Role of plasma osteocalcin levels in blood glucose concentrations and insulin levels in type II diabetic Sudanese patients

A thesis submitted in fulfillment for the requirements of the Philosophy Degree in human Physiology

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DECLARATION OF AUTHORSHIP

I hereby declare that this thesis has been composed entirely by myself with the assistant of the supervisors, and is a result of my own interpretations and investigations. When I have consulted and quoted from the published work of others, this is always clearly attributed. It has neither been accepted nor submitted for any other degree in this university or any other academic institution. The data collection, analysis and interpretation were the sole work of the author, except where acknowledged.

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Dedication

This thesis is dedicated to the soul of my supervisor, from whom I learnt much and through his support made this work visible.

To the soul of my father, who taught me how be a righteous and valuable for all mankind.

To my beloved mother, who gave me the touch of sympathy and glamor.

To my wife, for her eternal love and simplicity.

To my daughter and son, whom I see my future through their innocent lovely eyes.

To my brothers, sisters, my wings to flight in this world.

To my colleagues, and intimate friends whom I will never forget throughout my entire life.

To you all, I kindly dedicate this piece of work.
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Abstract

BACKGROUND:
Osteocalcin, a bone-derived protein, has recently been reported to affect energy metabolism. This study aimed to evaluate the role of osteocalcin levels in blood glucose concentrations and insulin levels in newly diagnosed type II diabetic Sudanese patients.

OBJECTIVES:
Compare the differences in fasting blood glucose (FBG) concentration, fasting plasma insulin (FPI) level and lipid profile between newly diagnosed type II diabetic Sudanese patients and healthy controls, and whether these differences are affected by gender, age and body mass index (BMI). Compare the differences in total osteocalcin (TOC), undercarboxylated osteocalcin (ucOC) concentrations between newly diagnosed type II diabetics and controls, and whether the differences are affected by age, gender and BMI. Determine the correlation of age, gender, BMI with TOC, ucOC.

MATERIALS & METHODS:
115 subjects, who were referred for diagnosis of diabetes mellitus in Khartoum state for the first time, were included in this study. 65 normal subjects were randomly selected from the same area as a control.

5 ml of venous blood was withdrawn from all participants after an overnight fasting, and was immediately centrifuged to separate the plasma. The plasma was used to measure the levels of TOC, ucOC and FPI, by enzyme-linked immunosorbent assay (ELISA), while FBG and lipid profile were measured by automated blood chemistry analyzer.

Univariate analysis was used to determine correlation between diabetes, gender, age, BMI, with TOC, ucOC, FI, FBG and lipid profile.
RESULTS:

In spite of presence of interactions between status (diabetic or not) and gender, status and age, status and BMI affecting the results of TOC, ucOC, FPI, FBG and lipid profile, but all these interactions were not statistically significant (P > 0.05 for all).

The mean of plasma concentration of TOC was significantly less in newly diagnosed diabetic group (2.38 ± 1.39 ng/ml) compared to control one (14.83 ± 2.11 ng/ml) (P = 0.000). The mean of ucOC concentration was significantly higher in the controls (2.61 ± 0.49 ng/ml) compared to the diabetics (1.09 ± 0.57 ng/ml) (P = 0.000). The mean of plasma FI concentration was higher in diabetics (20 ± 6 U/L) compared to control one (9 ± 2 U/L) (P = 0.000). The mean of plasma FBG concentration was higher in diabetics (195 ± 59 mg/dl) compared to control one (85 ± 25 mg/dl) (P = 0.000). The mean of plasma total cholesterol (TCHOL) concentration was significantly higher in diabetics (160 ± 35 mg/dl) compared to controls (147 ± 22 mg/dl) (P = 0.003), while the mean of plasma high density lipoprotein (HDL) concentration was significantly lesser in diabetics (20 ± 6 mg/dl) compared to controls (33 ± 6 mg/dl) (P = 0.000).

There were statistically significant correlations for TOC and ucOC with FBG and FPI (P = 0.000 for all Spearman correlation). There were statistically significant correlations for TOC with TCHOL and HDL that is statistically significant (P= 0.003, 0.05 respectively), but the correlation for triglycerides (TG) and low density lipoprotein (LDL) were not statistically significant (P > 0.05).

CONCULSION:

In conclusion, osteocalcin may have an important role in glucose metabolism, probably through enhancing insulin secretion and improving insulin resistance. Osteocalcin may also play an important role in lipid metabolism in type 2 diabetes mellitus (T2DM) patients.

KEY WORDS:

Total Osteocalcin, Undercarboxylated Osteocalcin, Fasting Insulin, Fasting Blood Glucose Lipid profile and Type II diabetes mellitus.
المستخلص

خلفية:
الأستوكالسين، وهو بروتين مشتق من العظم، وجد أنه يؤثر على أيض الطاقة. هدفت هذه الدراسة إلى تقييم تأثيرات مستوى الأستوكالسين على تركز الجلوكوز في الدم. ومستوى الأنسولين لدى مرضى السكري السودانيين من النوع الثاني المشخصين حديثًا.

الأهداف:
مقارنة الاختلافات في تركز جلوكوز الدم للصائم، ومستوى الأنسولين، والدهون بين مرضى السكري من النوع الثاني الذين شاركوا حديثًا مع مجموعة التحكم، وما إذا كانت هذه الاختلافات تتأثر بالجنس والعمر وميزة كلة الجسم. مقارنة الاختلافات في الأستوكالسين بين مرضى السكري من النوع الثاني الذين شاركوا حديثًا مع مجموعة التحكم، وما إذا كانت الاختلافات تتأثر بالجنس وميزة كلة الجسم. تحديد قيمة الارتباط مع العمر والجنس وميزة كلة الجسم، وترابط الأستوكالسين ومؤشرات الدهون على مستوى السكر في البلازما أثناء الصيام، في مرضى السكري من النوع الثاني الذين شاركوا حديثًا عن طريق أداء الاستوكالسين بالسكري.

طرق البحث:

تضمن 115 شخصًا مريضاً، بالداء السكري، في ولاية الخرطوم لأول مرة شاركوا في هذه الدراسة. وآخرين 65 شخصًا طبيعياً بشكل عشوائي من نفس منطقة التحكم.

سحب 5 مل من الدم الوريدي من جميع المشاركين بعد صيام الليلة الماضية، وفرصت البلازما. استخدمت البلازما لقياس مستويات الأستوكالسين، ومستوى الأنسولين، بواسطة مقايضة المتر المكاني المرتبط بالإنزيم، بينما تم قياس مستوى السكر في الدم أثناء الصيام ومستوى الدهون بواسطة محلل كيمياء الدم الآلي.

تحديد وجود تفاعلات كبيرة بين السكري أو عدمه والجنس والحالة والعمر وميزة كلة الجسم، مما يؤثر على الفرق في الأستوكالسين، مستوى الأنسولين، مستوى السكر في الدم أثناء الصيام، ومستوى الدهون، أجري تحليل أحادي المتغير.

النتائج:

على الرغم من وجود تفاعلات بين مرضى السكري والأصحاء والجنس والحالة والعمر وميزة كلة الجسم التي تؤثر على نتائج الأستوكالسين، مستوى الأنسولين، مستوى السكر أثناء الصيام، ومستوى الدهون، لكن كل هذه التفاعلات لم تكن ذات دلالة إحصائية (قيمة P أقل من 0.05 للجميع).

كان متوسط تركيز البلازما أقل بشكل ملحوظ في مجموعة السكري المشخصة حديثًا (1.39 ± 2.38) نانوغرام لكل مل مقارنةً بمجموعة التحكم (11.09 ± 1.09) نانوغرام لكل مل (P = 0.000). كان متوسط تركيز الأستوكالسين تحت الكروبات أعلى بكثير في مجموعة التحكم (2.61 ± 0.49) نانوغرام لكل مل مقارنةً بمرضى السكري (0.57 ± 0.59) نانوغرام لكل مل (P = 0.000).

viii
متوسط تركيز الانسولين أعلى في مرضى السكري (6 ± 20) وحدة لكل لتر مقارنة بجموعة التحكم (2 ± 9) وحدة لكل لتر (P = 0.000).

كانت هناك علاقات ذات دالة إحصائية لكل من الدهون الكلي و المجتمعاً مع مستوى السكر أثناء الصيام و مستوى الانسولين (P = 0.000). كانت هناك ارتباطات ذات دالة إحصائية للأونستيوكالين الكلي و مستوى الكولسترول الكلي و مستوى الكولسترول عالي الكثافة والتي ذات دالة إحصائية (P = 0.003، 0.05 على التوالي) ، لكن العلاقة للفلافير干扰 و السليستيرويل المنخفض الكثافة لم تكن ذات دالة إحصائية p أكبر من (0.05).

خاتمة:

في الختام ، قد يكون للأونستيوكالين دور مهم في استقلال الجلوكوز ، ربما من خلال تعزيز إفراز الأنسولين وتحسين المقاومة.

الأوستيوكالين قد يلعب أيضاً دوراً هاماً في عملية التثبيط الغذائي للدهون في مرضى السكري من النوع الثاني.

الكلمات الدالة:

الأوستيوكالين الكلي، الأوستيوكالين تحت الكربونات، الأمثل أثناء الصيام، سكر الدم أثناء الصيام، مستوى الدهون، التحلل السكري النمط الثاني.
Table of Contents

BIBLIOGRAPHIC ENTRY .................................................................i
Ph.D. EXAMINATION COMMITTEE MEMBER ...........................................ii
DECLARATION .............................................................................. iii
DEDICATION.................................................................................... iv
ACKNOWLEDGEMENTS .....................................................................v
ABSTRACT ...................................................................................... vi
مستخلص ........................................................................................ vili
LIST OF TABLES............................................................................... xv
LIST OF FIGURES ............................................................................ xvi
LIST OF APPENDICES ....................................................................... xvii
LIST OF ABBREVIATIONS ................................................................... xvii

CHAPTER [1]

INTRODUCTION, RATIONALE & OBJECTIVES

1.1. Definition and epidemiology of diabetes mellitus ...........................................1
1.2. Rational for this study ...........................................................................2
1.3. Objectives of the study ......................................................................3
1.3.1. General objectives .......................................................................3
1.3.2. Specific objectives .......................................................................3

CHAPTER [2]

LITERATURE REVIEW

2.1. Pathophysiology of type 2 diabetes mellitus .........................................4
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1.1</td>
<td>β-cell dysfunction and insulin resistance</td>
<td>4</td>
</tr>
<tr>
<td>2.1.1.</td>
<td>Environment, genes, and development of type 2 diabetes</td>
<td>5</td>
</tr>
<tr>
<td>2.1.2</td>
<td>Roles of the intestine and brain</td>
<td>8</td>
</tr>
<tr>
<td>2.1.3</td>
<td>Systemic and islet inflammation</td>
<td>9</td>
</tr>
<tr>
<td>2.2</td>
<td>Classification and diagnosis of diabetes mellitus</td>
<td>10</td>
</tr>
<tr>
<td>2.2.1</td>
<td>Type 1 diabetes mellitus</td>
<td>10</td>
</tr>
<tr>
<td>2.2.2</td>
<td>Type 2 diabetes mellitus</td>
<td>11</td>
</tr>
<tr>
<td>2.2.3</td>
<td>Gestational diabetes mellitus</td>
<td>11</td>
</tr>
<tr>
<td>2.2.4</td>
<td>Specific types of diabetes mellitus</td>
<td>11</td>
</tr>
<tr>
<td>2.3</td>
<td>Type 2 diabetes and obesity</td>
<td>11</td>
</tr>
<tr>
<td>2.4</td>
<td>Type 2 diabetes and lipid profile</td>
<td>12</td>
</tr>
<tr>
<td>2.5</td>
<td>Diabetes mellitus and bones</td>
<td>13</td>
</tr>
<tr>
<td>2.6</td>
<td>Osteocalcin</td>
<td>13</td>
</tr>
<tr>
<td>2.7</td>
<td>Role of osteocalcin in mineralization</td>
<td>15</td>
</tr>
<tr>
<td>2.8</td>
<td>Regulation of glucose metabolism by osteocalcin</td>
<td>16</td>
</tr>
<tr>
<td>2.9</td>
<td>Regulation of osteocalcin by insulin</td>
<td>17</td>
</tr>
<tr>
<td>2.10</td>
<td>Evidence of an endocrine function for osteocalcin in humans</td>
<td>18</td>
</tr>
<tr>
<td>2.11</td>
<td>Osteocalcin and its relations to BMI, the incidence of obesity and lipid profile</td>
<td>18</td>
</tr>
<tr>
<td>2.12</td>
<td>Effects of age on osteocalcin</td>
<td>19</td>
</tr>
<tr>
<td>2.13</td>
<td>Osteocalcin and bones</td>
<td>20</td>
</tr>
<tr>
<td>2.14</td>
<td>Osteocalcin and calcium</td>
<td>21</td>
</tr>
<tr>
<td>2.15</td>
<td>Osteocalcin metabolism</td>
<td>22</td>
</tr>
</tbody>
</table>
CHAPTER [3]

MATERIALS AND METHODS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1. Study design</td>
<td>25</td>
</tr>
<tr>
<td>3.2. Study area and population</td>
<td>25</td>
</tr>
<tr>
<td>3.2.1. Inclusion criteria</td>
<td>26</td>
</tr>
<tr>
<td>3.2.2. Exclusion criteria</td>
<td>26</td>
</tr>
<tr>
<td>3.3. Data collection Techniques</td>
<td>26</td>
</tr>
<tr>
<td>3.3.1. Questionnaire and consent</td>
<td>26</td>
</tr>
<tr>
<td>3.3.2. Blood sample collection</td>
<td>26</td>
</tr>
<tr>
<td>3.3.3. Clinical examination</td>
<td>26</td>
</tr>
<tr>
<td>3.4. Instruments</td>
<td>27</td>
</tr>
<tr>
<td>3.5. Chemicals and reagents</td>
<td>27</td>
</tr>
<tr>
<td>3.6. Diagnosis of diabetes mellitus</td>
<td>27</td>
</tr>
<tr>
<td>3.7. Human insulin kits</td>
<td>28</td>
</tr>
<tr>
<td>3.8. Human total osteocalcin kits</td>
<td>28</td>
</tr>
<tr>
<td>3.9. Human undercarboxylated osteocalcin kits</td>
<td>29</td>
</tr>
<tr>
<td>3.10. Reagents for glucose and lipid profile</td>
<td>29</td>
</tr>
<tr>
<td>3.10.1. Glucose</td>
<td>29</td>
</tr>
<tr>
<td>3.10.1.1. Reagents</td>
<td>29</td>
</tr>
<tr>
<td>3.10.2. Cholesterol</td>
<td>30</td>
</tr>
<tr>
<td>3.10.2.1. Reagents</td>
<td>30</td>
</tr>
<tr>
<td>3.10.3. Triglyceride</td>
<td>30</td>
</tr>
<tr>
<td>3.10.3.1. Reagents</td>
<td>30</td>
</tr>
<tr>
<td>3.10.4. High density lipoprotein</td>
<td>31</td>
</tr>
<tr>
<td>3.10.4.1. Reagents</td>
<td>31</td>
</tr>
<tr>
<td>3.10.5. Low density lipoprotein</td>
<td>31</td>
</tr>
</tbody>
</table>
3.10.5.1. Reagents.................................................................31
3.10.6. Very low density lipoprotein ........................................31
3.11. Essay for human insulin ..................................................31
3.11.1. Principle and methods ...............................................31
3.11.2. Procedure ..............................................................32
3.11.3. Calculation ..............................................................33
3.12. Assay for plasma total osteocalcin ....................................33
3.12.1. Principle and methods ...............................................34
3.12.2. Procedure ..............................................................34
3.12.3. Calculation ..............................................................35
3.13. Assay for plasma undercarboxylated osteocalcin ................35
3.13.1. Principle and methods ...............................................36
3.13.2. Procedure ..............................................................36
3.13.3. Calculation ..............................................................37
3.14.1. Procedure ..............................................................38
3.15. Principle of determination of triglycerides concentration ....38
3.15.1. Procedure ..............................................................39
3.16. Principle of determination of total cholesterol concentration ..39
3.16.1. Procedure ..............................................................40
3.17. Principle of determination of HDL-cholesterol concentration ..40
3.17.1. Procedure ..............................................................41
3.18. Principle of determination of LDL-cholesterol concentration ..41
3.18.1. Procedure ..............................................................42
3.19. Data analysis.................................................................42
## CHAPTER [4]

RESULTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Study group</td>
<td>43</td>
</tr>
<tr>
<td>4.2</td>
<td>Total osteocalcin</td>
<td>45</td>
</tr>
<tr>
<td>4.3</td>
<td>Undercarboxylated osteocalcin</td>
<td>50</td>
</tr>
<tr>
<td>4.4</td>
<td>Lipid profile</td>
<td>54</td>
</tr>
<tr>
<td>4.5</td>
<td>Fasting insulin, FBS</td>
<td>62</td>
</tr>
<tr>
<td>4.5.1</td>
<td>Prediction of FI using sex, age, BMI, TOC, ucOC, FBG, TCHOL, LDL, HDL and TG</td>
<td>64</td>
</tr>
<tr>
<td>4.5.2</td>
<td>Prediction of FBG using sex, age, BMI, TOC, ucOC, FI, TCHOL, LDL, HDL and TG</td>
<td>66</td>
</tr>
</tbody>
</table>

## CHAPTER [5]

DISCUSSION, CONCLUSION AND RECOMMENDATION

<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>Discussion</td>
<td>68</td>
</tr>
<tr>
<td>5.2</td>
<td>Conclusion</td>
<td>71</td>
</tr>
<tr>
<td>5.3</td>
<td>Recommendations</td>
<td>72</td>
</tr>
</tbody>
</table>

## CHAPTER [6]

REFERENCES AND APPENDICES

<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1</td>
<td>References</td>
<td>73</td>
</tr>
<tr>
<td>6.2</td>
<td>Appendices</td>
<td>101</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 4.1: Some characters of study group ..........................................................43
Table 4.2: Comparison of mean concentrations of TOC, ucOC, insulin, TC, TG, HDL and LDL in diabetic and control subjects. .................................................................44
Table 4.3: Number of diabetic and control subjects in each BMI group by gender........44
Table 4.4: Comparison of mean concentrations of TOC, ucOC, insulin, TC, TG, HDL and LDL in diabetic and control subjects. .................................................................45
Table 4.5: Correlation of total osteocalcin concentration and BMI ..........................49
Table 4.6: Pearson’s correlation between total osteocalcin (TOC), FI and FBG..........50
Table 4.7: Pearson's correlation between undercarboxylated osteocalcin (ucOC), FI and FBG.........................................................................................................................54
Table 4.8: Mean plasma concentrations of lipid profile (mg/dl) in control and diabetic subjects by sex.................................................................54
Table 4.9: Pearson's correlation between plasma TOC, ucOC concentrations and the parameter of lipid profile.................................................................56
Table 4.10: Mean concentrations of FI and FBG in the diabetic and the control by sex........62
Table 4.11: Values of R, R2 and adjusted R2 in the model predicting FI......................64
Table 4.12: Standardized (β) and unstandardized (B) coefficients for different predictors of plasma fasting insulin (FI)..................................................................................65
Table 4.13: Values of R, R2 and adjusted R2 in the model predicting ucOC ..................66
Table 4.14: Standardized (β) and unstandardized (B) coefficients for different predictors of plasma undercarboxylated osteocalcin..........................................................67
# LIST OF FIGURES

| Figure 4.1: Comparison of mean plasma concentrations of total osteocalcin between controls and diabetics by gender | 46 |
| Figure 4.2: Mean plasma concentrations of TOC (ng/ml) in different age groups in control and diabetic by gender | 47 |
| Figure 4.3: Mean plasma concentrations of (TOC) in control and diabetic subjects by BMI subgroups | 48 |
| Figure 4.4: Comparison of mean plasma concentrations of ucOC osteocalcin between controls and diabetics by gender | 51 |
| Figure 4.5: Mean plasma concentrations of TOC (ng/ml) in different age groups in control and diabetic subjects | 52 |
| Figure 4.6: Mean plasma concentrations of (ucOC) in control and diabetic subjects by BMI subgroups | 53 |
| Figure 4.7: Mean plasma concentrations of TCHOL among different BMI subgroups in control and diabetic subjects | 57 |
| Figure 4.8: Mean plasma concentrations of TG among different BMI subgroups in diabetic and control subjects | 58 |
| Figure 4.9: Mean plasma concentrations of HDL among different BMI subgroups in diabetic and control subjects | 59 |
| Figure 4.10: Mean plasma concentrations of LDL among different BMI subgroups in control and diabetic subjects | 60 |
# LIST OF APPENDICES

<table>
<thead>
<tr>
<th>Appendix</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participant consent form</td>
<td>102</td>
</tr>
<tr>
<td>Questionnaire form</td>
<td>102</td>
</tr>
<tr>
<td>Total osteocalcin measurements leaflets</td>
<td>104</td>
</tr>
<tr>
<td>Undercarboxylated osteocalcin measurements leaflets</td>
<td>110</td>
</tr>
<tr>
<td>Human insulin measurements leaflets</td>
<td>118</td>
</tr>
<tr>
<td>Glucose measurements leaflets</td>
<td>124</td>
</tr>
<tr>
<td>Total cholesterol measurements leaflets</td>
<td>127</td>
</tr>
<tr>
<td>LDL-cholesterol measurements leaflets</td>
<td>129</td>
</tr>
<tr>
<td>HDL-cholesterol measurements leaflets</td>
<td>131</td>
</tr>
<tr>
<td>Triglycerides measurements leaflets</td>
<td>133</td>
</tr>
<tr>
<td>Published paper</td>
<td>135</td>
</tr>
</tbody>
</table>

# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone mineral density</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>BGLAP</td>
<td>Bone Gamma-Carboxyglutamate Protein</td>
</tr>
<tr>
<td>CE</td>
<td>Cholesterol esterase</td>
</tr>
<tr>
<td>CETP</td>
<td>Cholesterol Ester Transport Protein</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>CO</td>
<td>Cholesterol oxidase</td>
</tr>
<tr>
<td>DHAP</td>
<td>Dihydroxyacetone phosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>DM</td>
<td>Diabetes Mellitus</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FBG</td>
<td>Fasting blood glucose</td>
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<tr>
<td>FOXO1</td>
<td>Forkhead box transcription factor O1</td>
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<tr>
<td>FFAs</td>
<td>Free fatty acids</td>
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<tr>
<td>FGF 19</td>
<td>Fibroblast growth factor 19</td>
</tr>
<tr>
<td>FI</td>
<td>Fasting insulin</td>
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<tr>
<td>GK</td>
<td>Glycerol kinase</td>
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<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide 1</td>
</tr>
<tr>
<td>GOD</td>
<td>Glucose oxidase</td>
</tr>
<tr>
<td>GPO</td>
<td>Glycerol phosphate oxidase</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Glycated hemoglobin</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>HOMA</td>
<td>The homeostatic model assessment</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatograph</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>IDDM</td>
<td>Insulin-dependent diabetes mellitus</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
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<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
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<tr>
<td>MGP</td>
<td>Matrix Gla protein</td>
</tr>
<tr>
<td>MODY</td>
<td>Maturity-onset diabetes of the young</td>
</tr>
<tr>
<td>Ng</td>
<td>Nano gram</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-esterified fatty acids</td>
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<tr>
<td>NIDDM</td>
<td>Non-insulin-dependent diabetes mellitus</td>
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<tr>
<td>O2</td>
<td>Oxygen</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>OGGT</td>
<td>Oral glucose tolerance test</td>
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<tr>
<td>OG1</td>
<td>Osteocalcin gene 1</td>
</tr>
<tr>
<td>OG2</td>
<td>Osteocalcin gene 2</td>
</tr>
<tr>
<td>OP</td>
<td>Osteoporosis</td>
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<tr>
<td>OST-PTP</td>
<td>Osteotesticular protein tyrosine phosphatase</td>
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<tr>
<td>ORG</td>
<td>Osteocalcin-related gene</td>
</tr>
<tr>
<td>POD</td>
<td>Peroxidase</td>
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<tr>
<td>PPARG</td>
<td>Peroxisome proliferator-activated receptor</td>
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<tr>
<td>R</td>
<td>Reagent</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>TG</td>
<td>Triglycerides</td>
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<tr>
<td>T1DM</td>
<td>Type 1 diabetes mellitus</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TCHOL</td>
<td>Total cholesterol</td>
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<tr>
<td>TOC</td>
<td>Total Osteocalcin</td>
</tr>
<tr>
<td>ucOC</td>
<td>Undercarboxylated osteocalcin</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>4-AA</td>
<td>4-aminoatipyrene</td>
</tr>
<tr>
<td>4-PL</td>
<td>Four-parameter logistic</td>
</tr>
</tbody>
</table>
Introduction

1.1. Definition and epidemiology of diabetes mellitus (D.M.):

Due to aging, accelerated population growth, urbanization and high prevalence of obesity and inactive lifestyle, the number of people with diabetes is increasing globally at a rapid speed. Important differences have been reported in the occurrence of DM and its complication between countries and between ethnic, cultural and even age groups within the same country. The World Health Organization (WHO) predicts that the current diabetic population of 177 million (in 2000) people will increase to 370 million by the year 2030 (1).

