown of Control Group Patients

Control Patient	**Patient Code**	**Age**	**Sex**	**Smoking Status**	**TCS**	**ND**	**LD**	**HD**
1	C001	62	F	30 a day	200	0	0	100
2	C002	58	F	Non-smoker	133	28	11	61
3	C003	44	M	Non-smoker	200	0	0	100
4	C004	61	M	Non-smoker	142	27	4	69

5	C005	56	F	Non-smoker	131	27	15	58
6	C006	57	M	Non-smoker	163	18	1	81

6 – DISCUSSION

This, to the knowledge of the student, is the first instance of Alkaline Comet Assay methodology being set up and conducted in any capacity in North Cyprus. This powerful method of DNA damage analysis is an important development in a country like the TRNC.

North Cyprus has a relatively high prevalence of hereditarily linked diseases and studies of the environment; especially exposure to potentially mutagenic, carcinogenic or teratogenic agents has historically been an area of insufficient coverage. Thus, the establishment of easy, simple and fast methodologies such as the Alkaline Comet Assay will allow more frequent studies investigating this type of exposure.The high frequency of hereditary diseases such as diabetes, hyperlipidemia, hypertension or cancer does not at first hint at the presence of DNA damaging factors being at play.

However, the frequency of these disorders is at very high levels. Of all the 20 years old or older adults in the TRNC, 11% have Type I or II Diabetes Mellitus, whereas another 18% have Pre-diabetes (DBNSH Centre for Diabetes Statistics). A further 5,000 adults and children are currently being treated for cancer (Help Those with Cancer Assoc.). Environmental pollution is a problem emerging rapidly in the collective Turkish Cypriot mind-set, and the sheer amount of areas where Alkaline Comet Assay can be applied (Cyprus Mining Corporation waste at Lefkas, unfiltered exhaust from Teknecik power station, uncontrolled pesticide use, widespread cigarette smoking, use of contaminated water, mine quarries at the Kyrenia mountains etc.) give North Cyprus unparalleled potential as a country where Alkaline Comet Assay can be used to improve the lives of the population and provide insight into how a change in environment can result in changes in the people that occupy it. Environmental factors are known to affect the genomic integrity of a person.

The Alkaline Comet Assay is used in a large variety of manners to check for DNA damage. It can be done using human peripheral blood lymphocytes (Naidoo et al., 2016), in non-human animals (Battal et al., 2013) and in cultured cells (Khoei et al., 2016).

The results obtained from the Assay Validation Group were in remarkable quality. For the purposes of the Assay, the division between "healthy" and "unhealthy" was set at an arbitrary TCS score of 20. This, corresponding to a maximum of 20% lightly damaged cells or 10% heavily damaged cells was not chosen as an experimentally-based limit, but was rather chosen to establish a strict limit with which to select an assay validation group. The untreated group gave an average Alkaline Comet Assay TCS of 5.33, compared to the test group's average of 128.17. Statistical analysis (Student's Paired t-test) gives out a p value of 0.0001, indicating a strong causation between H_2O_2 exposure and DNA damage.

The treatment with H_2O_2 was specifically chosen as its DNA damage calibration curve is well known in genotoxicology and provides a well-founded DNA damage "ladder" with which samples can be compared (Dai et al., 2015). This allows the extrapolation of the concept of DNA damage into phenotypic change i.e. It allows us to answer the question "How much of a phenotypic malfunction can this much DNA damage cause?"

There was originally a 7^{th} individual in the assay validation group, a non-smoker 32 year old female, however she was excluded from the assay validation group when she was diagnosed with Acute Lymphoblastic Leukaemia, and her Comet Assay results, average of 3 different counts, (100 cells each from 3 slides, then average of the 3 taken) showed a TCS reading of 174.