Diabetes Mellitus (DM) is a group of metabolic disorders in which the patient has hyperglycemia, either because the body does not produce enough insulin, or because cells do not respond to the insulin that is produced (i.e. Insulin resistance). The hyperglycemia produces the classical symptoms of polyuria, polydipsia, and polyphagia (2).

The prevalence of DM in African communities is increasing due to the increased aging population and lifestyle change associated with rapid urbanization and westernization. Traditional rural communities still have a very low prevalence, at most 1-2 %, except in some specific high-risk group, whereas up to 13% or more adults in urban communities have DM. Type II diabetes is the predominant form (70-90%), the rest being represented by typical Type I patients and patients with atypical presentation. Due to the high urban growth rate, unhealthy dietary changes, reduction in physical activity and increasing obesity it is estimated that the prevalence of diabetes is going to triple within the next 25 years. In addition, long–term complications occur early in the course of diabetes and affect a high proportion of patients, and that could be partly explained by uncontrolled hypertension, poor metabolic control, and possible ethnic predisposition. The combination of the rising prevalence of diabetes and the high rate of long-term complications in Africans will lead to a drastic increase in the burden of diabetes on health care systems of Africans countries (3).

The prevalence and incidence rates of DM in Sudan, as in many other low- income countries, are increasing to epidemic proportions, leading to the emergence of public health
problem of major socio-economic impact. In the northern states, the crude prevalence in 1992 reached 3.4% in those ≥ 25 years of age (4). It was found to be 5.5% in the Northern state and 8% in Khartoum state (5). Type I DM is not rare in Sudan, the prevalence being approximated to 0.1% among children 7-14 years of age (6).

DM in Sudan is associated with poor glycemic control, a high prevalence of complications, and low quality of life and particularly with morbidity (7). Patients with a median duration of diabetes of 9 years showed a high prevalence of micro- and macrovascular complications (8). Retinopathy was evidenced in approximately 43%, dipstick proteinuria in 22% and neuropathy in 37%. Cardiovascular disease was reported in 28%. Peripheral vascular disease was reported in 10% and cerebrovascular accident in 5.5%. As expected, patients with complications were significantly older, had longer disease duration and had higher serum cholesterol and triacylglycerol concentrations. The glycemic control was only acceptable (HbA1c < 7.5%) in 12.5% of the patients.

The rising prevalence of diabetes, its increasing morbidity and mortality, its disproportionate effect on disadvantaged individuals, communities and nations, and its high human and economic cost clearly establish diabetes as a significant global public health problem (9, 10).

1.2. Study rationale:

Recently a new protein hormone osteocalcin, secreted from osteoclast has shown a role in glucose metabolism. There are limited data supporting the relationship between low levels of under-carboxylated osteocalcin with abnormal glucose metabolism in humans, this does not clearly distinguish between associations of insulin resistance and diabetes with under-carboxylated osteocalcin, specifically as opposed to bone turnover reflected in total osteocalcin levels. Therefore, this proposed study aims to determine the relation of osteocalcin levels with glucose metabolism.
1.3. Study objectives:

1.3.1. General objective:
The objective of this study is to identify the role of circulating osteocalcin with blood insulin level in Type II diabetes.

1.3.2. Specific objectives:

1. To estimate the circulating total osteocalcin (both γ-carboxylated and undercarboxylated osteocalcin) in Type II diabetic patients and matched control subjects to assess its role in the pathogenesis of the disease.

2. To correlate total osteocalcin with insulin and blood glucose level in patients with Type II diabetes and with healthy control subjects to show their effect on insulin secretion and glucose metabolism.

3. To correlate total osteocalcin with lipid profile (showing association of osteocalcin level with cardiovascular risk) in patients with Type II diabetes and with healthy control subjects.

4. To assess age, gender and body mass index (BMI) in relation with osteocalcin level and their effect in the pathogenesis of Type II diabetes mellitus.
2.1. Pathophysiology of type 2 diabetes:

2.1.1. β-cell dysfunction and insulin resistance:

Development of the insulin radioimmunoassay led to the finding that patients with early-maturity-onset diabetes produced insulin and secreted this hormone in response to nutrient ingestion (11). Subsequently, defects in the ability of islet β cells to respond to intravenous secretagogues (including glucose) were reported in these patients (12). Additionally, these patients did not respond well to insulin, (13) and were thus deemed to be insulin insensitive. This insulin insensitivity was shown to contribute to increased production of glucose by the liver and decreased uptake of glucose in muscle and adipose tissue (14). Nowadays, some of these abnormalities are attributed to adiposity, especially adiposity within the intra-abdominal cavity (15).

The crucial role of β cells to glucose homeostasis by feedback regulation The importance of insulin resistance and β-cell dysfunction to the pathogenesis of type 2 diabetes was debated for a long time; many thought that insulin resistance was the main abnormality in type 2 diabetes, and that inability to secrete insulin was a late manifestation (14). This notion changed with the finding that, as with most endocrine systems in human beings, a feedback loop operates to ensure integration of glucose homeostasis and maintenance of glucose concentration in a narrow range (16). This feedback loop relies on the crosstalk between β cells and insulin-sensitive tissues. Insulin released in response to β-cell stimulation mediates uptake of glucose, amino acids, and fatty acids by insulin-sensitive tissues. In turn, these tissues feedback information to islet cells about their need for insulin. The mediator of this process has not been identified, but probably includes integration between the brain and humoral system. If insulin resistance is present, as often happens in people with obesity, β cells increase insulin output to maintain normal glucose tolerance.

However, if β cells are incapable of this task, plasma concentrations of glucose increase. Although the distinction between impaired fasting glucose and impaired glucose tolerance (sometimes together referred to as prediabetes) and diabetes is established by testing of 2 hours glucose concentrations after fasting and a standardized load of oral glucose, (17) these
disturbances form a continuum in which the magnitude of reduction in β-cell function establishes the degree of increase in plasma glucose. Insulin resistance is already well established if impaired glucose tolerance is present rises in glucose concentrations, even within the normal range, are due to a continuous fall in β-cell function (18). Further progressive deterioration of β-cell function accounts for the evolving natural history of the disease, from impaired glucose tolerance to type 2 diabetes (19,20). Reduced β-cell function is already present in groups at increased risk of diabetes (eg, first-degree relatives of patients with diabetes, (21) women with gestational diabetes (22) or polycystic ovary syndrome, (23) and elderly people (24) and underlies progression to the disease. Furthermore, the β-cell function is heritable (25) and is crucially important to differences in glucose intolerance and rates of type 2 diabetes between various racial and ethnic groups (26). Despite advances in the understanding of the importance of insulin resistance and β-cell dysfunction to the pathogenesis of type 2 diabetes and high-risk states, the disease process is clearly heterogeneous and includes other pathogenic factors.

2.1.2. Environment; genes and development of type 2 diabetes:

Environment and the genes together are important determinants of insulin resistance and β-cell dysfunction. Because changes in the gene pool cannot account for the rapid increase in the prevalence of type 2 diabetes in recent decades, environmental changes are essential to an understanding of the epidemic. Advances in technology and analytical approaches have identified genes linked with type 2 diabetes. With the use of candidate-gene approaches, peroxisome proliferator-activated receptor (PPARG) was the first gene identified (27). Subsequently, mostly with the use of genome-wide association studies, more than 50 gene loci have been linked with type 2 diabetes (28). Furthermore, 53 loci have been linked with concentrations of insulin and glucose (however, not always with both fasting and 2-hour concentrations of glucose), of which 33 are also associated with type 2 diabetes (29,30). Although some loci are associated with obesity and insulin resistance, most are linked with β-cell function.21 Gene products for most of these loci have not been definitively identified. Together, these genes do not explain much of the genetic basis of type 2 diabetes; the use of genotype risk scores only slightly improves prediction of subsequent diabetes compared with more frequently used clinical risk factors (31,32). Aside from obvious increases in caloric intake and decreased
energy expenditure, other environmental factors seem to be important. Nutrient composition, specifically increased amounts of dietary fat (particularly saturated fat), are important to the development of obesity, insulin resistance, β-cell dysfunction, and glucose intolerance (33). Furthermore, an aging-associated reduction in the responsiveness of β cells to carbohydrate partly underlies the fall in glucose tolerance with aging (34). The in-utero environment, established partly by the mother’s body size, could produce epigenetic and gene expression changes that affect the risk of development of obesity and type 2 diabetes for the offspring (35).

Recent discussion has also focused on the role of environmental chemicals in the epidemics of obesity and diabetes (36).

Further delineation of the roles of reduced β-cell numbers and α-cell dysfunction. The reduction of β-cell numbers in type 2 diabetes is well known (37–40). The basis for this loss is multifactorial and includes glucomlipotoxicity (41) and amyloid deposition that result in β-cell apoptosis through oxidative and endoplasmic-reticulum stress (40). This loss is not counterbalanced by replacement with new β cells, because the human pancreas seems to be incapable of renewing these cells after 30 years of age (42). Although a reduction in β-cell mass occurs in type 2 diabetes, the magnitude of this abnormality is clearly insufficient to explain the degree of impairment in insulin release. Whether the underlying defect in β-cell function is important as an initiator of β-cell loss, and whether increasing secretory demand on each individual β cell as numbers decrease causes ongoing loss of β cells, remains to be defined. Elucidation of the importance of β-cell function compared with mass could have important implications for the development of approaches to preserve β cells and help to maintain or improve glucose tolerance. Although less well studied, the dysregulated release of glucagon by α cells, which manifests as increased concentrations of fasting glucagon and failure to adequately suppress glucagon release after meal ingestion, contributes to the development of hyperglycemia (43). Whether this dysregulation is a primary change in α cells or is secondary to an abnormality in β-cell function is not yet resolved. However, islet blood flows from β cells to α cells and then to somatostatin producing δ cells; (44) high concentrations of insulin bathing α cells are capable of suppression of glucagon release (45). Other β-cell products eg, zinc, γ-aminobutyric acid, or glutamate—might also regulate glucagon release (45). Approaches that reduce glucagon release
or impair its action to raise glucose concentrations could represent additional therapeutic alternatives for type 2 diabetes (43).

Although understanding of the genetics of type 2 diabetes has advanced rapidly, much remains unknown. How genes interact with the environment to cause progressive loss of β-cell function is unclear. Environmental factors and hyperglycemia could contribute to epigenetic changes in deoxyribonucleic acid (DNA) and histones, thereby modifying gene expression in organs implicated in the pathogenesis and progression of type 2 diabetes, including in β cells (41,45). Whether such changes contribute to the increased risk of type 2 diabetes and the progression of the disease will be of interest. Finally, because only a small proportion of the risk of type 2 diabetes can be attributed to identified genetic loci, the search for rarer variants with approaches such as exome sequencing might provide additional insights and possible therapies. The so-called omics (eg, metabolomics, lipidomics, proteomics, genomics, and transcriptomics) are based on the study of constituents of the cell or body in a collective way. The findings made with the use of these approaches are being integrated to better understand the pathophysiology of type 2 diabetes and the heterogeneity of responses to different glucose-lowering therapies. Findings from studies that used metabolomics and lipidomics showed that increases in branched-chain and aromatic amino acids were associated with obesity and type 2 diabetes (46,47). Furthermore, patients with high concentrations of specific six-carbon sugars, amino acids, and fatty acids, and low concentrations of other amino acids and fatty acids, had an increased risk of developing type 2 diabetes over a 7-year follow-up (45). Whether all or some of these substrate markers are associated with genetic determinants, dietary factors, or the actions of gut microbes has not been established.

In the long term, these new approaches should identify additional genes and metabolic markers. Profiles obtained through these assessments could provide the level of detail needed to establish the mediator (or mediators) of the feedback loop that interconnects β cells with insulin-sensitive tissues, and help to unravel the heterogeneity of the disease. Furthermore, these assessments should complement and advance the present understanding of the best approaches to treat the dysregulated metabolic milieu in type 2 diabetes, which includes not only glucose but also fatty acids and amino acids.
2.1.3. The intestine, the nervous system and D.M:

The gastrointestinal tract produces various peptides, not all of which directly modulate nutrient absorption. Glucagon-like peptide 1 (GLP-1) and glucose-dependent insulino-tropic polypeptide (GIP), collectively known as incretins, act on the pancreatic islet. GLP-1 is more important and acts both on β cells to enhance insulin secretion and on α cells to suppress glucagon secretion (46). Plasma concentrations of GLP-1 generally do not differ in individuals with normal glucose tolerance, impaired glucose tolerance, or type 2 diabetes (47). Therefore, the β-cell response to GLP-1 after meal ingestion has to be deficient, as noted after intravenous administration of GLP-1 under controlled conditions (48). This deficient response is consistent with a model of global deficiency in β-cell responsiveness to many secretagogues (eg, sulfonylurea antidiabetics, amino acids, and β-adrenoreceptor agonists) (49). Although GLP-1 acts directly on α cells to suppress glucagon release, the effect of this mechanism compared with modulation by β-cell products is uncertain, both in healthy people and in those with type 2 diabetes (in which glucagon is inadequately suppressed during meals). Increased concentrations of GLP-1 have been reported after bariatric surgery, and are thought to account for many of the beneficial effects of the intervention, particularly in patients with type 2 diabetes (50). However, increased GLP-1 is not the only mechanism by which glucose lowering can occur after this surgical procedure (51,52). Bile acids are also important in the regulation of glucose metabolism. They are endogenous ligands of the farnesoid X receptor, and activation of the receptor results in the release of the fibroblast growth factor 19 (FGF) (52). Bile acids also activate G-protein-coupled bile-acid receptor 1 located on intestinal L cells, leading to GLP-1 secretion (53). In human beings, infusion of bile acids intraduodenally in a dose-dependent manner increases plasma concentrations of FGF19, with smaller effects on concentrations of GLP-1 and cholecystokinin (54). Because FGF19 has insulin-like effects by inducing synthesis of glycogen and proteins and inhibiting glucose production (52). The biliary system might have an underappreciated role in modulation of glucose homeostasis. The intestinal microbiome also seems to be important to the pathophysiology of type 2 diabetes (55). The microbiome has about 100 times more genetic information than has the human genome, together comprising the human metagenome. Many products of the microbiome provide functions beyond that of the host.
genome, thereby serving an important role in human physiology. These gut communities are thought to play an important part in several conditions and disorders (eg, obesity and type 2 diabetes), although which bacterial species cause changes to human metabolism is not clear (56). Findings from two studies that used fecal samples suggested that functional changes in the gut microbiome might be directly linked to the development of type 2 diabetes; (57,58) however, metagenomic markers differ between populations, suggesting that their ability to predict the development of diabetes will probably vary (58). Findings from a recent proof-of-concept study (59) showed improvements in insulin sensitivity in patients with metabolic syndrome 6 weeks after infusion of intestinal microbiota from lean individuals. Lastly, different gut flora might affect nutrient absorption, because in human beings nutrient load can alter the fecal bacterial community in a short time (60). The nervous system is another important regulator of metabolic processes. Both sympathetic and parasympathetic nervous systems control glucose metabolism, directly through neuronal input, and indirectly through the circulation to affect the release of insulin and glucagon (61) and production of hepatic glucose (62). In human beings, the vagus is important in the regulation of islets, because severing of this nerve results in impaired insulin secretion (63). The hypothalamus is an important integrator because its ablation in rats results in dysregulation of β cells and development of hyperinsulinemia (64). This brain region also regulates hepatic production of glucose through the actions of insulin, glucose, and fatty acids (65–67). Insulin action at this site is also essential in the regulation of body weight, with decreased activity leading to obesity (68). Inflammation induced neuronal injury occurs rapidly in rodents fed a high-fat diet (69). Findings from imaging studies of obese and lean people suggest that structural changes occur in the hypothalamus, consistent with the occurrence of gliosis in obesity (69). Clock genes expressed in the brain are important in the establishment of circadian rhythmicity and, together with sleep, have become a focus of investigation because changes in diurnal patterns and quality of sleep can have important effects on metabolic processes (70, 71).

2.1.4. Systemic and islet inflammation:

Obesity is often characterized by systemic inflammation, and preclinical evidence links systemic inflammation to β-cell dysfunction (72,73). Markers of systemic inflammation,
including C-reactive protein and its upstream regulator interleukin 6, are cross-sectionally associated with insulin sensitivity and β-cell function (74,75). Lifestyle change and pharmacological drugs improve markers of inflammation (75–77) and have been associated with improvements in β-cell function in patients with type 2 diabetes (78,79). Direct effects of inflammation on β cells arise from activation of the intraislet immune response (80). Glucose and fatty acids increase the production of interleukin 1β in islets, (81,82) and naturally occurring antagonists (particularly interleukin 1 receptor antagonist) balance and regulate the action of interleukin 1β in islets and other tissues (83).

Circulating concentrations of interleukin 1β and interleukin 1 receptor antagonist are increased in patients with type 2 diabetes (84,85). Lower concentrations at the time of initiation of interleukin 1 receptor antagonist treatment could predict maintenance of improved β-cell function after an intervention to reduce islet inflammation (86). Expansion of adipose tissue is associated with accumulation of activated macrophages that express several pro-inflammatory genes, including cytokines (eg tumor necrosis factor α) that locally impair insulin signaling. (86,87) A feed-forward process, in which activation of transcription factors causes further production of pro-inflammatory cytokines, also plays a part (88). When production of these cytokines is sufficient, they are released into the circulation where they can act at distant sites (eg, the liver and skeletal muscle) to worsen insulin resistance. A similar process can occur in the liver with Kupffer cells (resident macrophages) and recruited macrophages (89). Hypothalamic inflammation might also contribute to central leptin resistance and weight gain (90).

2.2. Classification and diagnosis of diabetes mellitus:

Diabetes is classified into the following types (1):

2.2.1. Type I diabetes (T1DM):

Results from the body's failure to produce insulin, and presently requires the patient to be injected with insulin. Also known as insulin-dependent diabetes mellitus (IDDM).
2.2.2. **Type II diabetes (T2DM):**

Results from insulin resistance, a condition in which cells are unable to use insulin properly, sometimes combined with an absolute insulin deficiency. This type is known as non-insulin-dependent diabetes mellitus (NIDDM). It often results from obesity and laziness.

2.2.3. **Gestational diabetes:**

Is when pregnant women, who have never had diabetes before, present with a high blood glucose level during pregnancy. It may precede the development of Type II diabetes mellitus.

2.2.4. **Specific types of diabetes:**

Is a huge band of conditions, which includes genetic problems in insulin secretion (such as in maturity-onset diabetes of the young (MODY) and insulinopathies), genetic problem in insulin action (e.g. syndromes of severe insulin resistance), pancreatitis and other exocrine disorders, hormone-secreting tumours such as acromegaly (growth hormone) and Cushing’s syndrome (cortisol). Some cases are triggered by the administration of drugs such as glucocorticoids. Some genetic syndromes are occasionally associated with diabetes (e.g. Down’s syndrome, Klinefelter’s syndrome) (91, 92).

2.3. **Type 2 diabetes and obesity:**

Obesity is defined as body mass index (≥30.0 kg/m²). Body mass index (BMI), the most commonly used index of body mass, is calculated by dividing the weight in kilograms by the square of the height in meters (93). According to the World Health Organization BMI classification system. It is classified into the following four grades: underweight (<18.5 kg/m²), normal weight (18.5 kg/m²–24.9 kg/m²), overweight (25.0 kg/m²–29.9 kg/m²), and obese (≥30.0 kg/m²). It has been shown that being overweight or obese are risk factors for diabetes mellitus (94).

Obesity is a known risk factor for many diseases like ischemic heart disease, non-insulin dependent diabetes mellitus (NIDDM), obstructive sleep apnoea, osteoarthritis, and some malignancies. These complications are most commonly prevalent in patients suffering from the metabolic syndrome which is a combination of diabetes mellitus, hypertension, hypercholesterolemia and hypertriglyceridemia (95,96). However, most of these complications
are not caused directly by obesity; they may be caused by mechanisms sharing a common cause with obesity such as a poor diet or a sedentary lifestyle. The link between obesity and these complications varies in strength. One of the strongest associations is the link between obesity and type 2 diabetes. The increase in body fat alter the body's response to insulin, potentially leading to insulin resistance. It was found that free fatty acids (FFAs) cause insulin resistance in muscle and liver and increase hepatic gluconeogenesis and lipoprotein production and perhaps decrease the hepatic clearance of insulin (96).

2.4. Type 2 diabetes and lipid profile:

A variety of interrelated lipid and lipoprotein abnormalities is known to be associated with type 2 diabetes mellitus T2DM, commonly termed as diabetic dyslipidemia (97). Hypertriglyceridemia, low high density lipoprotein cholesterol (HDL-c), small dense of low density lipoprotein (LDL) particles is commonly associated with T2DM (98). Deficiency of insulin decreases lipolysis and increases the hydrolysis of stored triglyceride, resulting in greater release of non-esterified fatty acids (NEFA), which is delivered to the liver leading to increased hepatic triglyceride production, which will lead to hepatic very low density lipoprotein (VLDL) production (99). The action of insulin on the lipolytic enzyme lipoprotein lipase is reduced which will lead to decreased clearance of triglyceride-rich lipoproteins, VLDL and chylomicrons, thus contributing to hypertriglyceridemia in T2DM (100). Insulin resistance increases the catabolism of HDL in the presence of normal levels of Cholesterol Ester Transport Protein (CETP) and Hepatic Lipase, which promotes the movement of cholesterol ester from HDL to VLDL. In the presence of higher VLDL- TG level. HDL-c production is also decreased secondary to impaired catabolism of VLDL – c and impaired lipoprotein lipase activity (101). Reduction in lipid abnormalities might preserve glomerular filtration rate, decrease proteinuria, thus reducing the rate of cardiac and renal complications of diabetes mellitus (102).

2.5. Diabetes mellitus and bones:

Osteoporosis (OP) is another public disease affecting millions of people worldwide. OP is defined as “a disease that is characterized by low bone mass, microarchitectural deterioration of bone tissue leading to enhanced bone fragility, and consequent increase in
fracture risk”. (103) OP is a silent disease until a fracture occurs, why primary prevention is problematic. OP often remains underdiagnosed and undertreated. Accordingly, secondary prevention is the most common approach to fracture prevention. The presence of OP related to DM has been less acknowledged and its clinical relevance less obvious than that of other DM complications. However, there is a growing awareness that T1DM and T2DM both predispose to bone fracture. (104) The lifetime risk of an osteoporotic fracture in the general population in Rochester, Minnesota, has been estimated to almost 40% in white women and 13% in men from the age of 50 years onward. (105) However, a Swedish study reported substantially higher fracture incidence in an age- and sex standardized material. (106) A higher incidence is found in northern compared to southern Europe with differences in the epidemiologic pattern of hip fracture. (107) In subjects with T1DM there is an about six-fold increase in hip fracture risk and an about twofold increase in vertebral fracture risk compared to non-diabetic individuals. (108) T2DM is associated with an about two- to threefold increase in hip fracture risk compared with non-diabetic individuals, despite normal or high bone mineral density (BMD). (109) In the ageing population, the likelihood to develop DM and/or OP are both increased.

2.6. Osteocalcin:

Osteocalcin has been used as a serum marker of osteoblastic bone formation and believed to act in the bone matrix to regulate mineralization, but new genetic and pharmacologic evidence now points to a hormonal role for the protein. These newly discovered actions link the energy demands of bone to global homeostasis (110,111) and close some open endocrine loops associated with the impact of nutrient availability (112, 113), leptin (114,115), adiponectin (116) and insulin (117) on skeletal metabolism.

Osteocalcin also referred to as bone γ-carboxyglutamic acid protein, is a 46–50 amino acid, 5.6 kilo Daltons (kDa) secreted protein that is produced primarily by osteoblasts (118). Smaller amounts are also been produced by odontoblasts of the teeth and hypertrophic chondrocytes. The protein was first isolated by Price et al (119,120) from bovine and human bone and shown to represent the major fraction of Gla containing protein in bone. A second Gla-protein, isolated later by the same group, was termed matrix Gla protein or MGP (121,122).
Together, these two proteins belong to a distinct subgroup of the larger vitamin K-dependent protein family, the constituents of which are primarily involved in coagulation.