Due to the low number of control group patients, it is not appropriate to draw comparisons between the two groups at this point. Similarly, the sub-divisions of the metformin group are also too few in number to draw conclusions based on statistics. What can be done on these results now is speculation. However, with the improvements given in Appendix B and further acquisition of samples, it will become possible to compare the control group with the metformin group with high

statistical power. Smoking is known to induce DNA damage (Beyoglu et al., 2010).. However, based on the limited number of samples available, the average TCS for metformin using smokers (153.5) and the average TCS for metformin using non-smokers (151.6) do not seem to be significantly different. These are both comparable to the average TCS for non-smoking control group, with an average TCS of 153.8.This will be one of the future foci of this study, as a lack of difference between the groups could mean that metformin or some other medication might be protecting against DNA damage, even in smokers. At this point, this is purely speculative, as the data currently at hand is insufficient to draw such conclusions; however a potential link between metformin and the prevention of DNA damage could give science a deeper perspective into how metformin conducts its anti-cancer effects and potentially produce more information on how cancer develops. It must, however, be noted that the average age of the non-smoking group was slightly lower than the smoker and quitter groups, therefore, with further data, if this age difference does not disappear, it will need to be taken into consideration as DNA damage can accumulate as age progresses. In a similar vein, females ranked lower than males in all TCS measures. Whether this is a significant difference or not requires further investigation. Males in both groups averaged very closely (168.5 in metformin group vs. 168.3 in control group) whereas metformin taking females ranked considerably lower than their control group counterparts (154.7 vs. 140.1 TCS). This putative discrepancy may simply be a fluke of statistics or might indicate a sexual dimorphism in metformin-induced cancer protection. This, however, also necessitates further study.

Otherwise, the relatively high total comet scores for both groups require an explanation. An average TCS in the vicinity of 150 is quite unexpected in relatively healthy individuals, although Type II Diabetes is a known cause of DNA damage (Sardas et al., 2001).

The easiest explanation would have been experimental error. Such high TCS values are unlikely to be seen in individuals without severe haematological abnormalities. However, the patients were specifically selected to exclude such effects. Therefore, it is unlikely that the results could be due to naturally-occurring factors.. However,

both the methodology used in the assay validation group and the two patient groups is precisely the same; therefore experimental error is also an unlikely explanation.

The most likely explanation is that the lymphocytes were already damaged by the time they were isolated from other blood constituents. Although 67 patient samples were collected over the duration of the study, only 23 samples could be recovered. During the majority of the study, especially during the late summer, temperatures in North Cyprus mostly riseduring daytime above 40°C. The time difference between the withdrawal of blood from the patient's bloodstream to the isolation and cryopreservation of lymphocytes at the Genotoxicology Laboratory can be as much as 6 hours. During this entire period, the blood samples are in a high temperature, low nutrient and highly hypoxic environment. All of these factors could be contributors to DNA damage or complete lymphocyte loss.

The preliminary results of the study indicate that the DNA damage levels in the Turkish Cypriot population are very high. This should be investigated by the implementation of large-scale studies to investigate potential sources and causes for these high levels of DNA damage.

Exposure to environmental agents such as pesticides or heavy metals can cause detectable levels of DNA damage (Cok et al., 2004). Lifestyle factors such as drinking and smoking are also known determinants of genetic damage in an individual (Kadioglu et al., 2012) and The 2005 Obesity Report of the Turkish Cypriot Diabetes Association reported that 17.2% of all adults are obese, whereas a further 36.3% were overweight.Obesity and overweightness are associated with the emergence of Type II Diabetes. The emergence of DM Type II is itself associated with a number of co-morbidities commonly called Metabolic Syndrome, which give rise to increased risk of developing cancer (Rani et al., 2016). Therefore, it is possible that environmental pollution, unhealthy lifestyle and habits and the emerging obesity epidemic within the Turkish Cypriot community are all causing rising levels of DNA damage in susceptible individuals.

In any case, the reason or reasons behind the high DNA damage level in Turkish Cypriots will be elaborated further and factors giving rise to this risk will need to be mitigated for the sake of public health.