The human osteocalcin gene, bone gamma-carboxyglutamate protein (BGLAP), is located on chromosome 1 at 1q25-q31 (123) and encodes an 11kD, 98 amino acid pre-protein. The mature peptide is generated by sequential cleavage events that remove an endoplasmic reticulum signal sequence and the pro-sequence followed by γ-carboxylation of three glutamic acid residues at positions 17, 21, and 24. All three of the glutamate carboxylation events occur during a single binding of the immature peptide to γ-glutamyl carboxylase (124). This enzyme utilizes carbon dioxide (CO$_2$), oxygen (O$_2$), and vitamin K, supplied by the vitamin K cycle and circulation, as cofactors. With each γ-carboxylation cycle, vitamin K is converted to an epoxide, which is then reduced by vitamin K epoxide reductase to allow another round of carboxylation (125). The mature carboxylated osteocalcin protein is packaged into intracellular vesicles for secretion into the bone matrix (118,126).

The osteocalcin and matrix Gla protein (MGP) genes appear to have diverged from an ancestral gene that first emerged 500 million years ago (127). MGP appeared first with the development of cartilaginous structures. In most organisms, including humans, osteocalcin is encoded by a single gene that is highly conserved across species. However, mice contain a cluster of three osteocalcin genes, indicative of an additional duplication late in rodent evolution (128). Two of these genes, osteocalcin gene 1 (OG1) and osteocalcin gene 2 (OG2), are expressed predominantly in a bone. The third gene, osteocalcin-related gene (ORG), is expressed primarily in the kidney. Although the exact function of ORG remains unknown (129), it has been suggested that ORG encodes nephrocalcin, a calcium-binding Gla protein important in calcium homeostasis (128).

2.7. Role of osteocalcin in mineralization:

Mature osteocalcin is secreted into the bone micro-environment and then undergoes a conformational change that aligns its calcium-binding Gla residues with the calcium ions in hydroxyapatite. This property was initially proposed as a mechanism that enables osteocalcin to initiate the formation of hydroxyapatite crystals (120). Osteocalcin could act as an inhibitor of
bone mineralization by inhibiting the precipitation of calcium salts from saturated solutions (130), and chronic treatment of rodents with warfarin, an inhibitor of vitamin K-dependent \(\gamma\)-carboxylation, results in over-mineralization and the premature closure of the growth plate (131).

To more fully study the role of osteocalcin in bone formation, Ducy \textit{et al.} (132) produced an osteocalcin null mouse lacking both osteocalcin genes expressed in bone. These mice exhibited an age-dependent increase in bone formation rate and bone mass compared to controls, without an impact on bone resorption. Subsequent analysis of the bone matrix using Fourier transform infrared microspectroscopy indicated that the mineral to matrix ratio was increased in older knockout animals. In addition, the hydroxyapatite crystal size was larger, suggesting that osteocalcin might regulate the rate of mineral maturation (133). However, the deposition of dentin by odontoblasts was normal in osteocalcin null mice (134), and the analysis of mice overexpressing osteocalcin in bone revealed a relatively normal state of mineralization (135). Therefore, the precise role of osteocalcin within the bone matrix remains unclear, and osteocalcin’s inhibitory effect on bone mineralization is likely to be considerably lower than that of MGP since the removal of this gene results in the calcification of the aorta and the progressive mineralization of the growth plate (135,136).

Other studies have led to the hypothesis that osteocalcin exerts a mechanical function within the bone matrix. As a result of its ability to tightly bind hydroxyapatite and form a complex with collagen through the matrix protein osteopontin (137-139), osteocalcin was proposed as a means to bridge the matrix and mineral fractions of bone tissue. Such an arrangement is compatible with the formation of dilatational bands that are seen when bone fractures. In this situation, osteocalcin and osteopontin might serve to prevent crack growth by stretching and dissipating energy (140,141). In accordance with this idea, fracture toughness is substantially reduced in osteocalcin null mice, osteopontin null mice, and double transgenic mice.

2.8. Regulation of glucose metabolism by osteocalcin:

Recent work from several groups has now clearly demonstrated a role for osteocalcin in the regulation of glucose metabolism. Studies by the Karsenty group showed that mice lacking
osteocalcin accumulate body fat and exhibit dramatic impairments in glucose metabolism (111). Since osteocalcin is mainly produced by osteoblasts and can enter the circulation, Karsenty speculated that osteocalcin functions as a hormone in a manner analogous to leptin and adiponectin.

Lee and colleagues (111) performed a screen of osteoblast-enriched genes that could regulate osteocalcin levels in the serum. These analyses identified the Esp gene, that encodes osteotesticular protein tyrosine phosphatase (OST-PTP), as a powerful regulator of glucose metabolism in mice. Mice lacking Esp exhibited a metabolic phenotype opposite of osteocalcin null mice, which was characterized by marked hypoglycemia and reduced fat mass secondary to hyperinsulinemia, increased β-cell proliferation, and increased insulin sensitivity. The effect on glucose metabolism was indeed so severe that a portion of Esp−/− mice did not survive until weaning. To demonstrate a functional linkage between osteocalcin and OST-PTP, Esp−/− mice were crossed with those deficient for osteocalcin. Removal of even a single allele of osteocalcin rescued the metabolic phenotypes of Esp−/− mice. While the mechanism was not known, OST-PTP appeared to regulate the carboxylation and bio-availability of the hormonal form of osteocalcin. Under-carboxylated osteocalcin increased the expression of adiponectin in adipose tissue and insulin and cyclin D1 expression in β-cells, but γ-carboxylated osteocalcin did not. In this way, bio-activation of osteocalcin appears to mimic the activation of prothrombin, which is also activated when its carboxylated residues are removed (142). However, work by another group has suggested that both carboxylated and undercarboxylated osteocalcin can stimulate a response in adipocytes and myoblasts (143).

To further examine the biological role of osteocalcin, Ferron et al (144) assessed the hormone’s influence on glucose metabolism in wild-type mice. In vitro, recombinant osteocalcin dose-dependently increased the expression of insulin and markers of proliferation in primary β-cells as well as cell models. In vivo, infusion of recombinant osteocalcin via subcutaneous mini-pump improved glucose tolerance and insulin sensitivity. Several recent studies have confirmed this effect and demonstrated that daily injections (145) or oral administration (146,147) of osteocalcin can abrogate the deleterious effects of a high-fat diet on metabolism. The precise mechanism by which osteocalcin administration improves glucose metabolism is still not known.
but it may be related to the protein’s ability to stimulate the release of glucagon-like peptide-1, an incretin released by intestinal endocrine cells that stimulates insulin secretion (146,147). Other beneficial actions of osteocalcin appear to involve its ability to reverse autophagic dysfunction and endoplasmic reticulum stress resulting from diet-induced obesity (148,149).

2.9. **Regulation of osteocalcin by insulin:**

The discovery of osteocalcin’s role in regulating glucose metabolism was independently established by Fulzele et al (117,150) in the course of studies designed to examine insulin actions in osteoblasts. In these studies, mice lacking the insulin receptor specifically in osteoblasts developed a metabolic phenotype reminiscent of the osteocalcin null mice described by the Karsenty group (111). Insulin receptor (IR) knockout mice accumulated body fat and exhibited hyperglycemia with reductions in serum insulin, insulin sensitivity, and glucose tolerance. Profiling of RNA from IR null osteoblasts identified osteocalcin as a major insulin-responsive gene in osteoblasts, which supported the hypothesis that insulin receptor signaling in the osteoblast is required for osteocalcin production. Consistent with this idea, administration of osteocalcin to the insulin receptor mutant mice improved glucose metabolism, suggesting that insulin and osteocalcin form a bone-pancreas endocrine loop (111).

The transcriptional mechanism through which insulin stimulates osteocalcin production has also been investigated. FoxOs are major transcriptional mediators of insulin action, and their phosphorylation and subsequent nuclear export is a primary response in many insulin target cells, including β-cells, adipocytes, and hepatocytes (151). Rached and colleagues identified three FoxO-binding sites in the osteocalcin gene and found that FoxO1 is a potent suppressor of osteocalcin expression (152). Mice rendered deficient for the transcription factor in osteoblasts (FoxO1\textsuperscript{flox}; Collagen1-Cre) exhibited increased pancreatic β-cell proliferation, insulin secretion, and insulin sensitivity secondary to increased osteocalcin expression and bio-activation (152). The suppression of osteocalcin expression by FoxO1 may also be mediated by its interaction with the transcription factor Atf4 (153), which also suppresses osteocalcin bio-activation (154), and through its inhibition of Runx2 activity (155).
2.10. Evidence of an endocrine function for osteocalcin in humans:

Since the initial discovery that osteocalcin impacts metabolism in the mouse, a number of studies have attempted to address the function of osteocalcin in humans. To date, the vast majority of studies have used a cross-sectional design to examine the association of circulating levels of total and/or undercarboxylated osteocalcin with altered glucose metabolism. These studies indicate that the levels of osteocalcin are negatively correlated with fasting glucose, fasting insulin, HOMA-IR (a representation of insulin resistance), body mass index, and hyperlipidemia (156-160). Untreated diabetics and pre-diabetics have decreased levels of undercarboxylated osteocalcin, while higher serum levels were associated with higher HOMA-β, a representation of enhanced β-cell function (161,162).

To date, only one study has attempted to directly examine the effects of insulin on osteocalcin and bone turnover in humans. Basu and colleagues (163) analyzed serum samples collected during a hyperinsulinemic-euglycemic clamp in healthy patients and measured the effect of this procedure on serum total and undercarboxylated osteocalcin as well as other bone markers. No relationship between insulin levels and osteocalcin levels or bone turnover markers was apparent, but measures of insulin sensitivity, including glucose disposal rates, were positively correlated with serum levels of the C-terminal telopeptide of type 1 collagen, a bone resorption marker, suggesting that insulin might elicit factors that promote enhanced insulin sensitivity in the periphery (163).

2.11. Osteocalcin and its relations to BMI, the incidence of obesity and lipid profile:

It is an established fact that high body mass index (BMI) is protective of osteoporosis (164). An increase in BMI is often associated with improvement in bone health, as assessed by bone mineral density and calcaneal quantitative ultrasonography (165,166). This is attributed to the ability of our skeletal system to adapt to changes in mechanical loading (167). Bone mass is also influenced by body fat via leptin, a hormone secreted by white adipocytes (168). Systemic infusion of leptin was shown to increase bone mass of rats, but intracerebroventricular infusion exerted negative effects on bone mass of rats (114,169). Hence, it is clear that our body composition exerted regulatory control on bone health by mechanical loading (via BMI) and
leptin (via body fat). Since our body operates on the principle of homeostasis, it is reasonable to consider that bone will also exert a reciprocal regulation on body composition. Recently, this was demonstrated by two separate in vivo studies, and the key mediator involved was shown to be osteocalcin (170, 171). Osteocalcin is a marker of bone formation, and it is used together with bone resorption markers to assess bone turnover (172). Mutant mice lacking the gene coding osteocalcin, were shown to be abnormally fat, have high fat pad mass, higher triglyceride level and were glucose intolerant (171). Infusion of osteocalcin in wild type mice was also proven to prevent obesity caused by high-fat diet and gold thioglucose-induced hyperphagia (170). The association between osteocalcin and fat mass and lipid parameters in humans was first demonstrated by Kindblom and colleagues in Osteoporotic Fractures in Men Study conducted on the Swedish male population (156). This was followed by subsequent reports on other populations, such as the Korean (172), the Chinese (173) and the American populations (174). The majority of the studies agreed that osteocalcin level was associated with obesity and fat mass, but the findings on lipid parameters were less clear (156, 174). For example, HDL cholesterol was found to be negatively (173), positively (156) or not associated at all (174) with osteocalcin level in different populations.

2.12. Effects of age on osteocalcin:

In the human bone samples, it was found that the proportion of osteocalcin which is glycated was lowest in children, relatively constant throughout the adults years, and increased linearly after the age of 60. These results are consistent not only with decreased glucose tolerance and chronic mild hyperglycemia of the elderly, but also with the slower bone turnover and increased vascularity of bone in the aged. In contrast, in younger adults in whom bone resorption is coupled to bone formation, the amount of glycation is constant. (175).

2.13. Osteocalcin and bone:

Osteocalcin is not only present in bone tissue (176) but also synthesized in bone cell cultures (177) and the secreted protein is identical with the one isolated from bone (178). The expression of osteocalcin appears to be unique to bone tissue and predominantly limited to the cells of osteoblastic lineage. It is strongly expressed by mature osteoblasts (179, 180) but also
by osteocytes (181, 182) as well as hypertrophic chondrocytes (183), cementoblasts (184) and odontoblasts (185, 186). The intracellular distribution of osteocalcin in osteoblasts and odontoblasts resembles that of a secreted protein, being in the rough endoplasmic reticulum and Golgi apparatus (187). There is also some evidence for low levels of osteocalcin expression in non-osteoid tissues (188), such as brain (180), bone marrow megakaryocytes and peripheral blood platelets (189), bone marrow stromal adipocytes (190), and a subpopulation of vascular smooth muscle cells isolated from aortic media. The expression levels in bone are, however, several orders of magnitude higher (191, 192). Although osteocalcin messenger RNA has been detected in other tissues than bone, it appears to be processed properly to yield a functional protein only in the bone microenvironment suggesting the existence of putative bone-specific splicing factors (193). The two mouse osteocalcin genes OG1 and OG2 appear to be expressed at a different level. OG1 is the major (80%) transcript in mouse bone tissue in vivo whereas OG1 and OG2 are expressed at equal levels in osteoblastic cells in vitro (194). The expression of osteocalcin appears to be stringently regulated in a developmental-stage-specific manner during differentiation of the osteoblast phenotype. Osteocalcin is not expressed during early stages of osteoblastic differentiation but it is prominently a marker of late, mature osteoblasts (195). During the differentiation of fetal calvarial osteoblasts in vitro, osteocalcin expression increases up to 200-fold in cells producing a mineralizing matrix when compared to confluent osteoblasts in a non-mineralizing matrix (196). Osteocalcin appears in calcifying tissues approximately two weeks after mineral deposition, at the same time than the maturation of bone mineral to hydroxyapatite is thought to occur (197) and is particularly present in mineralized bone matrix but not observed in non-mineralized, newly formed osteoid (198). Furthermore, the expression of osteocalcin is induced in vitro at the onset of mineralization, clearly after the expression of other osteoblastic markers such as alkaline phosphatase and type I collagen (199). Thus, the expression of osteocalcin is considered a specific feature of the late phases of bone formation. At tissue level, staining for osteocalcin is predominantly seen in the bone matrix and to some extent in osteoblasts and osteocytes (200, 201, 202). Osteocalcin is associated especially with mineralized regions of extracellular matrices of bone and cartilage (203). In normal lamellar human bone, osteocalcin is detected along the lamellar bone matrix in fine granular deposits, and under pathological conditions the provisory woven bone is devoid of osteocalcin (200). In tense,
but regionally variable, staining for osteocalcin can be seen in mineralized bone matrix and in small mineralization loci dispersed throughout the osteoid while unmineralized osteoid shows weak to moderate staining indicating that osteocalcin synthesis is low (198). Osteocalcin expression is concentrated at the mineralization front i.e. just ahead of the mineralized matrix both in vitro (204) and in vivo (202). Pattern of osteocalcin distribution within osteons changes with age and gender (205) and weaker expression of osteocalcin is seen in newborn bone compared to the adult (179). In humans, cortical bone contains approximately 30 times more osteocalcin than trabecular bone, probably reflecting the differences in age and maturity of these two bone types (206). Osteocalcin is also detected in dental tissues (186, 187) and in the hypertrophic zone of growth plate cartilage throughout the calcified cartilage matrix (198) but not in the primary spongiosa near the growth plate (179). Increased expression of osteocalcin is seen with the onset of development of the hypertrophic phenotype also in vitro (183). Interestingly, staining for osteocalcin is also seen at the cell borders of osteoclasts and the bone margins of resorption cavities, probably indicating osteocalcin released from bone matrix during resorption (200). In pathological circumstances, osteocalcin has been found in sites of ectopic calcification of cardiovascular tissues (207, 208).

2.14. Osteocalcin and calcium (Ca$^{2+}$):

Osteocalcin has a property to bind calcium (209). Studies indicate that in the absence of Ca$^{2+}$ osteocalcin exists primarily in the random coil (210) or in extended, unstructured conformation except for the turn required by the disulfide bridge Cys23-Cys29 (211). Due to the unstructured properties, a single structure for a pro-osteocalcin has not been solved (212). The addition of millimolar levels of Ca$^{2+}$ induces an alpha-helical structure. In the original study by Hauschka and Carr, the presence of physiological levels of Ca$^{2+}$ resulted in a significant increase in alpha-helical structure, from 8% in apo-osteocalcin to 38% after addition of Ca$^{2+}$ (210). Studies on bovine osteocalcin on solution also indicated a transition to a folded state on the addition of physiological concentrations of Ca$^{2+}$ with an increase in helical structure from 19% to 31% (211). Titration with Ca$^{2+}$ indicated that three moles of calcium per one mole of bovine osteocalcin is responsible for the conformational change. The three calcium ions are coordinated to the three Gla residues within each osteocalcin molecule and the oxygen atoms in
side chains as well as water molecules are involved in coordination (212). Ca$^{2+}$ mediates a structural transition also in other Gla proteins such as prothrombin in which the three-dimensional structure of the Gla-rich domain is induced and stabilized by metal ions (213). However, binding of calcium to osteocalcin is not associated with a clear transition from a disordered to an ordered structure as in the Gla domain of prothrombin (214). This property of osteocalcin may be associated with the potential to bind Ca$^{2+}$ on a crystal surface. The structure might be induced in the presence of hydroxyapatite and flexibility could thus have a functional value (211).

2.15. Metabolism of osteocalcin:

Osteocalcin is rapidly cleared from the circulation. The clearance of radiolabeled osteocalcin after single injection into rats demonstrated that the serum half-life of administrated protein was less than 5 minutes (215). Most of the cleared radioactivity was recovered in the kidney which appears to be the major clearance mechanism for serum osteocalcin. The role of the kidney was further emphasized by a seven-fold elevation in plasma osteocalcin in rats after nephrectomy (215). Kidney tissue homogenates degraded labeled osteocalcin by 60% in 20 minutes indicating that renal degradation may be a more predominant mechanism for clearance than renal excretion (216). Although osteocalcin clearance occurs predominantly in the kidney (215) some metabolism is also seen in the liver but the degradation of the protein is clearly slower (216). Furthermore, osteocalcin levels are elevated in patients with renal failure but not in patients with liver disease (217). Either the uptake of osteocalcin by the kidney and liver cells differ or that the cells that degrade osteocalcin are not affected by liver disease. After two days of injections, only negligible label from injected osteocalcin was found in circulation and the label had entered the organs (215). The remaining label was removed more slowly with a half-life of 6 days and it appeared to be predominantly accumulated in the skulls and long bones, revealing bone tissue as an additional organ for clearance of serum osteocalcin. Thermally decarboxylated osteocalcin accumulated with a substantially lower rate suggesting that the accumulation to bone was dependent on Gla residues. A significantly longer half-life of 91 minutes for osteocalcin has been reported in sheep after cessation of constant infusion of labeled osteocalcin (218). This may, however, be related to different half-lifes of protein metabolized in kidney and the one
entering bone. Also, the lung is a possible site for osteocalcin catabolism. Serum osteocalcin measured from the pulmonary and radial artery of human patients undergoing aorto-coronary bypass demonstrated that the proteolytic activity of pulmonary vessel endothelium involves about 5% of circulating osteocalcin (219). Osteocalcin is known to be sensitive to several common proteases such as trypsin, plasmin, and cathepsins, which could promote the metabolism in the vascular system. Intact osteocalcin is also rapidly degraded by serum proteases in vitro and the degradation can be prevented by protease inhibitors (220). Osteocalcin may, however, be protected from degradation by a larger molecular weight carrier(s) in the circulation but such a complex has not yet been identified. Since the main route for clearance is the glomerular filtration in the kidney, remnants of osteocalcin should be excreted into urine. This was first suggested when Gla residues were detected in urine samples (221). However, Gla is found also in other proteins and urinary Gla may not be derived exclusively from osteocalcin. Taylor and co-workers were the first to demonstrate the presence of immunoreactive osteocalcin fragments in urine (222). Urine did not contain intact osteocalcin and thus, only assays recognizing osteocalcin fragments were applicable for detection. They characterized urine osteocalcin by comparing the high-performance liquid chromatograph (HPLC) profiles of osteocalcin isolated from the serum and urine of healthy individuals and patients with renal disease or Paget’s disease. Normal serum did not contain peaks that corresponded to those found in urine suggesting that breakdown of intact osteocalcin molecules is a normal step in the metabolism and clearance of osteocalcin. However, serum from patients with renal failure contained fragments that corresponded to those found in normal urine. This indicated that the production of some of the urinary fragments occurred before renal clearance and was not a result of it and these fragments were not cleared from serum due to renal impairment. Serum sample obtained from a patient with Paget’s disease also contained fragments that corresponded to some of those found in urine and in addition, “a Paget’s disease specific peak” was seen in both serum and urine samples. Direct isolation and characterization methods have subsequently been used to clarify the identity of urine osteocalcin. The predominant urine osteocalcin fragments isolated from the urine sample of a pubertal boy and detectable by two-site assays spanned residues Leu6-Asp30 and Gly7-Asp30 (223). Trace amounts of intact osteocalcin were detected in the solid phase of urine associated with epithelial cells or crystals of calcium oxalate. Some years
later a structure for osteocalcin fragment isolated from Pagetic urine was resolved sequenced as Asp14-Asp28 (224). Furthermore, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-MS) was applied to elucidate the molecular weight of osteocalcin fragments in pooled urine samples collected either from healthy premenopausal women, postmenopausal women or postmenopausal women after 30-day-treatment with alendronate. The observed molecular weight (1921) fitted to the theoretical mass of osteocalcin fragment Asp14-Asp28 identified from Pagetic urine (1914) and to the epitope mapping of the antiserum, suggesting that this fragment was a predominant fragment of osteocalcin in urine. The peak was suppressed in women receiving alendronate leading to the conclusion that this predominant urine fragment might be of resorptive origin (224). The composition of urine osteocalcin may, however, be more complex (225).
Materials and methods:

3.1. Study design:
Hospital based- case-control cross-sectional study.

3.2. Study area and population:

One hundred fifteen adults who were referred for diagnosis of diabetes mellitus for the first time in Khartoum state participated in this study; with age ranging between 18 – 94 years. The patients were confirmed to have diabetes mellitus by measuring fasting plasma glucose (fasting plasma glucose ≥ 126 mg/dl) (2, 3). Sixty five healthy adults from the same area without history of diabetes mellitus were randomly selected as a control group.

All participants were subjected to thorough clinical examination and detailed history to exclude recent infection, taking insulin supplement, bone diseases and fracture (up to one year).

All participants were classified into two groups by gender (male and female), three groups by age (young 18-43 years, middle age 44-69 years and old 70-94 years), and four groups by body mass index group (underweight, normal weight, overweight and obese), to determine their effect on various variables measured in our work, such as fasting insulin, both γ-carboxylated and undercarboxylated osteocalcin, fasting blood glucose and lipid profile.

The samples size was calculated according to the following formula (226).

\[ N = \frac{Z^2Pq}{d^2} \]

N = Minimum sample size
Z= Normal standard deviation 95% confidence interval (Z = 1.96)
P = Prevalence of the disease
q= 1 – Prevalence
d= Margin of error (0.05)

The prevalence of diabetes in Sudan range between 2.6% in rural areas and 8% in urban areas (5). In this study, the prevalence of D.M was considered (8%).
3.2.1. **Inclusion criteria:**
- Type II diabetic patients, male, and females.
- Type II diabetic patients treated with Gilibenclamiad, Metformin, and Gliclaziad.

3.2.2. **Exclusion criteria:**
- Type II diabetic patients treated with insulin.
- Type II diabetic patients treated with warfarin and heparin, drugs for osteoporosis, 1,25-dihydroxy vitamin D₃, Glucocorticoids, and anticonvulsant drugs.
- Type II diabetes patients with multiple myeloma, hypercalcemia of malignancy, hyperthyroidism, fracture (up to one year), osteomalacia and Paget's disease.
- Pregnant ladies.

3.3. **Data Collection Techniques:**

3.3.1. **Questionnaire and consent:**

Full personal, medical and drug history was taken for every patient to determine the important correlated parameters; age, gender, history of severe disease, diabetes drugs and other drugs that patient received. A written consent that was approved by Shendi University was taken from all participants after explaining to him the project. (see appendix).

3.3.2. **Clinical examination:**

Clinical examination was carried out to exclude patients with one or more of the exclusion criteria. Height and weight of each participant were measured using standardized height and weight scales. The Body mass index (BMI) was calculated as weight (in kilograms)/height (in meters²).

3.3.3. **Blood sample collection:**

5 ml of venous blood were collected and transferred into fluoride oxalate test tubes after at least eight hours fasting for all participants, then the samples were centrifuged, plasma was separated and stored at – 20°C. The plasma was used to measure the level of fasting insulin, total osteocalcin and undercarboxylated osteocalcin, fasting blood glucose and lipid profile.
3.4. Instruments: Instruments used in this study included:

ELISA reader manufactured by (Biotek instruments, ELx 800; USA, SN). ELISA washing machine manufactured by (Biotek instruments, ELx 50; USA, SN237006). Bench centrifuge manufactured by (Hettich Centrifuge EBA 20 - Japan). ACCENT 200 blood chemistry analyzer manufactured by (Cormay; Poland. Water bath, Glassware, tips, pipettes and Eppendorf tubes.

3.5. Chemicals and reagents:

The ELISA kits used for measuring fasting Insulin, total osteocalcin, and undercarboxylated osteocalcin were produced by Sunlong Biotech (China). Glucose and lipid profile were measured using reagents and standard from ACCENT-200 CHOL (Lomianki, Poland 2015). All reagents and standards were stored in the refrigerator at 2 °C.

3.6. Diagnosis of diabetes mellitus:

Fasting plasma glucose (FBG) was measured for all participants to diagnose and classify them into a study group (diabetic) and control group (none-diabetic). Fasting is defined as no caloric intake for at least eight hours (1, 2). FPG ≥ 126 mg/dl was considered as diabetic, while FPG < 100 mg/dl was considered as normal (2, 3). Subjects referred for diagnosis of diabetes and had FPG between 100 mg/dl and 125 mg/dl were subjected to oral glucose tolerance test (OGTT) if the 2-h plasma glucose ≥ 200 mg/dl the subject was considered as diabetic (1, 2). Diabetic subjects were classified as type 2 by measuring their fasting plasma insulin (fasting plasma insulin > 0.08 nmol/l) (1).
3.7. Human Insulin – 2kits components:

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3.8. Human total osteocalcin (TOC)– 2kits components:

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3.9. Human undercarboxylated osteocalcin (ucOC): 2kit components:

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<td>1</td>
<td>Closure plate membrane.</td>
</tr>
<tr>
<td>2</td>
<td>Sealed bags.</td>
</tr>
<tr>
<td>3</td>
<td>Micro ELISA strip plate.</td>
</tr>
<tr>
<td>4</td>
<td>Standard : 2700pg/ml.</td>
</tr>
<tr>
<td>5</td>
<td>Standard diluent.</td>
</tr>
<tr>
<td>6</td>
<td>HRP-Conjugate reagent.</td>
</tr>
<tr>
<td>7</td>
<td>Sample diluent.</td>
</tr>
<tr>
<td>8</td>
<td>Chromogen Solution A</td>
</tr>
<tr>
<td>9</td>
<td>Chromogen Solution B</td>
</tr>
<tr>
<td>10</td>
<td>Stop Solution</td>
</tr>
<tr>
<td>11</td>
<td>wash solution</td>
</tr>
</tbody>
</table>

3.10. Reagents for glucose and lipid profile:

3.10.1. Glucose:
Glucose spectrum diagnostic liquizme is intended for the in-vivo quantitative diagnostic determination of glucose in human serum or plasma.
Glucose standard (St) 100 mg/dl 5.5mmol/L

3.10.1.1. Reagent (R):

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer (PH 7.0)</td>
<td>250 mmol/l</td>
</tr>
<tr>
<td>Phenol</td>
<td>5 mmol/l</td>
</tr>
<tr>
<td>Glucose oxidase (GOD)</td>
<td>&gt; 250 μkat/l</td>
</tr>
<tr>
<td>Peroxidase (POD)</td>
<td>&gt; 20 μkat/l</td>
</tr>
<tr>
<td>4-aminoantipyrine</td>
<td>500 μ mmol/l</td>
</tr>
</tbody>
</table>
3.10.2. Cholesterol:
Cholesterol standard (St) 200 mg/dl-5.17 mmol/l.

3.10.2.1. Reagent (R):

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Good’s buffer (PH 6.4)</td>
<td>100 mmol/l</td>
</tr>
<tr>
<td>Phenol</td>
<td>5 mmol/l</td>
</tr>
<tr>
<td>4-aminoantipyrine</td>
<td>0.3 mmol/l</td>
</tr>
<tr>
<td>Cholesterol esterase (CHE)</td>
<td>&gt; 3.2 µkat/l</td>
</tr>
<tr>
<td>Cholesterol oxidase (CHO)</td>
<td>&gt; 1.67 µkat/l</td>
</tr>
<tr>
<td>Peroxidase (POD)</td>
<td>&gt; 50 µkat/l</td>
</tr>
</tbody>
</table>

3.10.3. Triglyceride:
Standard (St) 200 mg/dl, 2.29 mmol/l.

3.10.3.1. Reagent (R):

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer TRIS (PH 8.0)</td>
<td>200 mmol/l</td>
</tr>
<tr>
<td>4-aminoantipyrine (4-AA)</td>
<td>&lt; 0.4 mmol/l</td>
</tr>
<tr>
<td>ATP</td>
<td>&lt; 1.5 mmol/l</td>
</tr>
<tr>
<td>Mg^{2+}</td>
<td>&lt; 1.6 mmol/l</td>
</tr>
<tr>
<td>4-chlorophenol</td>
<td>&lt; 2.5 mmol/l</td>
</tr>
<tr>
<td>Chlorophenicol</td>
<td>&lt; 1.6 mmol/l</td>
</tr>
<tr>
<td>Potassium hexacyanoferrate (11)</td>
<td>&lt; 1 mmol/l</td>
</tr>
<tr>
<td>FAD-2Na</td>
<td>&lt; 1 mmol/l</td>
</tr>
<tr>
<td>Glycerol kinase (GK)</td>
<td>2500 U/l</td>
</tr>
<tr>
<td>Glycerol phosphate kinase (GPK)</td>
<td>2500 U/l</td>
</tr>
<tr>
<td>Peroxidase (POD)</td>
<td>1900 U/l</td>
</tr>
<tr>
<td>Lipoprotein lipase (LPL)</td>
<td>2000 U/l</td>
</tr>
</tbody>
</table>
### 3.10.4. HDL-Cholesterol:

#### 3.10.4.1. Reagent (R):

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphotungstate</td>
<td>0.52 mmol/l</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>30 mmol/l</td>
</tr>
</tbody>
</table>

Reagents also contain non-reactive stabilizers and surfactants.
Supplementary reagents: A pack for spectrum liquizyme cholesterol reagent required.

### 3.10.5. LDL-Cholesterol:

#### 3.10.5.1 Reagent (R): 1 × 20 ml

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyvinylsulphate</td>
<td>3 g/L</td>
</tr>
<tr>
<td>Polyethylene glycol</td>
<td>3 g/L</td>
</tr>
</tbody>
</table>

### 3.10.6. VLDL-Cholesterol:

VLDL values not measured directly, it is calculated by dividing serum TG by 5 (TG/5).

### 3.11. Assay of Human Insulin:

The concentration of human insulin was measured by Enzyme-linked Immuno-Sorpant Assay (ELISA) method, which has been demonstrated by Sunlong Biotech Company.

#### 3.11.1. Principle of the method:

This ELISA kit uses Sandwich-ELISA as the method. The Microelisa strip plate provided in this kit has been pre-coated with an antibody specific to insulin. Standards of samples are added to the appropriate Microelisa strip plate wells and combined to the specific
antibody. Then a Horseradish Peroxidase (HRP) - conjugated antibody specific for insulin is added to each Microelisa strip plate well and incubated. Free components are washed away. Only those wells that contain insulin and HRP conjugated INS antibody will appear blue in color and then turn yellow after the addition of the stop solution. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm. The OD value is proportional to the concentration of insulin. The concentration of insulin in the samples was then determined by comparing the O.D. of the samples to the standard curve.

3.11.2 Procedure:

Preparing of the standard curve: 10 Standard wells were set on the ELISA plates coated, 100μl of the standard was added to the first and the second wells, then 50μl of standard dilution was added to the first and the second wells and mixed; 100μl was taken out form the first and the second wells then added to the third and the forth wells separately. Then 50μl Standard dilution was added to the third and the fourth wells and mixed ; then 50μl was taken from the third and the fourth wells and then discarded, then 50μl was added to the fifth and the sixth wells, then 50μl Standard dilution was added to the fifth and the sixth wells and mixed ; 50μl taken out from the fifth and the sixth wells and added to the seventh and the eighth wells, then Standard dilution 50μl was added to the seventh and the eighth wells and mixed ; 50μl was taken out from the seventh and the eighth wells and added to the ninth and the tenth wells, then 50μl Standard dilution was added to the ninth and the tenth wells and mixed, 50μl was taken out from the ninth and the tenth wells and discarded(Sample of 50μl was added to each well after diluting. (density: 18 mU/L, 12 mU/L, 6 mU/L, 3 mU/L, 1.5 mU/L)
The set of blank wells were separated (no sample and HRP-Conjugate reagent were added; other each step operation was the same).

For all testing sample wells 40μl of Sample dilution and 10μl of testing sample were added without touching the well wall as far as possible (sample final dilution was 5-fold), and Gently mixed. The plate was incubated for 30 min at 37°C. After closed with closure plate membrane. Then washed with washing solution (prepared by diluted washing solution 30-folds with distilled water and reserved) after removal of closure membrane and dried with a swing. The washing buffer was added to every well, left for the 30s then drained, repeated 5 times, dried by pat. The HRP-Conjugate reagent of 50μl was added to each well, except blank wells. The steps of incubation and washing were repeated as above. To each well 50μl of Chromogen Solution B was added left for 15 min at 37°C in a dark place. Then the reaction was stopped by addition of 50μl of stop solution to each well (the blue color changed to yellow color). The absorbance was read at 450 nm, taking the blank well as zero, within 15min. after addition of the stop solution.

3.11.3. Calculation:

The average zero standard optical density (O.D.) was administrated for each standard, control, and sample to create a standard curve by tumbling the data using a computer software able of generating a four-parameter logistic (4-PL) curve-fit. To get the mean absorbance, a standard curve was plotted of the Y-axis against the concentration on the X-axis to get the mean absorbance for each standard. This technique was used to draw a best-fit curve through the points on the graph. The data was linearized using the log of the human insulin concentration versus the log of the (O.D.) to get the best-fit line using regression analysis.


The concentration of Human total osteocalcin was measured by ELISA, the method adopted by Sunlong Biotech Company.
3.12.1 Principle:

This ELISA kit used sandwich-ELISA as the method. The Microelisa strip plate provided in this kit has been pre-coated with an antibody specific to TOC. Standard of samples are added to the appropriate Microelisa strip plate wells and combined to a specific antibody. Then a Horseradish Peroxidase (HRP) - conjugated antibody specific for TOC is added to each Microelisa strip plate well and incubated. Free components are washed away. Only those wells that contain TOC and HRP conjugated INS antibody will appear blue in color and then turn yellow after the addition of the stop solution. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm. The OD value is proportional to the concentration of TOC. The concentration of TOC in the samples was then determined by comparing the O.D. of the samples to the standard curve.

3.12.2 Procedure:

Preparing of the standard curve: 100µl of the standard and 50µl of Standard dilution were added to the first and the second wells and then mixed. Form the first and the second well 100µl was taken and added to the third and the forth wells separately. Then 50µl of Standard dilution added to the third and the fourth wells and mixed; then 50µl from the third and the forth well discarded. In same way 50µl to the fifth and the sixth wells, then 50µl of the Standard dilution and mixed. From fifth and sixth wells 50µl taken out and add to the seventh and the eighth wells, then 50µl Standard dilution and mixed. The ninth and tenth wells were prepared by taking 50µl from the seventh and the eighth wells and 50µl of Standard dilution, mixed and 50µl from each taken out discarded. After dilution 50µl sample was added to each well. (Density: 150 pg/ml, 100 µg/l, 50 pg/ml, 25 pg/ml, 12.5 pg/ml)
The set of blank wells were separated (no sample and HRP-Conjugate reagent were added; other each step operation was the same). For all testing sample wells 40μl of Sample dilution and 10μl of testing sample were added without touching the well wall as far as possible (sample final dilution was 5-fold), and Gently mixed. The plate was incubated for 30 min at 37°C after closed with closure plate membrane. Then washed with washing solution (prepared by diluted washing solution 30-folds with distilled water and reserved) after removal of closure membrane and dried with a swing. The washing buffer added to every well left for the 30s then drained, repeated 5 times, dried by pat. The HRP-Conjugate reagent of 50μl was added to each well, except blank wells. The steps of incubation and washing were repeated as above. To each well 50μl of Chromogen Solution B was added left for 15 min at 37°C in a dark place. Then the reaction was stopped by addition of 50μl of stop solution to each well (the blue color changed to yellow color). The absorbance was read at 450 nm, taking the blank well as zero, within 15min. after addition of the stop solution.

3.12.3 Calculation:
The average zero standard optical density (O.D.) was administrated for each standard, control, and sample to create a standard curve by tumbling the data using a computer software able of generating a four-parameter logistic (4-PL) curve-fit. To get the mean absorbance, a standard curve was plotted of the Y-axis against the concentration on the X-axis to get the mean absorbance for each standard. This technique was used to draw a best-fit curve through the points on the graph. The data was linearized using the log of the human TOC concentration versus the log of the (O.D.) to get the best-fit line using regression analysis.

3.13. Assay of plasma Undercarboxylated osteocalcin (ucOC):
The concentration of Human Undercarboxylated osteocalcin was measured by ELISA, the method adopted by Sunlong Biotech Company.
3.13.1. Principle:

This ELISA kit used sandwich-ELISA as the method. The Microelisa strip plate provided in this kit has been pre-coated with an antibody specific to ucOC. Standard of samples are added to the appropriate Microelisa strip plate wells and combined to a specific antibody. Then a Horseradish Peroxidase (HRP) - conjugated antibody specific for ucOC is added to each Microelisa strip plate well and incubated. Free components are washed away. Only those wells that contain ucOC and HRP conjugated INS antibody will appear blue in color and then turn yellow after the addition of the stop solution. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm. The OD value is proportional to the concentration of ucOC. The concentration of ucOC in the samples was then determined by comparing the O.D. of the samples to the standard curve.

3.13.2. Procedure:

Preparing of the standard curve: 100μl of the standard and 50μl of Standard dilution were added to the first and the second wells and mixed. Form the first and the second wells 100μl was taken and added to the third and the forth wells separately. Then 50μl of Standard dilution added to the third and the fourth wells and mixed; then 50μl from the third and the forth wells discarded. In same way 50μl to the fifth and the sixth well, then 50μl of the Standard dilution and mixed. From fifth and sixth wells 50μl taken out and add to the seventh and the eighth wells, then 50μl Standard dilution and mixed. The ninth and tenth wells were prepared by taking 50μl from the seventh and the eighth wells and 50μl of Standard dilution, mixed and 50μl from each taken out discarded. After dilution 50μl sample was added to each well.

(Density: 1800 pg/ml, 1200 pg/ml, 600 pg/ml, 300 pg/ml, 150 pg/ml)
The set of blank wells were separated (no sample and HRP-Conjugate reagent were added; other each step operation was the same).

For all testing sample wells 40μl of Sample dilution and 10μl of testing sample were added without touching the well wall as far as possible (sample final dilution was 5-fold), and Gently mixed. The plate was incubated for 30 min at 37°C after closed with closure plate membrane. Then washed with washing solution (prepared by diluted washing solution 30-folds with distilled water and reserved) after removal of closure membrane and dried with a swing. The washing buffer added to every well still for the 30s then drained, repeated 5 times, dried by pat. The HRP-Conjugate reagent of 50μl was added to each well, except blank wells. The steps of incubation and washing were repeated as above. To each well 50μl of Chromogen Solution B was added left for 15 min at 37°C in a dark place. Then the reaction was stopped by addition of 50μl of stop solution to each well (the blue color changed to yellow color). The absorbance was read at 450 nm taken the blank well as zero, within 15min. after addition of the stop solution.

3.13.3. Calculation:

The average zero standard optical density (O.D.) was administrated for each standard, control, and sample to create a standard curve by tumbling the data using a computer software able of generating a four-parameter logistic (4-PL) curve-fit. To get the mean absorbance, a standard curve was plotted of the Y-axis against the concentration on the X-axis to get the mean absorbance for each standard. This technique was used to draw a best-fit curve through the points on the graph. The data was linearized using the log of the human ucOC concentration versus the log of the (O.D.) to get the best-fit line using regression analysis.

3.14. Principle of determination of blood glucose:

Glucose level was determined after enzymatic oxidation by the glucose oxidase (GOD). The formed hydrogen peroxide reacts under the catalysis of peroxidase (GOD) with phenol and 4-aminatipyrene (4-AA) to form a red-violet quinoeimine dye as an indicator.

\[
\begin{align*}
\text{Glucose} + \text{H}_2\text{O} & \xrightarrow{\text{glucose oxidase}} \text{Gluconic acid} + \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 + \text{phenol} + 4\text{-AA} & \xrightarrow{\text{peroxidase}} \text{Quinoeimine} + 4\text{H}_2\text{O}
\end{align*}
\]
Red color
The color intensity was proportional to glucose concentration.

3.14.1. Procedure:
Glucose concentration was determined using the following assay protocol:
- All reagents and specimens were brought to room temperature.
- Three tubes were labeled as, blank, standard and specimen.
- In labeled test tubes the following were added by a pipette.
- 1 ml of the working reagent was added to the blank, standard and specimen tubes.
- 10 μl of the standard solution was added to the standard tube.
- 10 μl of the specimen solution was added to the specimen tube.
- All the three tubes were mixed and incubated for 10 minutes at room temperature.
- The absorbance of the specimen and standard were measured at 510 nm within 30 minutes against blank.

\[
\text{Glucose concentration} = \frac{(\text{Specimen})}{(\text{Standard})} \times 100 \text{ (mg/dL)}
\]

3.15. Principle of determination triglycerides (TG):
The method is based on the enzymatic hydrolysis of serum triglyceride and free fatty acid (FAA) by lipoprotein lipase (LPL) to glycerol.

\[
\begin{align*}
\text{Triglyceride} & \overset{\text{LPL}}{\longrightarrow} \text{Glycerol + fatty acid} \\
\text{Glycerol + ATP} & \overset{\text{GK}}{\longrightarrow} \text{Glycerol-3-phosphate + ADP} \\
\text{Glycerol-3-phosphate + O2} & \overset{\text{GOP}}{\longrightarrow} \text{dihydroxyacetone phosphate + H}_2\text{O}_2
\end{align*}
\]
A red color quinoneimine dye is produced by peroxidase (POD) catalyzed coupling of 4-chlorophenol and 4-amino antipyrine (4-AAP) with hydrogen peroxidase (H₂O₂) proportional to the concentration of triglyceride in a sample which is measured at 560 nm.

\[
\text{H}_2\text{O}_2 + \text{APP} + 4\text{-Cholesterol} \xrightarrow{\text{POD}} \text{Quinoneimine dye} + 4 \text{H}_2\text{O}
\]

3.15.1. Procedure:

Triglyceride was determined using the following assay protocol:

- All reagents and specimens were brought to room temperature.
- Three tubes were labeled as, blank, standard and specimen.
- In labeled test tubes the following were added by a pipette.
- 1 ml of working reagent was added to the blank, standard and specimen.
- 10 μl of the standard solution was added to the standard tube.
- 10 μl of the specimen solution was added to the specimen tube.
- All tubes were mixed and incubated for 5 minutes at room temperature.
- The absorbance of the specimen and standard were measured at 510 nm within 30 minutes against blank.

\[
\text{Triglyceride concentration} = \frac{(\text{Specimen}) \times 200}{(\text{Standard})} \text{ (mg/dL)}
\]

3.16. Principle of determination of total cholesterol (TCHOL):

The method for the measurement of total cholesterol in the serum is based on three enzymes: Cholesterol esterase (CE), Cholesterol oxidase (CO) and peroxidase (POD).

Cholesterol esters hydrolyzed by Cholesterol esterase (CE) to cholesterol and free fatty acids.

\[
\text{Cholesterol esters} \xrightarrow{\text{CE}} \text{cholesterol} + \text{Free Fatty Acid.}
\]

Free cholesterol oxidized by cholesterol oxidase (CO) to cholesterol-4-en-3-one and hydrogen peroxide.

\[
\text{Cholesterol} + \text{O}_2 \xrightarrow{\text{CO}} \text{Cholesterol-4-en-3-one} + \text{H}_2\text{O}_2
\]
The hydrogen peroxide combined with phenol and 4-amino antipyrine (4-AAP) in the presence of peroxidase (POD) to form a chromophore quinoneimine dye.

\[
\text{H}_2\text{O}_2 + \text{phenol} \quad \text{POD} \rightarrow \text{Quinoneimine dye} + 4\text{H}_2\text{O}_2
\]

3.16.1. Procedure:

- All reagents and specimens were brought to room temperature.
- Three tubes were labeled as, blank, standard and specimen.
- In labeled test tubes the following were added by a pipette.
- 1 ml of the working reagent was added to the blank, standard and specimen tubes.
- 10 μl of the standard solution was added to the standard tube.
- 10 μl of the specimen solution was added to the specimen tube.
- All tubes were mixed and incubated for 5 minutes at room temperature.
- The absorbance of the specimen and standard were measured at 510 nm within 30 minutes against blank.

\[
\text{Cholesterol concentration} = \frac{(\text{Specimen}) \times 200}{(\text{Standard})} \text{ (mg/dL)}
\]

3.17. Principle of determination of HDL-Cholesterol:

The method of measurement high-density lipoprotein (HDL) depends on the precipitation of low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL) in a sample with phosphotungstate and magnesium ion. After centrifugation, the cholesterol concentration in the HDL fraction which remains in the supernatant is determined.

\[
\text{Cholesterol ester} + \text{H}_2\text{O}_2 \quad \text{Chol. Esterase} \rightarrow \text{Cholesterol} + \text{Free Fatty Acid}
\]

\[
\text{Cholesterol} + \frac{1}{2} \text{O}_2 + \text{H}_2\text{O} \quad \text{Chol. Oxidase} \rightarrow \text{Cholestenone} + 4\text{H}_2\text{O}
\]

\[
2\text{H}_2\text{O} + 4\text{-aminoantipyrine} + \text{phenol} \quad \text{peroxidase} \rightarrow \text{Quinoneimine dye} + 4\text{H}_2\text{O}
\]
3.17.1. **Procedure:**

HDL-Cholesterol was determined using the following assay protocol:

- All reagents and specimens were brought to room temperature.
- Two test tubes were labeled as blank and specimen.
- In labeled test tubes 0.5 ml of the reagent and 0.2 ml of the specimen were added by a pipette.
- The tubes were mixed and incubated for 10 minutes at room temperature.
- Centrifuged for 10 minutes at 400 r.p.m.
- The supernatant was collected carefully.
- In label test tubes the following were added by a pipette.
  - 50 μl of distilled water was added to the blank tube.
  - 1 ml of cholesterol reagent was added to the blank and specimen tubes.
  - 50 μl of the specimen was added to the specimen tube.
- All tubes were mixed and incubated for 5 minutes at room temperature.
- The absorbance of the specimen was measured at 510 nm within 60 minutes against blank.

\[
\text{HDL-Cholesterol concentration} = (\text{Specimen}) \times \text{Sample Dilution Factor (570)} \\
\text{(mg/dL)}
\]

3.18. **Principle of determination of LDL-Cholesterol:**

Low-density lipoprotein (LDL) is the sample precipitation with polyvinyl sulfate. Their concentration is calculated from differences between the serum total cholesterol and cholesterol in supernatant centrifugation. The cholesterol is spectrophotometrically measured by means of the following coupled reaction.

\[
\begin{align*}
\text{Cholesterol ester} + \text{H}_2\text{O} & \xrightarrow{\text{Chol. Esterase}} \text{Cholesterol} + \text{Free Fatty Acid} \\
\text{Cholesterol} + \frac{1}{2} \text{O}_2 + \text{H}_2\text{O} & \xrightarrow{\text{Chol. Oxidase}} \text{Cholestenone} + \text{H}_2\text{O} \\
2\text{H}_2\text{O} + 4\text{-aminoantipyrine} + \text{phenol} & \xrightarrow{\text{peroxidase}} \text{Quinoneimine dye} + 4\text{H}_2\text{O}
\end{align*}
\]
3.18.1 Procedure:

- In labeled test tubes the following were added by a pipette.
- Two test tubes were labeled as blank and specimen.
- In labeled test tubes 0.2 ml of the reagent and 0.4 ml of the specimen were added by a pipette.
- The tubes were mixed and incubated for 15 minutes at room temperature.
- Centrifuged for 15 minutes at 400 r.p.m.
- The supernatant was collected carefully.
- In label test tubes the following were added by a pipette.
- 50 μl of distilled water was added to the blank.
- 1 ml of cholesterol reagent was added to the blank and specimen tubes.
- 50 μl of the specimen was added to the specimen tube.
- All tubes were mixed and incubated for 10 minutes at room temperature.
- The absorbance of the specimen and standard were measured at 500-550 nm within 60 minutes against blank.

\[ \text{LDL} = \frac{(\text{Specimen}) \times \text{Sample Dilution Factor}}{(mg/dL)} \]

3.19. Data analysis:

Results of this study were statistically analyzed using (IBM SPSS Statistics program – version 21 64 bits for windows 8). Standard multiple regression was run, the dependent variables (FBG, F1), while the independent variables (sex, age, BMI, TOC, ucOC, FBG, TCHOL, HDL, LDL and TG levels). To determine whether there is an interaction affecting the results of F1, FPG and TOC, ucOC between status and age, status and gender, status and BMI a univariate analysis was run. Significant differences between groups were assessed by Independent t-test and one-way analysis of variance (ANOVA), while correlations between independent variables assumed in our work (gender, age, BMI, TOC, ucOC, FBG, TCHOL, HDL, LDL and TG) and the dependents variable which were FPG, fasting insulin was measured by Spearman correlation. Pearson's correlation was run to determine the relationship between TOC level and BMI and lipid profile parameters. Significance levels were set at (P ≤ 0.05).
Results

4.1. Study group:

The total number of subjects recruited for this study were 180, of which 115 were diabetic (63.9%) and 65 were used as control (36.1%). Some of the characteristics of the diabetic and control groups are shown in (Table 4.1).