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8.1– APPENDIX A - INFORMED CONSENT FORMS (TURKISH)

ARAŞTIRMA AMAÇLI ÇALIŞMA İÇİN AYDINLATILMIŞ ONAM FORMU

(Araştırmacının Açıklaması)

Tip 2 Diyabet hastalığı tedavisinde kullanılan Metformin adlı ilaç ile ilgili yeni bir araştırma yapmaktayız. Araştırmanın ismi "Tip 2 Diyabet Hastalarında Kronik Metformin Kullanımının DNA Hasarı İle İlişkisinin Araştırılması"dir.

Sizin de bu araştırmaya katılmanızı öneriyoruz. Bu araştırmaya katılıp katılmamakta serbestsiniz. Çalışmaya katılım gönüllülük esasına dayalıdır. Kararınızdan önce araştırma hakkında sizi bilgilendirmek istiyoruz. Bu bilgileri okuyup anladıktan sonra araştırmaya katılmak isterseniz formu imzalayınız.

Bu araştırmayı yapmak istememizin nedeni, diaybet hastalığının tedavisinde yaygın olarak kullanılan Metformin adlı ilaca dair son yıllarda deney hayvanlarında ve doku kültürleri üzerinde yapılan çalışmalarda kromozomun yapı taşı olan DNA adlı yapılar üzerinde olumsuz etkileri olabileceği hakkında yayınlar mevcuttur. Bu tür çalışmaların her hangi bir spekülasyona yol açmaması için, veya gerçekten böyle bir etkisinin olup olmadığının aydınlatılması gerekir. Hasta güvenliği açısından tedavi dozunda kullanılan bu ilacın, siz hastalarımızın onayı ile araştırılması çok önemlidir. Yakın Doğu Üniversitesi Toksikoloji Anabilim Dalı ve Dr. Burhan Nalbantoğlu Devlet hastanesi Endokrinolojik ve Metabolik Hastalıklar Servisi ile ortaklaşa gerçekleştirilecek bu çalışmaya katılımınız araştırmanın başarısı için önemlidir.Eğer araştırmaya katılmayı kabul ederseniz, sizden 5 ml kan örneği alınacak ve bir araştırma formu doldurmanız istenecektir. Bu çalışmaya katılmanız için sizden herhangi bir ücret istenmeyecektir. Çalışmaya katıldığınız için size ek bir ödeme de yapılmayacaktır.Sizinle ilgili tıbbi bilgiler gizli tutulacak, ancak çalışmanın kalitesini denetleyen görevliler, etik kurullar ya da resmi makamlarca gereği halinde incelenebilecektir.

Bu çalışmaya katılmayı reddedebilirsiniz. Bu araştırmaya katılmak tamamen isteğe bağlıdır ve reddettiğiniz takdirde size uygulanan tedavide herhangi bir değişiklik olmayacaktır. Yine çalışmanın herhangi bir aşamasında onayınızı çekmek hakkına da sahipsiniz.

Katılımcı	**Görüşme tanığı**	**Araştırmacı**
Adı, soyadı:	Adı, soyadı:	Adı soyadı, unvanı:
Adres:	Adres:	Adres:
Tel.	Tel.	Tel.
İmza	İmza:	İmza

ARAŞTIRMA AMAÇLI ÇALIŞMA İÇİN AYDINLATILMIŞ ONAM FORMU

(Katılımcının / Hastanın Beyanı)

Sayın Uzm. Dr. Hasan SAV tarafından Yakın Doğu Üniversitesi Toksikoloji Anabilim Dalı ve Dr. Burhan Nalbantoğlu Devlet Hastanesi Endokrinoloji ve Metabolik Hastalıklar Servisi'nde "Tip 2 Diyabet Hastalarında Kronik Metformin Kullanımının DNA Hasarı İle İlişkisinin Araştırılması" konusunda bir araştırma yapılacağı belirtilerek bu araştırma ile ilgili yukarıdaki bilgiler bana aktarıldı. Bu bilgilerden sonra böyle bir araştırmaya "katılımcı" olarak davet edildim.

Eğer bu araştırmaya katılırsam araştırmacı ile aramda kalması gereken bana ait bilgilerin gizliliğine bu araştırma sırasında da büyük özen ve saygı ile yaklaşılacağına inanıyorum. Araştırma sonuçlarının eğitim ve bilimsel amaçlarla kullanımı sırasında kişisel bilgilerimin ihtimamla korunacağı konusunda bana yeterli güvence verildi.