All ages ranged between 18 and 94 years old. The mean ages ± standard deviation (SD) in years of males and females in the diabetic group were 65 ± 14 and 59.7 ± 15 respectively, while the respective values in the sample from the control group were 39.0 ± 16 and 43 ± 16. There were significant differences in the mean ages between diabetics and controls (P = 0.000) and between diabetic males and females compared to their control peers (P = 0.000) for both. (Table 4.1).

Table 4.1

<table>
<thead>
<tr>
<th></th>
<th>Diabetics (63.9%)</th>
<th></th>
<th>Controls (36.1%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Total</td>
<td>Male</td>
</tr>
<tr>
<td>Number</td>
<td>34 (29.56%)</td>
<td>81 (70.40%)</td>
<td>115 (100%)</td>
<td>26 (40%)</td>
</tr>
<tr>
<td>Mean age (Year) ± SD</td>
<td>65 ± 14</td>
<td>59.7 ± 15</td>
<td>61 ± 15</td>
<td>39 ± 16</td>
</tr>
<tr>
<td>Mean weight (Kg) ± SD</td>
<td>73.3 ± 11.9</td>
<td>75.1 ± 13.7</td>
<td>74.6 ± 13.2</td>
<td>66.5 ± 10.9</td>
</tr>
<tr>
<td>Mean height (m) ± SD</td>
<td>1.65 ± 0.08</td>
<td>1.62 ± 0.08</td>
<td>1.63 ± 0.08</td>
<td>1.68 ± 0.12</td>
</tr>
<tr>
<td>Mean BMI (Kg/m²) ± SD</td>
<td>27.0 ± 4.2</td>
<td>28.7 ± 5.0</td>
<td>28.2 ± 4.8</td>
<td>23.7 ± 3.3</td>
</tr>
</tbody>
</table>

To determine the effect of age on different parameters of the study, subjects in both control and diabetic groups were divided into different age groups including 18-43, 44-69 and 70-94 corresponding to young adults, middle age and old people. Table 4.2 shows the numbers and percentages in each age group by sex. (Table 4.2).
Table 4.2

Number and percentage of diabetic and control subjects in each age group by gender

<table>
<thead>
<tr>
<th>Age group</th>
<th>Diabetic males</th>
<th>Diabetic female</th>
<th>Total</th>
<th>Control males</th>
<th>Control females</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 – 43 years</td>
<td>3(2.60%)</td>
<td>12(10.43%)</td>
<td>15(13.04%)</td>
<td>19(29.23%)</td>
<td>23(35.38%)</td>
<td>42(64.71%)</td>
</tr>
<tr>
<td>44 – 69 years</td>
<td>16(13.91%)</td>
<td>45(39.13%)</td>
<td>61(53.04%)</td>
<td>42(64.61%)</td>
<td>13(20%)</td>
<td>19(29.23%)</td>
</tr>
<tr>
<td>70 – 94 years</td>
<td>15(13.04%)</td>
<td>24(20.86%)</td>
<td>39(33.91%)</td>
<td>19(29.23%)</td>
<td>3(4.61%)</td>
<td>4(6.15%)</td>
</tr>
</tbody>
</table>

The diabetic patients and healthy control subjects were classified into four groups according to their weight categories: underweight, ideal weight, overweight and obese. Two subjects (1.73%) in the diabetic group and three (4.61%) in the control group were found to be underweight. Most of the subjects in the diabetic group 29 (25.21%) had normal weight compared to 37 (56.92%) in the control group. The numbers and percentages of participants in the diabetic group who were overweight and obese were 43 (37.39%) and 33 (35.65%) respectively, while the respective numbers and percentages in the control group were 23 (25.38%) and 2 (3.07%). (Table 4.3).

Table 4.3

Number of diabetic and control subjects in each BMI group by gender

<table>
<thead>
<tr>
<th>BMI group</th>
<th>Diabetic males</th>
<th>Diabetic female</th>
<th>Total</th>
<th>Control males</th>
<th>Control females</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Underweight</td>
<td>0(0.0%)</td>
<td>2(1.73%)</td>
<td>2(1.73%)</td>
<td>2(3.07%)</td>
<td>1(1.53%)</td>
<td>3(4.61%)</td>
</tr>
<tr>
<td>Nor. weight</td>
<td>13(11.30%)</td>
<td>16(13.91%)</td>
<td>29(25.21%)</td>
<td>15(13.04%)</td>
<td>22(33.84%)</td>
<td>37(56.92%)</td>
</tr>
<tr>
<td>Overweight</td>
<td>13(11.30%)</td>
<td>30(26.08%)</td>
<td>43(37.39%)</td>
<td>8(12.30%)</td>
<td>15(23.07%)</td>
<td>23(25.38%)</td>
</tr>
<tr>
<td>Obese</td>
<td>8(6.95%)</td>
<td>33(28.69%)</td>
<td>41(35.65%)</td>
<td>1(1.53%)</td>
<td>1(1.53%)</td>
<td>2(3.07%)</td>
</tr>
</tbody>
</table>
4.2 Total osteocalcin:

The mean plasma concentration of total osteocalcin level for diabetic patients \((n = 115)\) and control subjects \((n = 65)\). The results suggest that the average diabetic patients level are \((\text{mean} \pm \text{SD}) = (2.38 \pm 1.39 \text{ ng/ml})\) was significantly less than the average control subjects level \((\text{mean} \pm \text{SD}) = (14.83 \pm 2.11 \text{ ng/ml})\) and \((P = 0.000)\) (Table 4.4).

Table 4.4:

Comparison of mean concentrations of TOC, ucOC, insulin, TC, TG, HDL and LDL in diabetic and control subjects

<table>
<thead>
<tr>
<th></th>
<th>Diabetic</th>
<th>Control</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td></td>
</tr>
<tr>
<td>TOC ng/ml</td>
<td>2.38 ± 1.39</td>
<td>14.83 ± 2.11</td>
<td>0.000</td>
</tr>
<tr>
<td>ucOC ng/ml</td>
<td>1.09 ± .57</td>
<td>2.61 ± .49</td>
<td>0.000</td>
</tr>
<tr>
<td>FI ml U/L</td>
<td>20 ± 6</td>
<td>9 ± 2</td>
<td>0.000</td>
</tr>
<tr>
<td>FBG mg/dl</td>
<td>195 ± 59</td>
<td>85 ± 25</td>
<td>0.000</td>
</tr>
<tr>
<td>TCHOL mg/dl</td>
<td>160 ± 35</td>
<td>147 ± 22</td>
<td>0.003</td>
</tr>
<tr>
<td>TG mg/dl</td>
<td>130 ± 48</td>
<td>118 ± 41</td>
<td>0.077</td>
</tr>
<tr>
<td>HDL mg/dl</td>
<td>20 ± 6</td>
<td>33 ± 6</td>
<td>0.000</td>
</tr>
<tr>
<td>LDL mg/dl</td>
<td>52 ± 14</td>
<td>49 ± 12</td>
<td>0.030</td>
</tr>
<tr>
<td>BMI kg/m²</td>
<td>23.8 ± 3</td>
<td>28.2 ± 4.8</td>
<td>0.000</td>
</tr>
</tbody>
</table>

The mean plasma concentrations of total osteocalcin level were significantly lesser in both diabetic females \((2.33\pm 1.29 \text{ ng/ml})\) and diabetic males \((2.52\pm 1.62 \text{ ng/ml})\) compared to control females \((15.18\pm2.54 \text{ ng/ml})\) and control males \((14.31\pm 1.04 \text{ ng/ml})\) \((P = 0.000\) for both) (Figure 4.1).
Figure 4.1

Comparison of mean plasma concentrations of total osteocalcin between controls (C) and diabetics (D) by gender

The plasma total osteocalcin concentration was higher in controls compared to diabetics in all age groups, and was statistically significant in all age groups (P = 0.000 for age groups 18-43 and 44-69) and (P = 0.003 for the age group 70-94). (Figure 4.2). In the control, the plasma total osteocalcin concentration was as follows, in the age group 18-43 years (14.72 ± 2.13 ng/ml), 44-69 years (14.73 ±1.7 ng/ml) and among age group 70-94 years (16.58 ± 3.36 ng/ml). In the diabetics, the value of plasma total osteocalcin concentration was increased gradually with age. The highest value was noted among age group 70-94 years (2.45 ± 1.32 ng/ml), followed by age group 44-69 years (2.37 ±1.49 ng/ml) and the least value was noted in age group 18-43 years (2.27 ± 2.13 ng/ml). Comparing each age group against the others within controls or diabetics led to differences which were not statistically significant in both cases. (Figure 4.2).
In the control group, the highest mean plasma concentration of total osteocalcin was noted among normal-weight subjects (14.97 ± 2.4 ng/ml). It decreased progressively in the underweight subjects (14.79 ± 1.99 ng/ml) and underweight subjects (14.78 ± 0 ng/ml) to reach a minimum value in obese subjects (13.79 ± 1.24 ng/ml). (Figure 4.3). In the diabetic group, the highest mean plasma concentration of total osteocalcin was noted among underweight subjects (2.45 ± 0.74 ng/ml). It decreased to (2.44 ± 1.5 ng/ml) in the obese subjects and the values in normal-weight and overweight subjects were identical (2.37 ± 1.38 ng/ml). (Figure 4.3). In all BMI groups, the mean plasma concentration of total osteocalcin was significantly higher in the control groups than in their respective diabetic groups (P = 0.003 for underweight subjects, P = 0.000 for normal-weight, overweight and obese subjects). (Figure 4.3).
A Pearson's product-moment correlation was run to assess the relationship between plasma TOC concentration and BMI in the controls and the diabetics.

In the control group, there was a weak non-significant correlation between TOC and BMI, $r = 0.112$ and $P$-value $= 0.375$.

In the diabetic group, there is a weak correlation that is statistically not significant between plasma total osteocalcin and BMI, $r = 0.057$ and $P$-value $= 0.546$. (Table 4.5).
### Table 4.5:

**Correlation of total osteocalcin concentration and BMI**

<table>
<thead>
<tr>
<th>Status</th>
<th>TOC ng/ml</th>
<th>BMI Kg/m²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pearson correlation</td>
<td>1</td>
</tr>
<tr>
<td>Control</td>
<td>Sig. (2-tailed)</td>
<td>0.375</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>Pearson correlation</td>
<td>0.112</td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>0.375</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>65</td>
</tr>
<tr>
<td>Diabetic</td>
<td>TOC ng/ml</td>
<td>Pearson correlation</td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>0.546</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>Pearson correlation</td>
<td>0.057</td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>0.546</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>115</td>
</tr>
</tbody>
</table>

**Correlation is significant at the 0.01 level (2-tailed).**

A Pearson's product-moment correlation was run to assess the relationship between plasma TOC concentration and FI, FBG in the controls and the diabetics.

In the control group, there was a weak non-significant correlation between TOC and FI, FBG concentrations, $r = 0.022$,- $0.042$ and P-value = 0.860, 0.739 respectively.

In the diabetic group, there is a moderate negative correlation that is statistically significant between plasma TOC concentration and FI, FBG level. For insulin $r = 0.530$, P-value = 0.000, regarding FBG $r = 0.373$, P-value = 0.001.(Table 4.6).
Table 4.6:

Pearson's correlation between total osteocalcin (TOC), FI and FBG.

<table>
<thead>
<tr>
<th>Status</th>
<th>TOC ng/ml</th>
<th>FI ml U/L</th>
<th>FBG mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pearson correlation</td>
<td>1</td>
<td>0.022</td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>-</td>
<td>0.860</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>Control</td>
<td>TOC ng/ml</td>
<td>Pearson correlation</td>
<td>0.022</td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>0.860</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>Control</td>
<td>FI ml U/L</td>
<td>Pearson correlation</td>
<td>-0.042</td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>0.739</td>
<td>0.372</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>Control</td>
<td>FBG mg/dl</td>
<td>Pearson correlation</td>
<td>0.373**</td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>0.001</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>115</td>
<td>115</td>
</tr>
<tr>
<td>Diabetic</td>
<td>TOC ng/ml</td>
<td>Pearson correlation</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>-</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>115</td>
<td>115</td>
</tr>
<tr>
<td>Diabetic</td>
<td>FI ml U/L</td>
<td>Pearson correlation</td>
<td>0.530**</td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>0.000</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>115</td>
<td>115</td>
</tr>
<tr>
<td>Diabetic</td>
<td>FBG mg/dl</td>
<td>Pearson correlation</td>
<td>0.373**</td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>0.001</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>115</td>
<td>115</td>
</tr>
</tbody>
</table>

** Correlation is significant at the 0.01 level (2-tailed).

4.3: Undercarboxylated osteocalcin:

The mean plasma concentration of undercarboxylated osteocalcin level for diabetic patients (n = 115) and control subjects (n = 65). The results suggest that the average diabetic patients level are (mean ± SD) = (1.09 ± 0.57 ng/ml) was significantly less than the average control subjects level (mean ± SD) = (2.61 ± 0.49 ng/ml) and (P = 0.000) (Table 4.4).
The mean plasma concentrations of ucOC level were significantly lesser in both diabetic females (1.1± 0.56 ng/ml) and diabetic males (1.07±0.60 ng/ml) compared to control females (2.59 ± 0.50 ng/ml) and control males (2.63 ± 0.48 ng/ml) (P = 0.000 for both) (Figure 4.4).

**Figure 4.4**

**Comparison of mean plasma concentrations of ucOC osteocalcin between controls (C) and diabetics (D) by gender**

The plasma undercarboxylated osteocalcin concentration was higher in controls compared to diabetics in all age groups, these differences were statistically significant in all age groups (P = 0.000 for age groups 18-43 and 44-69) and (P = 0.003 for the age group 70-94). (Figure 4.5). In the control, the plasma ucOC concentration was as follows, in the age group 18-43 years (2.58 ± 0.48 ng/ml), 44-69 years (2.67 ±0.53 ng/ml) and among age group 70-94 years (2.63 ± 0.38 ng/ml). In the diabetics, the value of plasma ucOC concentration was similar in age groups 18-43, 44-69 years (1.11 ± 1.6 ng/ml) and was found to be (1.08 ±0.56 ng/ml) in the age
group 70-90. Comparing each age group against the others within controls or diabetics led to differences which were not statistically significant in both cases. (Figure 4.5).

![Figure 4.5](image)

**Figure 4.5**

Mean plasma concentrations of ucOC (ng/ml) in different age groups in control (C) and diabetic (D) subjects

In the control group, the highest mean plasma concentration of ucOC was noted among normal-weight subjects (2.63 ± 0.63 ng/ml). It decreased progressively in the overweight subjects (2.61 ± 0.43 ng/ml). It is higher in the obese subjects (2.59 ± 0 ng/ml) than in the underweight subjects (2.31 ± 0.55 ng/ml). (Figure 4.6). In the diabetic group, the highest mean plasma concentration of ucOC was noted among normal-weight and overweight subjects (1.10 ± 0.52 ng/ml) for both. It decreased to (1.09 ± 0.56 ng/ml) in the obese and least concentration was noticed in the underweight subjects (0.87± 0.58 ng/ml). (Figure 4.6). In all BMI groups, the mean plasma concentration of ucOC was significantly higher in the control groups than in their respective diabetic groups (P = 0.00) for underweight subjects and normal-weight, (P = 0.01) for the obese subjects, but it is not significant for the underweight subjects (P = 0.065 ). (Figure 4.6).
A Pearson's product-moment correlation was run to assess the relationship between plasma ucOC concentration and FI, FBG in the controls and the diabetics. In the control group, there was a weak negative significant correlation between ucOC and FI, \( r(98) = -0.307 \) and P-value = 0.013. Regarding FBG, there was a weak negative non-significant correlation between ucOC and FBG, \( r(98) = -0.117 \) and P-value = 0.354. In the diabetic group, there is a moderate negative correlation that is statistically significant between plasma ucOC concentration and FI, \( r(98) = -0.654 \) and P-value = 0.000. Regarding FBG level, there was a weak negative significant correlation between ucOC and FBG, \( r(98) = -0.297 \) and P-value = 0.001. (Table 4.7).

**Table 4.7:**

<table>
<thead>
<tr>
<th>Status</th>
<th>ucOC ng/ml</th>
<th>FI ml U/L</th>
<th>FBG mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>underweight</td>
<td>2.31</td>
<td>1.10</td>
<td>1.09</td>
</tr>
<tr>
<td>Normal weight</td>
<td>2.63</td>
<td>1.10</td>
<td>1.10</td>
</tr>
<tr>
<td>Overweight</td>
<td>2.61</td>
<td>1.10</td>
<td>1.09</td>
</tr>
<tr>
<td>Obese</td>
<td>2.59</td>
<td>1.09</td>
<td>1.09</td>
</tr>
</tbody>
</table>

**Figure 4.6:**

Mean plasma concentrations of (ucOC) in control (C) and diabetic (D) subjects by BMI subgroups
<table>
<thead>
<tr>
<th></th>
<th>ucOC ng/ml</th>
<th><strong>Pearson correlation</strong></th>
<th>Sig. (2-tailed)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td>65</td>
</tr>
<tr>
<td>ucOC</td>
<td></td>
<td>1</td>
<td>0.307*</td>
<td>-0.117</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td></td>
<td>-</td>
<td>0.013</td>
<td>0.354</td>
</tr>
<tr>
<td>N</td>
<td></td>
<td>65</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>FI ml U/L</td>
<td></td>
<td>0.307*</td>
<td>1</td>
<td>0.113</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td></td>
<td>0.013</td>
<td>-</td>
<td>0.372</td>
</tr>
<tr>
<td>N</td>
<td></td>
<td>65</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>FBG mg/dl</td>
<td></td>
<td>-0.0117</td>
<td>0.113</td>
<td>1</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td></td>
<td>0.354</td>
<td>0.372</td>
<td>-</td>
</tr>
<tr>
<td>N</td>
<td></td>
<td>65</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>Diabetic</td>
<td></td>
<td><strong>0.634</strong></td>
<td><strong>-0.297</strong></td>
<td></td>
</tr>
<tr>
<td>ucOC</td>
<td></td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td></td>
<td>-</td>
<td>0.000</td>
<td>0.001</td>
</tr>
<tr>
<td>N</td>
<td></td>
<td>115</td>
<td>115</td>
<td>115</td>
</tr>
<tr>
<td>FI ml U/L</td>
<td></td>
<td><strong>0.634</strong></td>
<td>1</td>
<td><strong>0.253</strong></td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td></td>
<td>0.000</td>
<td>-</td>
<td>0.006</td>
</tr>
<tr>
<td>N</td>
<td></td>
<td>115</td>
<td>115</td>
<td>115</td>
</tr>
<tr>
<td>FBG mg/dl</td>
<td></td>
<td><strong>0.297</strong></td>
<td><strong>0.253</strong></td>
<td>1</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td></td>
<td>0.001</td>
<td>0.006</td>
<td>-</td>
</tr>
<tr>
<td>N</td>
<td></td>
<td>115</td>
<td>115</td>
<td>115</td>
</tr>
</tbody>
</table>

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

### 4.4. Lipid profile:

When diabetics were compared to controls, the mean plasma concentration of total cholesterol (TCHOL) was significantly higher in the diabetic group (160 ± 35 mg/dl) compared to control one (147 ± 22 mg/dl) (P = 0.003). The mean plasma concentration of high-density lipoproteins (HDL) was significantly higher in the control group (33 ± 6 mg/dl) compared to the diabetic group (20 ± 6 mg/dl) (P = 0.000). The mean plasma concentration of triglycerides (TG) was higher in diabetics (130 ± 38 mg/dl), compared to controls (118 ± 41 mg/dl), but this difference was not significantly significant (P = .077). The mean plasma concentration of
low-density lipoproteins (LDL) was significantly higher in the control (52.0 ± 14 mg/dl), compared to diabetic subjects (49± 12 mg/dl). (Table 4.4)

The same results were observed with a changed level of statistical significance when diabetic males and diabetic females were compared to their respective controls (Table 4.7). In males, mean TG was significantly higher in diabetics (134 ± 43 mg/dl) compared to controls (110 ± 47 mg/dl) (P =0.042). TCHOL was statistically not significantly higher in diabetic males (159± 30 mg/dl) compared to control ones (146 ± 23 mg/dl) (P = 0.055), and LDL was higher in diabetic males (51 ± 13 mg/dl) compared to control males (46 ± 12 mg/dl) (P = 0.092). But, mean HDL was significantly higher in control males (134 ± 43 mg/dl) compared to diabetics (19 ± 4 mg/dl) (P = 0.000). In females, mean HDL was significantly higher in controls (32 ± 6 mg/dl) compared to diabetics (21 ± 6 mg/dl) (P = 0.000), while TCHOL was significantly higher in diabetics (160.0 ± 37 mg/dl) compared to controls (148 ± 22 mg/dl) (P = 0.028). In females, both TG and LDL were higher in diabetics compared to controls but these differences were not statistically significant (P = 0.524 & 0.620 respectively). TG values were (129 ± 50 mg/dl) in diabetic females and (123 ± 37 mg/dl) in control ones, while LDL values were (52 ± 15 mg/dl) in diabetic females and (51 ± 11 mg/dl) in controls. (Table 4.8).
Table 4.8

Mean plasma concentrations of lipid profile parameters (mg/dl) in control and diabetic subjects by sex:

<table>
<thead>
<tr>
<th>Sex</th>
<th>Variable</th>
<th>Status</th>
<th>N</th>
<th>Mean</th>
<th>St deviation</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCHOL mg/dl</td>
<td>Control</td>
<td>26</td>
<td>146</td>
<td>23</td>
<td>0.055</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>34</td>
<td>159</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG mg/dl</td>
<td>Control</td>
<td>26</td>
<td>110</td>
<td>47</td>
<td>0.042</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>34</td>
<td>134</td>
<td>43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL mg/dl</td>
<td>Control</td>
<td>26</td>
<td>33</td>
<td>7</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>34</td>
<td>19</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL mg/dl</td>
<td>Control</td>
<td>26</td>
<td>46</td>
<td>12</td>
<td>0.092</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>34</td>
<td>51</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>female TCHOL mg/dl</td>
<td>Control</td>
<td>39</td>
<td>148</td>
<td>22</td>
<td>0.028</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>81</td>
<td>160</td>
<td>37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG mg/dl</td>
<td>Control</td>
<td>39</td>
<td>123</td>
<td>37</td>
<td>0.524</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>81</td>
<td>129</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL mg/dl</td>
<td>Control</td>
<td>39</td>
<td>32</td>
<td>6</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>81</td>
<td>21</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL mg/dl</td>
<td>Control</td>
<td>39</td>
<td>51</td>
<td>11</td>
<td>0.620</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>81</td>
<td>52</td>
<td>15</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

When grouping was based on BMI, in controls mean TCHOL concentrations were highest in underweight (156 ± 5 mg/dl) and overweight subjects (154 ± 18 mg/dl) compared to normal weight (143 ± 23 mg/dl) and obese subjects (130 ± 49 mg/dl). These differences were statistically significant in normal weight (P = 0.005) and were not statistically significant in underweight subjects (P = 0.887), overweight (P = 0.490) and obese (P = 0.211). In diabetics mean TCHOL concentration was highest in normal weight (161 ± 28 mg/dl), and were nearly
similar in overweight subjects (159 ± 42 mg/dl) and obese subjects (160 ± 32 mg/dl) and the least value was observed in underweight subjects (151 ± 43 mg/dl), also these differences were not statistically significant (P = 0.887). Mean TCHOL concentrations were higher in all BMI groups of diabetic subjects compared to their control peers except in underweight subjects group, and the differences were only significant between normal-weight groups (P = 0.005), but not statistically significant in overweight (P = 0.490) and obese subjects (P = 0.211). (Figure 4.7)

![Figure 4.7: Mean plasma concentrations of TCHOL among different BMI subgroups in control (C) and diabetic (D) subjects](image)

The differences in mean plasma concentrations of TG were not statistically significant, if the BMI groups were compared within controls or diabetics separately or if the groups were compared as diabetics versus controls.

The interesting observation was that the mean TG concentration decreased progressively from underweight subjects towards obese subjects in both diabetic and control subgroups. The mean TG concentrations were higher in all diabetic BMI groups compared to
their respective controls. (Figure 4.8). The values in control subgroups were (141 ± 60 mg/dl) is underweight, (117 ± 45 mg/dl) in normal weight, (116 ± 36 mg/dl) in overweight and (106 ± 13 mg/dl) in obese, and the respective values in diabetic subgroups were (172 ± 118 mg/dl), (136 ± 53 mg/dl), (132 ± 41 mg/dl) and (123 ± 17 mg/dl). (figure 4.8)

A correlation between TOC concentration and TG showed a weak positive correlation that is statistically non-significant P = 0.674. The same test was done to assess the correlation between ucOC and TG, it revealed a weak positive correlation that is statistically non-significant P = 0.426.

![Figure 4.8: Mean plasma concentrations of TG among different BMI subgroups in control (C) and diabetic (D) subjects](image)

The mean HDL plasma concentrations in the subgroups of BMI of control subjects showed nearly inverted U-shaped, but in the diabetic subgroups, these concentrations increased
progressively from underweight subjects toward obese one to reach its maximum. (Figure 4.9).

In controls, the highest levels were observed in normal weight (34 ± 7 mg/dl) and overweight subjects (31 ± 6 mg/dl) and the lowest levels were noticed in obese subjects (31 ± 1 mg/dl) and underweight (29 ± 7 mg/dl). In diabetics, the values of mean HDL concentrations increased progressively from underweight (15.0 ± 5 mg/dl) to normal weight (19 ± 6 mg/dl) to overweight (21 ± 5 mg/dl) to reach its maximum level in the obese group (21 ± 6 mg/dl). When the diabetic subgroups were compared versus their respective control one, the differences were statistically significant in normal-weight (P = 0.000), overweight (P = 0.000), obese (P = 0.034). (Figure 4.9)

A correlation between TOC concentration and HDL showed a weak negative correlation that is statistically significant P = 0.050. The same test was done to assess the correlation between ucOC and HDL, it revealed a weak negative correlation that is statistically non-significant P = 0.062.

Figure 4.9:

Mean plasma concentrations of HDL among different BMI subgroups in control (C) and diabetic (D) subjects
The pattern of LDL means in both control and diabetic were nearly similar as shown in (Figure 4.9). The highest values were noticed in underweights and the lowest was observed in obese subgroups, while normal weights and overweight had nearly similar values in both groups. In controls, the respective values were (56 ± 17 mg/dl) in underweight, (49 ± 12 mg/dl) in normal weight, (48 ± 10 mg/dl) in overweight and (41 ± 13 mg/dl) in obese subjects. In diabetics, the respective values were (61 ± 31 mg/dl) in underweight subjects, (52 ± 14 mg/dl) in normal weight, (53 ± 14 mg/dl) in overweight subjects, and (50 ± 14 mg/dl) in obese subjects. (Figure 3.9). When the subgroups of diabetics were compared against their respective controls, the mean LDL concentrations were higher in diabetic BMI subgroups, but these differences were not statistically significant in underweight (P = 0.836), in normal-weight (P = 0.325), in overweight (P = 0.161), and in the obese (P = 0.369). (Figure 4.10).

Figure 4.10:
Mean plasma concentrations of LDL among different BMI subgroups in control (C) and diabetic (D) subjects
A Pearson's product-moment correlation was run to assess the relationship between plasma TOC, ucOC concentration and the parameters of lipid profile. When the test was done between TOC and lipid profile, there was a moderate negative correlation between TOC and TCHOL. Which was statistically significant (P = 0.003). There was a moderate negative correlation between TOC and TG. Which was statistically non-significant (P = 0.057). There was a strong positive correlation between TOC and HDL. Which was statistically significant (P = 0.000). There was a weak positive correlation between TOC and LDL. That is statistically non-significant P = 0.105. When the test was done between ucOC and lipid profile, there was a moderate negative correlation between ucOC and TCHOL. Which was statistically significant (P = 0.004). There was a weak negative correlation between ucOC and TG. Which was statistically non-significant P = 0.200. There was a moderate positive correlation between ucOC and HDL. Which was statistically significant (P = 0.000). There was a weak negative non-significant correlation between ucOC and LDL, (P = 0.208). (Table 4.9).
**Table 4.9**

Pearson's correlation between plasma TOC, ucOC concentrations and the parameters of lipid profile:

<table>
<thead>
<tr>
<th></th>
<th>TOC ng/ml</th>
<th>TCHOL mg/dl</th>
<th>TG mg/dl</th>
<th>HDL mg/dl</th>
<th>LDL mg/dl</th>
<th>ucOC ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOC ng/ml</td>
<td>Pearson correlation</td>
<td>1</td>
<td>-0.220**</td>
<td>-0.142</td>
<td>0.715**</td>
<td>-0.121</td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>0.003</td>
<td>0.057</td>
<td>0.000</td>
<td>0.105</td>
<td>0.000</td>
</tr>
<tr>
<td>N</td>
<td>180</td>
<td>180</td>
<td>180</td>
<td>180</td>
<td>180</td>
<td>180</td>
</tr>
<tr>
<td>TCHOL mg/dl</td>
<td>Pearson correlation</td>
<td>-0.220**</td>
<td>1</td>
<td>0.307**</td>
<td>-0.052</td>
<td>0.520**</td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>0.003</td>
<td>0.000</td>
<td>0.491</td>
<td>0.000</td>
<td>0.004</td>
</tr>
<tr>
<td>N</td>
<td>180</td>
<td>180</td>
<td>180</td>
<td>180</td>
<td>180</td>
<td>180</td>
</tr>
<tr>
<td>TG mg/dl</td>
<td>Pearson correlation</td>
<td>-0.142</td>
<td>0.307**</td>
<td>1</td>
<td>-0.277**</td>
<td>0.859**</td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>0.057</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.200</td>
</tr>
<tr>
<td>N</td>
<td>180</td>
<td>180</td>
<td>180</td>
<td>180</td>
<td>180</td>
<td>180</td>
</tr>
<tr>
<td>HDL mg/dl</td>
<td>Pearson correlation</td>
<td>0.715**</td>
<td>-0.052</td>
<td>-0.277**</td>
<td>1</td>
<td>-0.156</td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>0.000</td>
<td>0.491</td>
<td>0.000</td>
<td>0.037</td>
<td>0.000</td>
</tr>
<tr>
<td>N</td>
<td>180</td>
<td>180</td>
<td>180</td>
<td>180</td>
<td>180</td>
<td>180</td>
</tr>
<tr>
<td>LDL mg/dl</td>
<td>Pearson correlation</td>
<td>-0.121</td>
<td>0.520**</td>
<td>0.859**</td>
<td>-0.156</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>0.105</td>
<td>0.000</td>
<td>0.000</td>
<td>0.037</td>
<td>0.208</td>
</tr>
<tr>
<td>N</td>
<td>180</td>
<td>180</td>
<td>180</td>
<td>180</td>
<td>180</td>
<td>180</td>
</tr>
<tr>
<td>ucOC ng/ml</td>
<td>Pearson correlation</td>
<td>0.859**</td>
<td>-0.213**</td>
<td>-0.096</td>
<td>0.531**</td>
<td>-0.094</td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>0.000</td>
<td>0.004</td>
<td>0.200</td>
<td>0.000</td>
<td>0.208</td>
</tr>
<tr>
<td>N</td>
<td>180</td>
<td>180</td>
<td>180</td>
<td>180</td>
<td>180</td>
<td>180</td>
</tr>
</tbody>
</table>

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

**Table 4.4**

4.5. Fasting insulin, FPG:

The mean of fasting plasma insulin (FI) concentration was found statistically significantly higher in the diabetic group (20 ± 6 ml U/L) than in control group (9 ± 2 ml U/L) (P = 0.000). (Table 4.4). This fact persisted when both genders in the diabetic group were compared
to their respective controls. The mean plasma concentration of insulin was significantly higher in diabetic females (81 ± 20 ml U/L) and diabetic males (19 ± 6 ml U/L) compared to control females (10 ± 3 ml U/L) and control males (9 ± 2 ml U/L) respectively (P = 0.0001 for both sexes). (Table 4.10).

The mean of fasting blood glucose (FBG) concentration was found statistically significantly higher in the diabetic group (195 ± 59 mg/dl) than in control group (85 ± 25 mg/dl) (P = 0.000). (Table 4.4). This fact persisted when both genders in the diabetic group were compared to their respective controls. The mean plasma concentration of insulin was significantly higher in diabetic females (201 ± 63 mg/dl) and diabetic males (182 ± 45 ml U/L) compared to control females (87 ± 30 ml U/L) and control males (82 ± 14 ml U/L) respectively (P = 0.000 for both sex). (Table 4.10).

**Table 4.10**

**Mean concentrations of FI and FBG in the diabetic and the control by sex:**

<table>
<thead>
<tr>
<th>Sex</th>
<th>Variable</th>
<th>Status</th>
<th>NO.</th>
<th>Mean</th>
<th>STD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>male</td>
<td>FI ml U/L</td>
<td>Control</td>
<td>26</td>
<td>9</td>
<td>2</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diabetic</td>
<td>34</td>
<td>19</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FPG mg/dl</td>
<td>Control</td>
<td>26</td>
<td>82</td>
<td>14</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diabetic</td>
<td>34</td>
<td>182</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>female</td>
<td>FI ml U/L</td>
<td>Control</td>
<td>39</td>
<td>10</td>
<td>3</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>81</td>
<td>20</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FPG mg/dl</td>
<td>Control</td>
<td>39</td>
<td>87</td>
<td>30</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diabetic</td>
<td>81</td>
<td>201</td>
<td>63</td>
<td></td>
</tr>
</tbody>
</table>
4.5.1. Prediction of FI using sex, age, BMI, TOC, ucOC, FBG, TCHOL, LDL, HDL and TG:

Standard multiple regression was run to determine the prediction magnitude of plasma fasting insulin (FI) by sex, age, BMI, TOC, ucOC, FBG, TCHOL, HDL, LDL and TG in newly diagnosed type II diabetes. The overall model including sex, age, BMI, TOC, ucOC, FBG, TCHOL, HDL, LDL and TG was statistically highly significant (P = 0.000), with R = 0.809, R² = 0.654 and adjusted R² = 0.634. (Tables 4.11).

Table 4.11

Values of R, R² and adjusted R² in the model predicting FI:

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>R</th>
<th>R²</th>
<th>Adjusted R²</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FI</td>
<td>0.809</td>
<td>0.654</td>
<td>0.634</td>
<td>0.000</td>
</tr>
</tbody>
</table>

The overall model showed statistically highly significant negative correlation between plasma insulin and ucOC (P = 0.000). Also there was significant a negative correlation between plasma insulin and TG (P = 0.045). There was a negative correlation which is not significant between FI and TOC, age and LDL as shown in the coefficient table. (Table 4.10). Similarly the correlation between FI and TCHOL showed positive statistically significant correlation (P = 0.019). The correlations of other predictors with FI were positive, but all of them were not statistically significant as shown in (Table 4.12).
Table 4.12

Standardized (β) and unstandardized (B) coefficients for different predictors of plasma fasting insulin (FI)

<table>
<thead>
<tr>
<th>Predictor</th>
<th>B</th>
<th>β</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>21.195</td>
<td>-</td>
<td>0.000</td>
</tr>
<tr>
<td>ucOC ng/ml</td>
<td>-4.836</td>
<td>-0.617</td>
<td>0.000</td>
</tr>
<tr>
<td>TOC ng/ml</td>
<td>-0.099</td>
<td>-0.087</td>
<td>0.494</td>
</tr>
<tr>
<td>FBG mg/dl</td>
<td>0.012</td>
<td>0.007</td>
<td>0.091</td>
</tr>
<tr>
<td>Sex</td>
<td>0.190</td>
<td>0.013</td>
<td>0.789</td>
</tr>
<tr>
<td>Age years</td>
<td>-0.003</td>
<td>0.008</td>
<td>0.884</td>
</tr>
<tr>
<td>BMI kg/m²</td>
<td>0.069</td>
<td>0.046</td>
<td>0.365</td>
</tr>
<tr>
<td>TCHOL mg/dl</td>
<td>0.007</td>
<td>0.013</td>
<td>0.019</td>
</tr>
<tr>
<td>TG mg/dl</td>
<td>-0.014</td>
<td>0.015</td>
<td>0.045</td>
</tr>
<tr>
<td>LDL mg/dl</td>
<td>-0.012</td>
<td>-0.061</td>
<td>0.848</td>
</tr>
<tr>
<td>HDL mg/dl</td>
<td>0.011</td>
<td>-0.022</td>
<td>0.100</td>
</tr>
</tbody>
</table>

Model: included constant, TOC, ucOC, FBG, sex, age, BMI, TCHOL, HDL, LDL and TG
4.5.2. Prediction of FBG using sex, age, BMI, TOC, ucOC, FI, TCHOL, LDL, HDL and TG:

Standard multiple regression was run to determine the prediction magnitude of fasting blood glucose (FBG) by sex, age, BMI, TOC, ucOC, FI, FBG, TCHOL, HDL, LDL and TG in newly diagnosed type II diabetes. The overall model including sex, age, BMI, TOC, ucOC, FI, FBG, TCHOL, HDL, LDL and TG was statistically highly significant (P = 0.000), with R = 0.777, R² = 0.604 and adjusted R² = 0.581. (Tables 4.13).

Table 4.13:

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>R</th>
<th>R²</th>
<th>Adjusted R²</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBG</td>
<td>0.777</td>
<td>0.604</td>
<td>0.581</td>
<td>0.000</td>
</tr>
</tbody>
</table>

The overall model showed a statistically highly significant negative correlation between TOC and fasting blood glucose concentration (P = 0.000). Similarly, the correlation between FBG and ucOC, age, BMI, TCHOL, and HDL showed a negative correlation that is not significant as shown in (Table 4.14). The correlations of other predictors with FBG were positive, but all of them were not statistically significant as shown in (Table 4.14).
Table 4.1:

Standardized (β) and unstandardized (B) coefficients for different predictors of plasma undercarboxylated osteocalcin

<table>
<thead>
<tr>
<th>Predictor</th>
<th>B</th>
<th>β</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>4.091</td>
<td>-</td>
<td>0.000</td>
</tr>
<tr>
<td>FI ml U/L</td>
<td>-0.055</td>
<td>-0.430</td>
<td>0.000</td>
</tr>
<tr>
<td>FBG mg/dl</td>
<td>-0.001</td>
<td>-0.111</td>
<td>0.056</td>
</tr>
<tr>
<td>Sex</td>
<td>0.041</td>
<td>0.021</td>
<td>0.584</td>
</tr>
<tr>
<td>Age years</td>
<td>0.001</td>
<td>0.002</td>
<td>0.751</td>
</tr>
<tr>
<td>BMI kg/m²</td>
<td>0.008</td>
<td>0.043</td>
<td>0.309</td>
</tr>
<tr>
<td>TCHOL mg/dl</td>
<td>-0.001</td>
<td>-0.040</td>
<td>0.402</td>
</tr>
<tr>
<td>HDL mg/dl</td>
<td>0.004</td>
<td>-0.036</td>
<td>0.522</td>
</tr>
<tr>
<td>LDL mg/dl</td>
<td>0.002</td>
<td>0.028</td>
<td>0.752</td>
</tr>
<tr>
<td>TG mg/dl</td>
<td>0.00</td>
<td>0.017</td>
<td>0.833</td>
</tr>
</tbody>
</table>

Model: included constant, sex, age, FI, FBG, BMI, TCHOL, HDL, LDL and TG
5.1. Discussion

This study was designed to investigate the level of TOC, ucOC in diabetic patients compared to the healthy control and correlate it with the different metabolic changes in diabetes mellitus. Osteocalcin may have a role in the regulation of systemic energy metabolism, given the common origin of the osteoblasts with the two other cells implicated (adipocytes and muscle cells). Bioactivity of circulating human carboxylated and uncarboxylated osteocalcin should be characterized in-depth, especially in those patients with increased concentrations (renal failure). Osteocalcin is one of the clues in the interaction between calcium and glucose metabolism.

Following the previous studies (156-162) our study has shown that TOC was significantly less in newly diagnosed type II diabetic patients compared to control. The elevation in serum osteocalcin in the elder subjects, in both sexes that was found in our study, confirms earlier reports (228-232), although this effect is not seen in all studies (233).

Interestingly, the increase in bone and serum osteocalcin occurred at the period when trabecular bone loss or postmenopausal osteoporosis is accelerated in women (234, 235). Lian and Gundberg (236), reported that the bone osteocalcin content was higher in young children and bone osteocalcin concentrations appeared slightly higher in males at all ages. In our study, a significant serum osteocalcin increase in both sexes with aging was observed.

Although the mean age of diabetics is higher than the control in this study (table4.1) and has been shown that the elderly have higher osteocalcin levels (229), and it increased with age as shown in this study still osteocalcin level in diabetics is lower than in the control (fig. 4.2). The increased osteocalcin in the elderly could be a compensatory mechanism or due to decreased renal clearance. Which need further investigations, but why should it decrease in diabetics might be explained by the suggestion that hyperglycaemia suppresses osteoblast function and thereby decrease production and secretion of osteocalcin. It is also suggested that increased glucose metabolism may intoxicate osteoblasts.
The moderate negative correlation that is statistically significant between plasma TOC concentration and Fl, FBG level shown in this study these observations were consistent with previous reports that showed an inverse association of total serum osteocalcin with glucose and visceral fat mass, parameters of insulin secretion and its sensitivity in patients with type 2 diabetes (237).

Concerning BMI osteocalcin shown no significant correlation with BMI (table 4.5), and that our findings were inconsistent with many previous studies, as several studies have shown that osteocalcin is associated with lipid metabolism (238) and metabolic syndrome (239, 240). Moreover, it is reported that osteocalcin concentrations in obese people are lower than normal controls (241). In some studies, a negative and inversely relationship between osteocalcin with body mass index (BMI) and waist circumference has been shown (242-244) and in the others demonstrated a positive association between osteocalcin with BMI (245, 246).

Regarding the undercarboxylated osteocalcin, some recent studies have shown that serum ucOC is related to T2DM in different racial groups (247, 248) and that serum ucOC was inversely correlated with adiposity, blood glucose, insulin resistance, and triglycerides. Consistent with these results, we found that the serum ucOC concentrations were significantly lower in diabetics than in controls. The plasma undercarboxylated osteocalcin concentration was low in diabetics compared to controls in all age groups, these differences were statistically significant in all age groups. Comparing each age group against the others within controls or diabetics led to differences that were not statistically significant in both cases.

Notably, our results showed that there was a moderate negative correlation that was statistically significant between plasma ucOC level and fasting plasma insulin.

Alfadda AA, (249) reported that there was no correlation between plasma ucOC concentration and BMI. Our study revealed that there was a significant correlation between plasma ucOC level and BMI. There is a paucity of published data on undercarboxylated osteocalcin levels in humans and their possible changes in diabetes, so the estimated number might be too low to reveal the trend.

Our findings that decreased osteocalcin levels were associated with abnormal lipid profiles were in line with a recent animal study reported a beneficial effect of osteocalcin on fat mass and serum triglyceride concentration (250). When grouping was based on BMI, also there
was not statistically significant difference obtained. The differences in mean plasma concentrations of TG were not statistically significant, if the BMI groups were compared within controls or diabetics separately or if the groups were compared as diabetics versus controls.

The interesting observation was that the mean TG concentration decreased progressively from underweight subjects towards obese subjects in both diabetic and control subgroups. A correlation between TOC concentration and TG showed a weak positive correlation that is statistically non-significant \( p = 0.674 \). The same test was done to assess the correlation between ucOC and TG, it revealed a weak positive correlation that was statistically non-significant \( p = 0.426 \). In contrast to our findings, a few other reports also showed a negative association between osteocalcin levels and triglycerides in blacks and non-Hispanic whites (174) and in older men (157).

The mean HDL plasma concentrations in the subgroups of BMI of control subjects showed nearly inverted U-shaped, but in the diabetic subgroups, these concentrations increased progressively from underweight subjects toward obese one to reach its maximum. When the diabetic subgroups were compared versus their respective control one, the differences were statistically significant in normal-weight \( (p = 0.000) \), overweight \( (p = 0.000) \), obese \( (p = 0.034) \).

A correlation between TOC concentration and HDL showed a weak negative correlation that was statistically significant \( p = 0.050 \). The same test was done to assess the correlation between ucOC and HDL, it revealed a weak negative correlation that was statistically non-significant \( p = 0.062 \). In previous studies, HDL cholesterol was found to be negatively (156), positively (173) or not associated at all (174) with osteocalcin level in different populations.

The pattern of LDL means in both control and diabetic were nearly similar. The highest values were noticed in underweight and the lowest was observed in obese subgroups, while normal weight and overweight had nearly similar values in both groups. When the subgroups of diabetics were compared against their respective controls, the mean LDL concentrations were high in diabetic BMI subgroups, but these differences were not statistically significant in underweight \( (p = 0.836) \), in normal-weight \( (p= 0.325) \), in overweight \( (p = 0.161) \), and the obese \( (p = 0.369) \).
There was a strong positive correlation between TOC and HDL. That was statistically significant $p = 0.000$. Similar to our findings, a recent study in the Chinese male population indicated that osteocalcin was positively correlated with HDL-C (244).

Our findings revealed that the mean of fasting plasma insulin (FI) concentration was statistically significantly high in the diabetic group compared to the control group. The mean plasma concentration of insulin was significantly high in diabetic females and diabetic males compared to control females and control males. These results were supported by (154, 169, 210-214). Our results showed a statistically highly significant negative correlation between plasma insulin ucOC. Also, there was a significant negative correlation between plasma insulin TG. There was a negative correlation that was not significant between FI and TOC, age and LDL. Similarly, the correlation between FI and TCHOL showed positive statistically significant. The correlations of other predictors with FI were positive, but all of them were not statistically significant. Our results are similar to those reported by (251-255) and contraindicated to those reported by (256, 257).

Our findings showed a statistically highly significant negative correlation between TOC and fasting blood glucose concentration. Similarly, the correlation between FBG and ucOC, age, BMI, TCHOL, and HDL showed a negative correlation that was not significant. The correlations of other predictors with FBG were positive, but all of them were not statistically significant. Our findings are similar to those reported by (258-260).

5.2. Conclusion:

By estimating the circulating levels of TOC, ucOC, insulin and, FBS in patients with type II diabetes, we found that the TOC and ucOC levels of patients were lower than those of the healthy control subjects. Fasting serum insulin was higher in patients compared to healthy control subjects. TOC and ucOC negatively correlate with Fasting serum insulin and FBS, therefore, TOC and ucOC may play an important role in regulating blood glucose and improving insulin secretion and insulin sensitivity. So, bones are not just inert calcified structure, but it may play an important role in controlling obesity, energy, and sugar metabolism.
5.3. Recommendations:

1. Future researches that take into account the effects of osteocalcin in bone turnover, bone density and atherosclerosis were needed.

2. Experimental research at the tissue and cellular level were needed to examine the effects of total osteocalcin and undercarboxylated osteocalcin on cellular glucose transport, and insulin responses to various stimuli.
6.1. References


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Appendix:

Participant consent

أنا آمل أن أقرأ أهداف هذا البحث ومخاطر الناتجة عن أخذ عينة دم

الاسم .............................................
الأمضاء ............................................
التاريخ ............................................

101
Questionnaire form:

1. Sample ID: ……………………………
2. Name: ………………………………..
3. Gender: ♂ [ ] ♀ [ ]
4. Age: …………………………………..
5. Weight: ………………………………
6. Height: ………………………………
7. Tribe: ………………………………
8. Mobile: ………………………………
9. Treatment received to treat DM:
   1. ……………………………………
   2. ……………………………………
   3. ……………………………………
10. Do you medicate by one or more of these drugs:
    Warfarin [ ] Heparin [ ]
    Drugs for osteoporosis [ ] 1,25- dihydroxyvitamin D3 [ ]
    Glucocorticoids [ ] anticonvulsant drugs [ ]
    Others [ ]
    …………………………………………………………………………………………………………………
    …………………………………………………………………………………………………………………
11. Are you suffering from one of these diseases:
    Multiple myeloma [ ] Malignancy [ ]
    Hyperthyroidism [ ] Fracture (up to one year) [ ]
    Osteomalacia [ ] Paget`s disease [ ]
    Hypertension [ ] cardiovascular disease [ ]
Other diseases:

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Human total osteocalcin (OT) ELISA Kit
96 / 48 Tests
Catalogue Number:SL1328Hu
Store all reagents at 2-8°C
Validity Period: six months

For samples:
In Human serum, blood plasma, and other biological fluids.