Projenin yürütülmesi sırasında herhangi bir sebep göstermeden araştırmadan çekilebilirim. (Ancak araştırmacıları zor durumda bırakmamak için araştırmadan çekileceğimi önceden bildirmemim uygun olacağının bilincindeyim) Ayrıca tıbbi durumuma herhangi bir zarar verilmemesi koşuluyla araştırmacı tarafından araştırma dışı tutulabilirim.

Araştırma için yapılacak harcamalarla ilgili herhangi bir parasal sorumluluk altına girmiyorum. Bana da bir ödeme yapılmayacaktır.

İster doğrudan, ister dolaylı olsun araştırma uygulamasından kaynaklanan nedenlerle meydana gelebilecek herhangi bir sağlık sorunumun ortaya çıkması halinde, her türlü tıbbi müdahalenin sağlanacağı konusunda gerekli güvence verildi. (Bu tıbbi müdahalelerle ilgili olarak da parasal bir yük altına girmeyeceğim).

Araştırma sırasında bir sağlık sorunu ile karşılaştığımda; herhangi bir saatte, Uzm. Dr. Hasan SAV'ı 0392 22 82 272 (iş) veya 0533 861 80 90 (cep) no'lu telefonlardan ve Dr. Burhan Nalbantoğlu Devlet Hastanesi Endokrinoloji ve Metabolik Hastalıklar Servisi –Lefkoşa adresinden arayabileceğimi biliyorumBu araştırmaya katılmak zorunda değilim ve katılmayabilirim. Araştırmaya katılmam konusunda zorlayıcı bir davranışla karşılaşmış değilim. Eğer katılmayı reddedersem, bu durumun tıbbi bakımıma ve hekim ile olan ilişkime herhangi bir zarar getirmeyeceğini de biliyorum.

Bana yapılan tüm açıklamaları ayrıntılarıyla anlamış bulunmaktayım. Kendi başıma belli bir düşünme süresi sonunda adı geçen bu araştırma projesinde "katılımcı" olarak yer alma kararını aldım. Bu konuda yapılan daveti kabul ediyorum.

İmzalı bu form kâğıdının bir kopyası bana verilecektir.

Katılımcı	**Görüşme tanığı**	**Araştırmacı**
Adı, soyadı:	Adı, soyadı:	Adı soyadı, unvanı:
Adres:	Adres:	Adres:
Tel.	Tel.	Tel.
İmza	İmza:	İmza

8.1 – APPENDIX A - COMPLETE QUESTIONNAIRE FORM (ENGLISH)

DATE : ____/____/_____

INVESTIGATION OF METFORMIN USAGE AND DNA DAMAGE IN TYPE 2 DIABETES PATIENTS

QUESTIONNAIRE FORM

Name-Surname	
Age	
Gender	
Telephone	
Patient Code	

PLEASE ANSWER THE QUESTIONS BELOW AS FAITHFULLY TO THE TRUTH AS POSSIBLE

1. What is your profession and position ? How long have you been working at this position?

2. What is your education level ? Please tick the appropriate box.

Primary		University	
Secondary		Master's Degree	
High School		PhD / Doctorate	

3. Do you have a smoking habit ? If so, please give further information.

I've never smoked	
I used to smoke, but I quit	

When did you quit smoking ? .....

If you still smoke;

How many cigarettes do you smoke per day ?	
How long have you been smoking ?	

4. Are you frequently exposed to cigarette smoke ?

Yes	
No	

5. Do you consume alcohol ?

Yes	
No	

What kinds of alcohol do you consume ?

Whiskey, Rakı, Vodka	
Wine	
Beer	

How frequently do you drink ?

Rarely	
Once a week	
2-3 times a week	
4-5 times a week	
6-7 times a week	

6. Are there any medications, vitamins and antioxidants you have been using regularly or frequently in the last year ?

Yes	
No	

Please give further information on what they are, how frequently they are used, what dosages are used and how long you have been taking them:

7. Have you been exposed to radiating tests such as X-ray, Computed Tomography or Scintillography in thelast year ? If yes, please give further information on their dates and what they were.

Yes	
No	

8. Do you or any of your relatives suffer from any hereditary or genetically-linked diseases ? If yes, please give further information.

Yes	
No	

9. Do you have any relatives that suffer from or died due to diseases/disorders such as cancer, organ insufficiency, diabetes, cardiovascular diseases, neurodegenerative diseases (Alzheimer's, Parkinson's Multiple Sclerosis, Premature Senility) ?

Relationship with you	Disease/Disorder	Duration suffered	Date of death (if deceased)

10. Have you had any hormonal therapies (insulin, thyroid, birth control, menopause) in the last year ? If yes, please give further information.

Evet	
Hayır	

8.1 – APPENDIX A - COMPLETE QUESTIONNAIRE FORM (TURKISH)

TARİH : ____/____/_____

TİP 2 DİYABET HASTALARINDA KRONİK METFORMİN KULLANIMININ DNA HASARI İLE İLİŞKİSİNİN ARAŞTIRILMASI

ANKET FORMU

Ad-Soyad	
Yaş	
Cinsiyet	
Telefon	
Hasta Kodu	

LÜTFEN AŞAĞIDAKİ SORULARI DİKKATLİCE OKUYARAK EN DOĞRU ŞEKİLDE CEVAPLAYINIZ

1. Nerede Çalışmaktasınız ? Göreviniz Nedir ? Kaç yıldır bu pozisyonda çalışmaktasınız ?

2. Eğitim Düzeyiniz nedir ? Lütfen uygun kutuyu işaretleyiniz.

İlkokul		Üniversite	
Ortaokul		Yüksek Lisans	
Lise		Doktora	

3. Sigara içme alışkanlığınız var mıdır ? Var ise lütfen ileri bilgi veriniz.

Hiç içmedim	
İçiyordum bıraktım	

Ne zaman bıraktınız ? .....

Halen içiyorsanız;

İçme sıklığınız (günde kaç sigara)	
Kaç yıldır içiyorsunuz ?	

4. Sigara içilen ortamlarda sıkça bulunur musunuz ?

Evet	
Hayır	

5. Alkol kullanır mısınız ?

Evet	
Hayır	

Ne tür alkol kullanırsınız ?

Viski, Rakı, Vodka	
Şarap	
Bira	

Ne sıklıkta kullanırsınız ?

Nadiren	
Haftada 1 kez	
Haftada 2-3 kez	
Haftada 4-5 kez	
Haftada 6-7 kez	

6. Son 1 yıl içerisinde sürekli veya düzenli olarak kullandığınız ilaç, vitamin veya antioksidan var mıdır ?

Evet	
Hayır	

Lütfen ne oldukları, ne sıklıkta kullanıldıkları, ne dozda kullanıldıkları ve ne süreden beridir kullandığınız hakkında bilgi veriniz:

7. Son 1 yıl içerisinde röntgen, bilgisayarlı tomografi (CT) veya sintillografi gibi radyasyon içeren testlere tabi oldunuz mu ? Cevabınız evet ise lütfen bilgi veriniz.

Evet	
Hayır	

8. Sizde veya aile bireylerinizde genetik kökenli herhangi bir hastalık var mıdır ? Var ise lütfen bilgi veriniz.

Evet	
Hayır	

9. Aileniz içerisinde kanser, organ yetmezliği, diyabet, nörodejeneratif hastalık (Alzheimer, Parkinson, Mültipl Skleroz, Erken Bunama) gibi hastalıklardan mustarip veya vefat etmiş bireyler var mıdır ?

Yakınlık Derecesi	Hastalık	Kaç yıldır mustarip ?	Vefat tarihi (etmişse)

10. Son 1 yıl içerisinde herhangi bir hormon tedavisi (tiroid, insülin, doğum kontrol ilacı) gördünüz mü ? Tedavi görmüşseniz lütfen ileri bilgi veriniz.

Evet	
Hayır	