FOR RESEARCH USE ONLY!
NOT FOR THERAPEUTIC OR DIAGNOSTIC APPLICATIONS!
PLEASE READ THROUGH ENTIRE PROCEDURE BEFORE BEGINNING
Human total osteocalcin (OT) ELISA Kit

FOR RESEARCH USE ONLY

Drug Names

Generic Name: Human total osteocalcin (OT) ELISA Kit

Purpose

Our Human total osteocalcin (OT) ELISA kit is to assay OT levels in Human serum, plasma, culture media or any biological fluid.

Principle

This ELISA kit uses Sandwich-ELISA as the method. The Microtiter stripplate provided in this kit has been pre-coated with an antibody specific to OT. Standards or samples are added to the appropriate Microtiter stripplate wells and combined with the specific antibody. Then a Horseradish Peroxidase (HRP) conjugated antibody specific for OT is added to each Microtiter stripplate well and incubated. Free components are washed away. The TMB substrate solution is added to each well. Only those wells that contain OT and HRP conjugated OT antibody will appear blue in color and then turn yellow after the addition of the stop solution. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm. The OD value is proportional to the concentration of OT. You can calculate the concentration of OT in the samples by comparing the OD of the samples to the standard curve.

Materials provided with the kit
23 pg/ml and 12.5 pg/ml, respectively.

2. In the Microtiter stripplate, leave a well empty as blank control. In sample wells, 40 μl Sample dilution buffer and 10 μl sample are added (dilution factor is 3). Samples should be loaded onto the bottom without touching the well wall. Mix well with gentle shaking.

3. Incubation: Incubate 30 min at 37°C after sealed with Closure plate membrane.

4. Dilution: dilute the concentrated washing buffer with distilled water (20 times for 96T and 20 times for 48T).

5. Washing: carefully peel off Closure plate membrane, aspirate and refill with the wash solution. Discard the wash solution after waiting for 30 seconds. Repeat the washing procedure for 5 times.

6. Add 50 μl HRP-Conjugate reagent to each well except the blank control well.

7. Incubation as described in Step 3.

8. Washing as described in Step 5.

9. Coloring: Add 50 μl Chromogen Solution A and 50 μl Chromogen Solution B to each well, mix with gently shaking and incubate at 37°C for 15 minutes. Please avoid light during coloring.

10. Termination: Add 50 μl stop solution to each well to terminate the reaction. The color in the well should change from blue to yellow.

11. Read absorbance O.D. at 450nm using a Microtiter Plate Reader. The OD value of the blank control well is set as zero. Assay should be carried out within 15 minutes after adding stop solution.
Notes:

1. Store the kit at 4°C upon receipt. The kit should be equilibrated to room temperature before the assay. Remove any unused strips from Human OT Antibody-Coated plate, reseal them in zip-lock foil and keep at 4°C.

2. Precipitates may appear in concentrated washing buffer. Please heat the buffer to dissolve all the precipitates, which will not affect the results.

3. Accurate pipette should be used to avoid experimental error. Samples should be added to the Microplate in less than 5 minutes. If a large number of samples are included, multiple channel pipette is recommended.

4. Standard curve should be included in every assay. Replicate wells are recommended. If the OD value of the sample is greater than the first well of standards, please dilute the sample (in times) before test. When calculating the original OT concentration, please multiply the total dilution factor (OxX5).

5. In order to avoid cross-contamination, Microplate seals are for one-time use only.

6. Please keep Substrate away from light.

7. All the operation should be accordance with the manufacturer's instructions strictly. The results determined by the Microplate Reader.

8. All the samples, washing buffer and waste should be treated as infectious agents.

9. Reagents from different lots should not be mixed.

Calculation of Results
Precision

Intra-assay Precision (Precision within an assay): 3 samples with low, middle and high level
Human OT were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 samples with low, middle and high level
Human OT were tested on 3 different plates, 8 replicates in each plate.

$CV(\%) = \frac{SD}{mean} \times 100$

Intra-Assay: CV<10%
Inter-Assay: CV<12%

Assay range

3 pg/ml - 160 pg/ml

Sensitivity:

0.1 pg/ml

Storage and validity
1. Storage
2. Duration: 6 months
Human Undercarboxylated Osteocalcin (UcOC) ELISA Kit

96 / 48 Tests
Catalogue Number: SL2404Hu

Store all reagents at 2-8°C
Validity Period: six months

For samples:
In Human serum, blood plasma, and other biological fluids.

FOR RESEARCH USE ONLY!
NOT FOR THERAPEUTIC OR DIAGNOSTIC APPLICATIONS!
PLEASE READ THROUGH ENTIRE PROCEDURE BEFORE BEGINNING
Human Undercarboxylated Osteocalcin (UcOC) ELISA Kit

FOR RESEARCH USE ONLY

Drug Names

Generic Name: Human Undercarboxylated Osteocalcin (UcOC) ELISA Kit

Purpose

Our Human Undercarboxylated Osteocalcin (UcOC) ELISA kit is to assay UcOC levels in human serum, plasma, culture media or any biological fluid.

Principle

This ELISA kit uses Sandwich-ELISA as the method. The Microelisa stripplate provided in this kit has been pre-coated with an antibody specific to UcOC. Standards or samples are added to the appropriate Microelisa stripplate wells and combined with the specific antibody. Then a Horseradish Peroxidase (HRP)- conjugated antibody specific for UcOC is added to each Microelisa stripplate well and incubated. Free components are washed away. The TMB substrate solution is added to each well. Only those wells that contain UcOC and HRP conjugated UcOC antibody will appear blue in color and then turn yellow after the addition of the stop solution. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm. The OD value is proportional to the concentration of UcOC. You can calculate the concentration of UcOC in the samples by comparing the OD of the samples to the standard curve.
Materials provided with the kit

<table>
<thead>
<tr>
<th>Materials provided with the kit</th>
<th>96 determinations</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 User manual</td>
<td>1</td>
<td>RT</td>
</tr>
<tr>
<td>2 Closure plain membrane</td>
<td>2</td>
<td>RT</td>
</tr>
<tr>
<td>3 Sealed bags</td>
<td>1</td>
<td>RT</td>
</tr>
<tr>
<td>4 Microtitre stripplate</td>
<td>1</td>
<td>2-8°C</td>
</tr>
<tr>
<td>5 Standard: 2750pg/ml</td>
<td>0.3ml×1 bottle</td>
<td>2-8°C</td>
</tr>
<tr>
<td>6 Standard diluent</td>
<td>1.5ml×1 bottle</td>
<td>2-8°C</td>
</tr>
<tr>
<td>7 HRP- conjugate reagent</td>
<td>6ml×1 bottle</td>
<td>2-8°C</td>
</tr>
<tr>
<td>8 Sample diluent</td>
<td>6ml×1 bottle</td>
<td>2-8°C</td>
</tr>
<tr>
<td>9 Chromogen Solution A</td>
<td>6ml×1 bottle</td>
<td>2-8°C</td>
</tr>
<tr>
<td>10 Chromogen Solution B</td>
<td>6ml×1 bottle</td>
<td>2-8°C</td>
</tr>
<tr>
<td>11 Stop Solution</td>
<td>4ml×1 bottle</td>
<td>2-8°C</td>
</tr>
<tr>
<td>12 wash solution</td>
<td>20ml×1 bottle</td>
<td>2-8°C</td>
</tr>
</tbody>
</table>

Sample preparation

1. Specimen preparation

   After collection of the whole blood, allow the blood to clot by leaving it undisturbed at room temperature. This usually takes 10-20 minutes. Remove the clot by centrifuging at 2,000-3,000 rpm for 20 minutes. If precipitates appear during rehydration, the sample should be centrifuged again.

2. Plasma preparation

   Collect the whole blood into tubes with anticoagulant (EDTA or citrate). After incubated at room temperature for 10-20 minutes, tubes are centrifuged at 2,000-3,000 rpm. Collect the supernatant carefully as plasma samples. If precipitates appear during rehydration, the sample should be centrifuged again.

3. Urine samples

   Collect urine into aseptic tubes. Collect the supernatant carefully after centrifuging for 30 min at 2,000-3,000 rpm. If precipitates appear during rehydration, the sample should be centrifuged again. The preparation procedure of cerebrospinal fluid and pleural fluid is the same as that of urine sample.

4. Cell samples

   If you want to detect the sequestration of cells, collect culture supernatant into aseptic tubes.
Collect the supernatant carefully after centrifuging for 20 min at 2,000-3,000 rpm. If you want

to detect intracellular components, dilute the cells to 1X10^6/ml with PBS (pH 7.2-7.4). The
cells were destroyed to release intracellular components by repeated freezing and thawing.
Collect the supernatant carefully after centrifuging for 20 min at 2,000-3,000 rpm. If
precipitates appear during resolution, the sample should be centrifuged again.

5. Tissue samples

Tissue samples are cut, weighed, frozen in liquid nitrogen and stored at -80°C for future
use. The tissue samples were homogenized after adding PBS (pH 7.4). Samples should be
operated at 4°C. Collect the supernatant carefully after centrifuging for 20 min at 2,000-3,000
rpm. Aliquot the supernatant for ELISA assay and future use.

Notes:

1. Sample extraction and ELISA assay should be performed as soon as possible after sample
collection. The samples should be extracted according to the relevant literature. If ELISA
assay cannot be performed immediately, samples can be stored at -20°C. Repeated
freeze-thaw cycles should be avoided.

2. Our kits can not be used for samples with NaN3 which can inhibit the activity of ERP.

Procedure

1. Dilution of Standards

Ten wells are set for standards in a Microplate strip. In Well 1 and Well 2, 100μl
Standard solution and 50μl Standard Dilution buffer are added and mixed well. In Well 3
and Well 4, 100μl solution from Well 1 and Well 2 are added respectively. Then 50μl
Standard Dilution buffer are added and mixed well. 50μl solution is discarded from Well 5
and Well 4. In Well 5 and Well 6, 50μl solution from Well 3 and Well 4 are added
respectively. Then 50μl Standard Dilution buffer are added and mixed well. In Well 7 and
Well 8, 50μl solution from Well 5 and Well 6 are added respectively. Then 50μl Standard
Dilution buffer are added and mixed well. In Well 9 and Well 10, 50μl solution from Well
7 and Well 8 are added respectively. Then 50μl Standard Dilution buffer are added and
mixed well. 50μl solution is discarded from Well 9 and Well 10. After dilution, the total
volumes in all the wells are 50µl and the concentrations are 1800 pg/ml, 1200 pg/ml, 600 pg/ml, 300 pg/ml and 150 pg/ml, respectively.

2. In the Microtiter strip plate, leave a well empty as blank control. In sample wells, 40µl Sample dilution buffer and 10µl sample are added (dilution factor is 5). Samples should be loaded onto the bottom without touching the well wall. Mix well with gentle shaking.

3. Incubate: Incubate 30 min at 37°C after sealed with Closure plate membrane.

4. Dilution: Add the concentrated washing buffer with distilled water (50 times for 96T and 20 times for 48T).

5. Washing: Carefully peel off Closure plate membrane, aspirate and refill with the wash solution. Discard the wash solution after resting for 30 seconds. Repeat the washing procedure for 5 times.

6. Add 50 µl HRP- Conjugate reagent to each well except the blank control well.

7. Incubation: as described in Step 5.

8. Washing as described in Step 5.

9. Coloring: Add 50 µl Chromogen Solution A and 50 µl Chromogen Solution B to each well, mix with gently shaking and incubate at 37°C for 15 minutes. Please avoid light during coloring.

10. Termination: Add 50 µl stop solution to each well to terminate the reaction. The color in the well should change from blue to yellow.

11. Read absorbance O.D. at 450nm using a Microtiter Plate Reader. The OD value of the blank control well is set as zero. Assay should be carried out within 15 minutes after
adding step solution.

Notes:

1. Store the kit at 4°C upon receipt. The kit should be equilibrated to room temperature before the assay. Remove any unneeded strips from Human uOOC Antibody-Coated plate, reseal them in zip-lock foil and keep at 4°C.

2. Precipitates may appear in concentrated washing buffer. Please heat the buffer to dissolve all the precipitates, which will not affect the results.

3. Accurate pipette should be used to avoid experimental error. Samples should be added to the Microplate in less than 5 minutes. If a large number of samples are included, multiple channel pipette is recommended.

4. Standard curve should be included in every assay. Replicate wells are recommended. If the OD values of the sample is greater than the first well of standards, please dilute the sample (n times) before test. When calculating the original uOOC concentration, please multiply the total dilution factor (n×5).

5. In order to avoid cross-contamination, Microplate readers are for one-time use only.

6. Please keep substrate away from light.

7. All the operation should be accordance with the manufacturer's instructions strictly. The results determined by the Micronetix Plate Reader.

8. All the samples, washing buffer and wastes should be treated as infectious agents.

9. Reagents from different lots should not be mixed.
Calculation of Results

Known concentrations of Human UcOC Standard and its corresponding reading OD is plotted on the log scale (x-axis) and the log scale (y-axis) respectively. The concentration of Human UcOC in sample is determined by plotting the sample's O.D. on the Y-axis. The original concentration is calculated by multiplying the dilution factor.

Precision

Intra-assay Precision (Precision within an assay): 3 samples with low, middle and high level Human UcOC were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 samples with low, middle and high level Human UcOC were tested on 3 different plates, 8 replicates in each plate.

CV(%) = SD/mean x 100

Intra-Assay: CV<10%

Inter-Assay: CV<12.5%

Assay range

56pg/ml - 2000 pg/ml

Sensitivity:

18pg/ml
Storage and validity

1. Storage: 2-8°C.
2. Duration: 6 months
Human Insulin(INS) ELISA Kit

96 / 48 Tests
Catalogue Number: SL0933Hu
Store all reagents at 2-8°C
Validity Period: six months

For samples:
In Human serum, blood plasma, and other biological fluids.

FOR RESEARCH USE ONLY!
NOT FOR THERAPEUTIC OR DIAGNOSTIC APPLICATIONS!
PLEASE READ THROUGH ENTIRE PROCEDURE BEFORE BEGINNING
**Human Insulin(INS) ELISA Kit**

**FOR RESEARCH USE ONLY**

**Drug Names**
Generic Name: Human Insulin(INS) ELISA Kit

**Purpose**

Our human Insulin(INS) ELISA kit is to assay INS levels in serum, plasma, culture media or any biological fluid.

**Principle**

This ELISA kit uses Sandwich-ELISA as the method. The Microelisa stripplate provided in this kit has been pre-coated with an antibody specific to INS. Standards or samples are added to the appropriate Microelisa stripplate wells and combined to the specific antibody. Then a Horseradish Peroxidase (HRP)- conjugated antibody specific for INS is added to each Microelisa stripplate well and incubated. Free components are washed away. The TMB substrate solution is added to each well. Only those wells that contain INS and HRP conjugated INS antibody will appear blue in color and then turn yellow after the addition of the stop solution. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm. The OD value is proportional to the concentration of INS. You can calculate the concentration of INS in the samples by comparing the OD of the samples to the standard curve.

**Materials provided with the kit**

<table>
<thead>
<tr>
<th>Materials provided with the kit</th>
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</tr>
</thead>
<tbody>
<tr>
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<td>R.T.</td>
</tr>
<tr>
<td>2 Closure plate membrane</td>
<td>2</td>
<td>R.T.</td>
</tr>
<tr>
<td>3 Sealed bags</td>
<td>1</td>
<td>R.T.</td>
</tr>
<tr>
<td>4 Microelisa stripplate</td>
<td>1</td>
<td>2-8°C</td>
</tr>
</tbody>
</table>
Sample preparation

1. Serum preparation

After collection of the whole blood, allow the blood to clot by leaving it undisturbed at room temperature. This usually takes 10-20 minutes. Remove the clot by centrifuging at 2,000-3,000 rpm for 20 minutes. If precipitates appear during reservation, the sample should be centrifugated again.

2. Plasma preparation

Collect the whole blood into tubes with anticoagulant (EDTA or citrate). After incubated at room temperature for 10-20 minutes, tubes are centrifugated for 20 min at 2,000-3,000 rpm. Collect the supernatant carefully as plasma samples. If precipitates appear during reservation, the sample should be centrifugated again.

3. Urine samples

Collect urine into aseptic tubes. Collect the supernatant carefully after centrifuging for 20 min at 2,000-3,000 rpm. If precipitates appear during reservation, the sample should be centrifugated again. The preparation procedure of cerebrospinal fluid and pleuroperitoneal fluid is the same as that of urine sample.

4. Cell samples

If you want to detect the secretions of cells, collect culture supernatant into aseptic tubes. Collect the supernatant carefully after centrifuging for 20 min at 2,000-3,000 rpm. If you want to detect intracellular components, dilute the cells to 1X10^6/ml with PBS (pH 7.2-7.4). The cells were destroyed to release intracellular components by repeated freezing and thawing. Collect the supernatant carefully after centrifuging for 20 min at 2,000-3,000 rpm. If precipitates appear during reservation, the sample should be centrifugated again.
5. Tissue samples

Tissue samples are cut, weighed, frozen in liquid nitrogen and stored at -80°C for future use. The tissue samples were homogenized after adding PBS (pH 7.4). Samples should be operated at 4°C. Collect the supernatant carefully after centrifuging for 20 min at 2,000-3,000 rpm. Aliquot the supernatant for ELISA assay and future use.

Notes:

1. Sample extraction and ELISA assay should be performed as soon as possible after sample collection. The samples should be extracted according to the relevant literature. If ELISA assay can not be performed immediately, samples can be stored at -20°C. Repeated freeze-thaw cycles should be avoided.

2. Our kits can not be used for samples with NaN3 which can inhibit the activity of HRP.

Assay procedure

Procedure

1. Dilution of Standards

Take five tubes, marked No. 1, 2, 3, 4, 5. Add 110 μl of standard diluent buffer to each tube. Add 220 μl of Standard Solution to No. 1 and mix thoroughly, add 220 μl from No. 1 to No. 2, when the No. 2 mix thoroughly, discard 110 μl solution of No. 2 then add 110 μl to No. 3, mix thoroughly, add 110 μl from No. 3 to No. 4 and mix thoroughly, transfer 110 μl from No. 4 to No. 5, mix thoroughly and discard 110 μl solution of No. 5. Now each tube has 110 μl Standard Solution, and the concentrations are 18 mU/L, 12 mU/L, 6 mU/L, 3 mU/L, and 1.5 mU/L, respectively. Add 50 μl Standard Solution to two wells from each tube. Five different concentrations ten wells, each well has 50 μl Standard Solution.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Standard No.</th>
<th>Diluent</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 mU/L</td>
<td>Standard No.1</td>
<td>220 μl</td>
<td>220 μl Original + 110 μl Standard diluent</td>
</tr>
<tr>
<td>12 mU/L</td>
<td>Standard No.2</td>
<td>220 μl</td>
<td>220 μl Standard No.1 + 110 μl Standard diluent</td>
</tr>
<tr>
<td>6  mU/L</td>
<td>Standard No.3</td>
<td>110 μl</td>
<td>110 μl Standard No.2 + 110 μl Standard diluent</td>
</tr>
<tr>
<td>3  mU/L</td>
<td>Standard No.4</td>
<td>110 μl</td>
<td>110 μl Standard No.3 + 110 μl Standard diluent</td>
</tr>
<tr>
<td>1.5 mU/L</td>
<td>Standard No.5</td>
<td>110 μl</td>
<td>110 μl Standard No.4 + 110 μl Standard diluent</td>
</tr>
</tbody>
</table>
2. In the Microelisa stripplate, leave a well empty as blank control. In sample wells, 40µl Sample dilution buffer and 10µl sample are added (dilution factor is 5). Samples should be loaded onto the bottom without touching the well wall. Mix well with gentle shaking.

3. Incubation: incubate 30 min at 37°C after sealed with Closure plate membrane.

4. Dilution: dilute the concentrated washing buffer with distilled water (30 times for 96T and 20 times for 48T).

5. Washing: carefully peel off Closure plate membrane, aspirate and refill with the wash solution. Discard the wash solution after resting for 30 seconds. Repeat the washing procedure for 5 times.

6. Add 50 µl HRP-Conjugate reagent to each well except the blank control well.

7. Incubation as described in Step 3.

8. Washing as described in Step 5.

9. Coloring: Add 50 µl Chromogen Solution A and 50 µl Chromogen Solution B to each well, mix with gently shaking and incubate at 37°C for 15 minutes. Please avoid light during coloring.

10. Termination: add 50 µl stop solution to each well to terminate the reaction. The color in the well should change from blue to yellow.

11. Read absorbance O.D. at 450nm using a Microtiter Plate Reader. The OD value of the blank control well is set as zero. Assay should be carried out within 15 minutes after adding stop solution.
Notes:

1. Store the kit at 4°C upon receipt. The kit should be equilibrated to room temperature before the assay. Remove any unneeded strips from Human INS Antibody-Coated plate, reseal them in zip-lock foil and keep at 4°C.

2. Precipitates may appear in concentrated washing buffer. Please heat the buffer to dissolve all the precipitates, which will not affect the results.

3. Accurate pipette should be used to avoid experimental error. Samples should be added to the Microplate in less than 5 minutes. If a large number of samples are included, multiple channel pipette is recommended.

4. Standard curve should be included in every assay. Replicate wells are recommended. If the OD value of the sample is greater than the first well of standards, please dilute the sample (n times) before test. When calculating the original INS concentration, please multiply the total dilution factor (XnX5).

5. In order to avoid cross-contamination, Microplate sealers are for one-time use only.

6. Please keep Substrate away from light.

7. All the operation should be accordance with the manufacturer's instructions strictly. The results determined by the Microtiter Plate Reader.

8. All the samples, washing buffer and wastes should be treated as infectious agents.

9. Reagents from different lots should not be mixed.

Calculation of Results

Known concentrations of Human INS Standard and its corresponding reading OD is plotted on the log scale (x-axis) and the log scale (y-axis) respectively. The concentration of Human INS in sample is determined by plotting the sample's O.D. on the Y-axis. The original concentration is calculated by multiplying the dilution factor.

This diagram is for reference only
Precision

Intra-assay Precision (Precision within an assay): 3 samples with low, middle and high level human INS were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 samples with low, middle and high level human INS were tested on 3 different plates, 8 replicates in each plate.

\[ CV(\%) = \frac{SD}{mean} \times 100 \]

Intra-Assay: CV<10%

Inter-Assay: CV<12%

Assay range

0.3 mU/L - 20 mU/L

Sensitivity:

0.05 mU/L

Storage and validity

1. Storage: 2-8°C.
2. Duration: 6 months
DIAGNOSTIC KIT FOR DETERMINATION OF GLUCOSE CONCENTRATION

INTRODUCTION
Glucose is a simple six-carbon sugar. Oxidative metabolism of glucose provides the energy for most cellular processes. Glucose level in the blood is tightly controlled by several hormones. Elevated glucose level is the classic sign of diabetes mellitus. Glucose level abnormalities (hyper- or hypoglycemia) might be caused also by pancreatic tumors and diseases of liver, thyroid gland or adrenal glands.

METHOD PRINCIPLE
Colorimetric, enzymatic method with glucose oxidase.

\[
glucose + H_2O + O_2 \rightarrow\text{gluconic acid} + H_2O_2
\]

\[2H_2O_2 + \text{phenol} + 4-\text{aminooantipyrine} \rightarrow (p-\text{benzoquinonemonooimid})-\text{phenazone} + 4H_2O \quad \text{(red colour)}
\]

The colour intensity is proportional to the glucose concentration.

REAGENTS
Package
1-Reagent 4 x 35 ml

The reagent when stored at 2-8°C is stable up to expiry date printed on the package. Stability on board of the analyser at 2-10°C: ACCENT-200 - 11 weeks. ACCENT-200 II GEN - 11 weeks. Protect from light and avoid contamination!

Concentrations in the test
- phosphate buffer (pH 7.0) 250 mmol/l
- phenol 5 mmol/l
- glucose oxidase (GOD) > 250 µkat/l
- peroxidase (POD) > 20 µkat/l
- 4-aminooantipyrine (4-AA) 50 µmol/l

Warnings and notes
- Product for in vitro diagnostic use only.

SPECIMEN
- Serum, EDTA or heparinized plasma (recommended: heparine lithium, sodium or ammonium salt) free from hemolysis, cerebrospinal fluid.
- Specimen which is not assayed immediately after collection should be kept in tubes containing sodium fluoride or sodium iodidate. These compounds adding prevent glycolysis and stabilize glucose level.
- Glucose concentration in cerebrospinal fluid should be measured directly after specimen collection.
- Serum and plasma can be stored up to 3 days at 2-8°C.
- Nevertheless it is recommended to perform the assay with freshly collected samples!

PROCEDURE
- This reagent may be used in automatic analyser ACCENT-200 and ACCENT-200 II GEN.
- 1-Reagent is ready to use.
- For reagent blank deionized water is recommended.

APPLICATION for ACCENT-200 and ACCENT-200 II GEN

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Test Name</th>
<th>Test No</th>
<th>Full Name</th>
<th>Reference No</th>
<th>Analy. Type</th>
<th>Pri. Wave.</th>
<th>Sec. Wave.</th>
<th>Trend</th>
<th>Rec. Time</th>
<th>Incuba. Time</th>
<th>Unit</th>
<th>Precision</th>
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<tbody>
<tr>
<td></td>
<td>GLUC</td>
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<td>Glucose</td>
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<td>mg/dl</td>
<td>Integer</td>
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<td></td>
<td>R1 Blank</td>
<td>Mixed Reg. Blank</td>
<td>Concentration</td>
<td>Linear Limit</td>
<td>Substrate Limit</td>
<td>Factor</td>
<td></td>
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Calibration Rule
- Rule: One Point Linear
- Sensitivity: 1
- Replicates: 3
- Interval (day): 56
- Difference Limit: 0
- SD: 0
- Blank Response: 0
- Error Limit: 0
- Coefficient: 0

REFERENCE VALUES

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<th>mg/dl</th>
<th>mmol/l</th>
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</thead>
<tbody>
<tr>
<td>Cerebrospinal fluid</td>
<td>40 - 70</td>
<td>2.2 - 3.9</td>
</tr>
</tbody>
</table>

It is recommended for each laboratory to establish its own reference ranges for local population.

QUALITY CONTROL
For internal quality control it is recommended to use the CORMAY SERUM FN (Cat. No 5-172) and CORMAY SERUM HP (Cat. No 5-175) with each batch of samples.

For the calibration of automatic analyser systems the CORMAY MULTICALIBRATOR LEVEL 1 (Cat. No 5-174; 5-176) or LEVEL 2 (Cat. No 5-175; 5-177) is recommended.

ATTENTION: When the biological material is plasma collected in EDTA or heparin, for the calibration of automatic analyser systems only CORMAY MULTICALIBRATOR LEVEL 2 (Cat. No. 5-175; 5-177) is recommended.

The calibration curve should be prepared every 8 weeks (ACCENT-200, ACCENT-200 II GEN), with change of reagent lot number or as required e.g. quality control findings outside the specified range.

PERFORMANCE CHARACTERISTICS
These metrological characteristics have been obtained using automatic analyser ACCENT-200. Results may vary if a different instrument or a manual procedure is used.

- Sensitivity: 2.8 mg/dl (0.154 mmol/l)
- Linearity: up to 500 mg/dl (27.5 mmol/l).
- If glucose concentration exceeds the range of linearity, dilute sample with 0.9% NaCl and repeat the assay. Multiply the result by the dilution factor.
### Specificity / Interferences

Hemoglobin up to 2.5 g/dl, ascorbate up to 62 mg/dl, bilirubin up to 20 mg/dl and triglycerides up to 1000 mg/dl do not interfere with the test.

### Precision

<table>
<thead>
<tr>
<th></th>
<th>Level 1</th>
<th>Level 2</th>
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<tbody>
<tr>
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<td>Mean</td>
<td>SD</td>
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<tr>
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<td>mg/dl</td>
<td>mg/dl</td>
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<td>Reproducibility</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>(day to day)</td>
<td>mg/dl</td>
<td>mg/dl</td>
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<tr>
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<td>2.17</td>
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<td>299.74</td>
<td>5.71</td>
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</table>

### Method comparison

A comparison between glucose values determined at ACCENT-200 (y) and at ADVIA 1650 (x) using 30 serum samples gave following results:

\[
x = 1.0368x - 0.5293 \text{ mg/dl;}
R = 0.9959 \quad \text{(R – correlation coefficient)}
\]

A comparison between glucose values determined at ACCENT-200 (y) and at ADVIA 1650 (x) using 30 plasma samples gave following results:

\[
y = 0.9836x + 2.2108 \text{ mg/dl;}
R = 0.9995 \quad \text{(R – correlation coefficient)}
\]

### WASTE MANAGEMENT

Please refer to local legal requirements.

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**LITERATURE**


**Date of issue:** 09.2015.

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**MANUFACTURER**

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09/15.09.15
DIAGNOSTIC KIT FOR DETERMINATION OF TOTAL CHOLESTEROL CONCENTRATION

INTRODUCTION
Cholesterol is an essential structural component of cell membranes and precursor of bile acids and all steroid hormones. This is why cholesterol has an enormous significance for organ function and normal functioning. But there is also well established association between blood cholesterol concentration and coronary heart disease. Measurement of cholesterol serum level is valuable in prevention and monitoring cardiovascular disease. This determination is useful also for evaluation of intense absorption, liver and gallbladder function.

METHOD PRINCIPLE
Enzymatic, colorimetric method with cholesterol esterase and cholesterol oxidase.

\[ \text{cholesterol} + \text{H}_{2}\text{O} + \text{CH}_{3} \text{OH} \rightarrow \text{acetate} + \text{fatty acids} + \text{H}_{2}\text{O} \]

\[ 2 \text{H}_{2}\text{O}_{2} + 4\text{-aminoantipyrine } + \text{phenol } + \text{POD} \rightarrow 4\text{H}_{2}\text{O} + \text{quinonemine dye} \]

(red coloured)

The colour intensity is proportional to the cholesterol concentration.

REAGENTS

Package
1-Test Kit
4 x 35 ml

The reagent when stored at 2-8°C is stable up to expiry date printed on the package. Stability on board of the analyser is 3-10°C.

CONCENTRATIONS IN THE TEST

- Good's buffer (pH 6.4): 100 mmol/l
- Phenol: 5 mmol/l
- 4-aminoantipyrine: 0.3 mmol/l
- Cholesterol esterase (CHE): > 3.5 phkat/l
- Cholesterol oxidase (CHO): > 1.67 phkat/l
- Peroxidase (POD): > 50 phkat/l

WARNINGS AND NOTES
- Product for in vitro diagnostic use only.
- The reagent contains (< 0.1%) sodium azide as a preservative. Avoid contact with skin and mucous membranes.

QUALITY CONTROL

For internal quality control it is recommended to use the CORMAY SERUM HN (Cat. No. 5-172) and CORMAY SERUM HP (Cat. No. 5-173) with each batch of samples. For the calibration of automatic analyzers systems the CORMAY MULTICALIBRATOR LEVEL 1 (Cat. No. 5-174; 5-176) or LEVEL 2 (Cat. No. 5-178; 5-177) is recommended.

The calibration curve should be prepared every 10 weeks (ACCENT-200, ACCENT-200 II GEN), with change of reagent lot number or as required e.g. quality control findings outside the specified range.

PERFORMANCE CHARACTERISTICS

These metrological characteristics have been obtained using automatic analyser ACCENT-200. Results may vary if a different instrument or a manual procedure is used.

- Sensitivity: 3.2 mg/dl (0.083 mmol/l)

CALIBRATION RULE

One point linear

REFERENCE VALUES

<table>
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<tr>
<th>Serum</th>
<th>Range (mg/dl)</th>
<th>Range (mmol/l)</th>
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</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>50-120</td>
<td>1.3-4.4</td>
</tr>
<tr>
<td>Children &lt; 4 yr</td>
<td>60-200</td>
<td>1.6-4.9</td>
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<tr>
<td>Adults</td>
<td>110-230</td>
<td>2.8-6.0</td>
</tr>
<tr>
<td>&lt; 200</td>
<td>&lt; 5.2</td>
<td></td>
</tr>
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</table>
**Linearity:** up to 750 mg/dl (19.4 mmol/l).

**Specificity / Interferences**
Hemoglobin up to 0.31 g/dl, ascorbate up to 62 mg/dl, bilirubin up to 20 mg/dl and triglycerides up to 1000 mg/dl do not interfere with the test.

**Precision**

<table>
<thead>
<tr>
<th>Repeatability (run to run)</th>
<th>Mean [mg/dl]</th>
<th>SD [mg/dl]</th>
<th>CV [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 20</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>level 1</td>
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<table>
<thead>
<tr>
<th>Reproducibility (day to day)</th>
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<th>SD [mg/dl]</th>
<th>CV [%]</th>
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<tr>
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<td>2.88</td>
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<tr>
<td>level 2</td>
<td>260.25</td>
<td>7.88</td>
<td>3.03</td>
</tr>
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</table>

**Method comparison**
A comparison between cholesterol values determined at ACCENT-200 (y) and at COBAS INTEGRA 400 (x) using 36 samples gave following results:

\[ y = 0.9229x + 12.725 \text{ mg/dl} \]

\[ R = 0.9819 \quad (R = \text{correlation coefficient}) \]

**Waste Management**
Please refer to local legal requirements.

**LITERATURE**

**Date of Issue:** 03. 2012.

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03/12/03/12
INTRODUCTION
Plasma lipoproteins are spherical particles containing varying amounts of cholesterol, triglycerides, phospholipids, and proteins. The relative protein and lipid determine the density of these lipoproteins and provide the basis on which to begin their classification. The classes are: chylomicron, very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL).

LDL are synthesized in the liver by the action of various lipolytic enzymes on triglyceride rich VLDL. LDL-cholesterol concentration is considered to be the most important clinical predictor of all single parameters, with respect to coronary atherosclerosis. Accurate measurement of LDL-cholesterol is of vital importance in therapies which focus on lipid reduction to prevent atherosclerosis or reduce its progress and to avoid plaque rupture.

METHOD PRINCIPLE
The assay consists of 2 distinct reaction steps:
1. Elimination of cholest erase, VLDL and HDL by cholesterol esterase, cholesterol oxidase and subsequently catalase.

\[
\text{cholesterol ester} \xrightarrow{\text{cholesterol esterase}} \text{cholesterol + fatty acid}
\]

\[
\text{cholesterol + O}_2 \xrightarrow{\text{catalase}} \text{cholestone + H}_2\text{O}
\]

2. Specific measurement of LDL-Cholesterol after release of LDL-Cholesterol by detergents in 2-Reagent. In the second reaction catalase is inhibited by sodium azide in 2-Reagent.

\[
\text{cholesterol esterase} \xrightarrow{\text{cholesterol oxidase}} \text{cholesterol + fatty acid}
\]

\[
\text{cholesterol + O}_2 \xrightarrow{\text{peroxidase}} \text{cholestone + H}_2\text{O}
\]

\[
2\text{H}_2\text{O}_2 + 4\text{AA} + \text{TOOS} \rightarrow \text{quinone pigment + 4H}_2\text{O}
\]

The colour intensity is proportional to the LDL cholesterol concentration when measured at 690 nm.

REAGENTS
Package
1-Reagent \hspace{1cm} 1 x 30 ml
2-Reagent \hspace{1cm} 1 x 10 ml

The reagents are stable up to the kit expiry date printed on the package when stored at 2-8°C. The reagents are stable for 12 weeks on board the analyser at 2-10°C. Protect from light and avoid contamination.

Concentrations in the test
1-Reagent
- Good's buffer (pH 7.0) \hspace{1cm} 50 mmol/l
- Cholesterol esterase \hspace{1cm} 600 U/l

APPLICATION for ACCENT-200 and ACCENT-200 II GEN
Parameters
- LDI D
- R1
- R2
- Sample Volume
- R1 Blank
- Mixed Reag. Blank
- Concentration
- Linearity Limit
- Substrate Limit
- Factor
- Prozone check
- PC
- Abs

Calibration Rule
- One Point Linear

REFERENCES VALUES
- serum/plasma < 100 mg/dl
- < 2.59 mmol/l
As LDL cholesterol is affected by a number of factors such as smoking, exercise, hormones, age and sex, each laboratory should establish its own reference ranges for local population.

QUALITY CONTROL
For internal quality control it is recommended to use CORMAY LIPID CONTROL 1 (Cat. No. 5-179) and CORMAY LIPID CONTROL 2 (Cat. No. 5-180) on CORMAY SERUM 1HN (Cat. No. 5-172) and CORMAY SERUM 10H (Cat. No. 5-173) with each batch of samples.

For the calibration of automatic analysers the CORMAY HDL/LDL CALIBRATOR (Cat. No. 5-178) is recommended. The calibration curve should be prepared every 12 weeks, with change of reagent lot number or as required e.g. quality control findings outside the specified range.

PERFORMANCE CHARACTERISTICS
These metrological characteristics have been obtained using automatic analysers ACCENT-200 and ACCENT-200 II GEN.

Results may vary if a different instrument is used.

- Sensitivity (Accent-200): 20 mg/dl (0.518 mmol/l).
- Sensitivity (Accent-200 II GEN): 9.4 mg/dl (0.243 mmol/l).
- Linearity (Accent-200): up to 400 mg/dl (10.36 mmol/l).
- Linearity (Accent-200 II GEN): up to 230 mg/dl (8.55 mmol/l).

For higher concentration of LDL cholesterol dilute the sample with 0.9% NaCl and repeat the assay. Multiply the result by dilution factor.

- Specificity / Interferences
  trypsin up to 20 mg/dl, ascorbic up to 62 mg/l, haemoglobin up to 2,5 g/dl, and triglycerides up to 300 mg/dl do not interfere with the test.

- Precision (Accent-200)

<table>
<thead>
<tr>
<th>Repeatability (run to run)</th>
<th>n = 20</th>
<th>Mean [mg/dl]</th>
<th>SD [mg/dl]</th>
<th>CV [%]</th>
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<tbody>
<tr>
<td>level 1</td>
<td></td>
<td>135.70</td>
<td>5.99</td>
<td>4.41</td>
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<tr>
<td>level 2</td>
<td></td>
<td>135.70</td>
<td>5.99</td>
<td>4.41</td>
</tr>
</tbody>
</table>

- Reproducibility (day to day) | n = 80 | Mean [mg/dl] | SD [mg/dl] | CV [%] |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>level 1</td>
<td></td>
<td>105.22</td>
<td>13.43</td>
<td>11.45</td>
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<td>level 2</td>
<td></td>
<td>105.22</td>
<td>13.43</td>
<td>11.45</td>
</tr>
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</table>

- Method comparison
A comparison between LDL cholesterol values determined at ACCENT-200 (y) and at PRESTIGE 24 (x) using 20 samples gave following results:

\[ y = 0.9127x + 11.665 \text{ mg/dl} \]

R² = 0.9901

(R - correlation coefficient)

A comparison between LDL cholesterol values determined at ACCENT-200 II GEN (y) and at Hitachi 912 (x) using 21 samples gave following results:

\[ y = 1.0426x + 8.875 \text{ mg/dl} \]

R² = 0.981

(R - correlation coefficient)

WASTE MANAGEMENT
Please refer to local legal requirements.

LITERATURE

Date of issue: 06. 2013.
II GENERATION

DIAGNOSTIC KIT
FOR DETERMINATION OF HDL-CHOLESTEROL CONCENTRATION
(DIRECT METHOD)

INTRODUCTION
Plasma lipoproteins are spherical particles containing varying amounts of cholesterol, triglycerides, phospholipids and proteins. The relative protein and lipid determine the density of these lipoproteins and provide the basis on which to begin their classification. The classes are: chylomicron, very-low-density lipoprotein (VLDL), low-density-lipoprotein (LDL) and high-density lipoprotein (HDL). The principle rule of HDL is lipid metabolism is the uptake and transport of cholesterol from peripheral tissues to the liver. Low HDL cholesterol (HDL-C) levels are strongly associated with an increased risk of coronary artery disease. The HDL-C determination is used to identify high-risk patients.

METHOD PRINCIPLE
The assay is a homogeneous method for directly measuring HDL-cholesterol concentration in serum or plasma, without any off-line pretreatment or centrifugation steps. Accelerator selective detergent methodology.

During the first phase, LDL, VLDL particles and Chylomicrons generate free non-HDL cholesterol, which through an enzymatic reaction, produce hydrogen peroxide. The generated peroxide is consumed by a peroxidase reaction with DSBmT yielding a colourless product.

During the second phase, specific detergent solubilizes HDL-Cholesterol. In conjunction with cholesterol oxidase (CO) and cholesterol esterase (CE) action, peroxidase and 4-AAP develop a coloured reaction which is proportional to HDL-Cholesterol concentration.

REAGENTS
Packaging
1-Reagent 2 x 25 ml
2-Reagent 2 x 9 ml

The reagents are stable up to the kit expiry date printed on the package when stored at 2-8°C. Stability on board of the analyser at 2-10°C ACCENT-200 – 12 weeks, ACCENT-200 II GEN – 12 weeks. Protect from light and avoid contamination!

Concentrations in the test
1-Reagent Buffer
Cholesterol oxidase (E.coli) < 1000 U/l
Peroxidase (horseradish) < 1300 u/g
N.N-bis(2-hydroxyethyl)toluidine, disodium (DSBmT) < 1 mM
Accelerator < 1 mM
Preservative < 0.06 %
Ascorbic acid oxidase (Curcubita sp.) < 3000 U/l

2-Reagent Buffer
Cholesterol esterase (Pseudomonas sp.) < 1500 U/l
4-aminophenylpyrine (4-AAP) < 1 mM
Detergent < 2 %
Preservative < 0.06 %

Warnings and notes
• Product for in vitro diagnostic use only.

SPECIMEN
Serum, heparinized or EDTA plasma.
Anticoagulants containing citrate should not be used.
Blood should be collected only if the patient has been fasting for 12-14 hours.
Serum: Collect whole blood by venepuncture and allow to clot. Centrifuge and remove the serum as soon as possible after collection (within 3 hours).
Plasma: Specimens may be collected in EDTA or lithium or sodium heparin. Centrifuge and remove the plasma as soon as possible after collection (within 3 hours).
Serum and plasma should not remain at 15-30°C longer than 14 hours. If assays are not completed within 14 hours, serum or plasma should be stored at 2-8°C for up to 1 week. If specimens need to be stored for more than 1 week, they may be preserved at less than -20°C for up to 3 months. Samples may be frozen once.
Nevertheless it is recommended to perform the assay with freshly collected samples.

PROCEDURE
These reagents may be used in automatic analysers ACCENT-200 and ACCENT-200 II GEN.
1-Reagent and 2-Reagent are ready to use.
for reagent blank deionized water is recommended.

APPLICATION for ACCENT-200

Parameters
Test Name HDL D
Test No 25
Full Name HDL Direct
Reference No 25
Analy. Type
Pri. Wave. 578 nm
Sec. Wave. 620 nm
Trend
Reac. Time 0-15
Incuba. Time 10
Unit mg/dl
Precision 0.3

 Calibration Rule
Rule Two-Point Linear
Sensitivity 1
Repetitions 3
Interval (day) 84
Difference Limit 0
SD 0
Blank Response 0
Error Limit 0
Coefficient 0

PC Abs

ACCENT-200 HDL DIRECT II GENERATION page 1
### Application for ACCENT-200 II GEN

<table>
<thead>
<tr>
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<th>R2</th>
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<tr>
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<td>Precision</td>
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</table>

### Calibration Rule

- Two-Point Linear
- Sensitivity: 2
- Interval (day): 84
- Difference Limit: 0
- Blank Response: 0
- Error Limit: 0
- Coefficient: 0

### Reference Values

| | serum / plasma | 40 – 60 mg/dL | 1.04 – 1.55 mmol/l |
|-----------------|----------------|-----------------|

As HDL cholesterol is affected by a number of factors such as smoking, exercise, age and sex, each laboratory should establish its own reference range for local population.

### Quality Control

For annual quality control it is recommended to use CORMAX LIPID CONTROL 2 (Cat. No 5-180) or CORMAY SERUM FN (Cat. No 5-172) and CORMAY SERUM HP (Cat. No 5-173) with each batch of samples. For the calibration of automatic analysers the CORMAY HDL/IDL CALIBRATOR (Cat. No 5-178) is recommended. Deionised water should be used as a calibrator 0.

The calibration curve should be prepared every 12 weeks (ACCENT-200, ACCENT-200 II GEN), with change of reagents, lot number or as required e.g. quality control findings outside the specified range.

### Performance Characteristics

These methodological characteristics have been obtained using automatic analysers ACCENT-200 and ACCENT-200 II GEN. Results may vary if a different instrument or a manual procedure is used.

- **Sensitivity (ACCENT-200):** 0.5 mg/dL (0.013 mmol/l).
- **Sensitivity (ACCENT-200 II GEN):** 0.8 mg/dL (0.021 mmol/l).
- **Linearity (ACCENT-200):** up to 200 mg/dL (5.18 mmol/l).
- **Linearity (ACCENT-200 II GEN):** up to 140 mg/dL (3.86 mmol/l).

For higher concentration dilute the sample with 0.9% NaCl and repeat the assay. Multiply the result by dilution factor.

- **Specificity / interference:** Bilirubin conjugated up to 60 mg/dL, bilirubin total up to 60 mg/dL, haemoglobin up to 1 g/dL, ascorbic acid up to 100 mg/dL, inositol up to 1800 mg/dL, triglycerides up to 2000 mg/dl and gamma-globulins up to 5000 mg/dL do not interfere with the test.

### Precision

<table>
<thead>
<tr>
<th>ACCENT-200</th>
<th>Mean [mg/dL]</th>
<th>SD [mg/dL]</th>
<th>CV [%]</th>
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<tr>
<td>n = 10</td>
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<td>Level 1</td>
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<td>Level 2</td>
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<tr>
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<tr>
<td>Level 2</td>
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<td>1.35</td>
<td>2.24</td>
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</table>

### Method Comparison

- **Comparison between HDL-cholesterol values determined at ACCENT-200 (y) and at Advia 1650 (x) using 54 samples gave following results:**

  \[ y = 0.8381 x + 4.7706 \text{ mg/dL}; \]

  \[ R = 0.978 \]  

  (R – correlation coefficient)

- **Comparison between HDL-cholesterol values determined at ACCENT-200 II GEN (y) and at Hitachi 912 (x) using 26 samples gave following results:**

  \[ y = 1.0790 x - 2.4566 \text{ mg/dL}; \]

  \[ R = 0.994 \]  

  (R – correlation coefficient)

### Waste Management

Please refer to local legal requirements.

### Literature


Date of issue: 03. 2015.

### Manufacturer

PZ CORMAY S.A.,
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05-092 Łomianki, POLAND
tel.: +48 (0) 22 751 79 10
fax: +48 (0) 22 751 79 14
http://www.cormay.pl

03/15/03/15
DIAGNOSTIC KIT FOR DETERMINATION OF TRIGLYCERIDES CONCENTRATION

INTRODUCTION
Triglycerides are built of glycerol molecule esterified with three fatty acids molecules. Triglycerides are delivered with food or are synthesized endogenously in liver. Triglycerides stored in adipose tissue constitute a reserve of energy. Elevated triglycerides serum level is a risk factor of atherosclerosis. Triglycerides measurement is useful for hyperlipidemia diagnosis and treatment or for estimation of atherosclerosis progression.

METHOD PRINCIPLE
Colorimetric, enzymatic method with glycerophosphate oxidase.

\[ \text{triglycerides} + H_2O \xrightarrow{LPL} \text{glycerol} + \text{fatty acids} \]

\[ \text{glycerol} + \text{ATP} \xrightarrow{\text{GKO}} \text{glycerol-3-phosphate} + \text{ADP} \]

\[ \text{glycerol-3-phosphate} + H_2 \xrightarrow{\text{GPO}} \text{dihydroxy-acetone-phosphate} + 2\text{H}_2\text{O} \]

\[ 2\text{H}_2\text{O} + 4\text{AA} + 4\text{chlorophenol} \xrightarrow{\text{POD}} \text{quinoneminimine dye} + 4\text{H}_2\text{O} \]

The colour intensity is proportional to the triglycerides concentration.

REAGENTS
Package
1- Reagent
6 x 31 ml

The reagent when stored at 2-8°C are stable up to expiry date printed on the package. Stability on board of the analyser at 2-10°C ACCENT-200 – 10 weeks, ACCENT-200 II GEN – 10 weeks. Protect from light and avoid contamination!

Concentrations in the test

<table>
<thead>
<tr>
<th>Reagent</th>
<th>200 mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>buffer TRIS (pH 8.0)</td>
<td>&lt; 0.4 mmol/l</td>
</tr>
<tr>
<td>4-aminopyridine (4-AA)</td>
<td>&lt; 1.5 mmol/l</td>
</tr>
<tr>
<td>ATP</td>
<td>1.6 mmol/l</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>&lt; 2.5 mmol/l</td>
</tr>
<tr>
<td>chlorophenol</td>
<td>&lt; 1 mmol/l</td>
</tr>
<tr>
<td>potassium hexacyanoferrate (II)</td>
<td>&lt; 1 mmol/l</td>
</tr>
<tr>
<td>FAD-2Na</td>
<td>&lt; 1 mmol/l</td>
</tr>
<tr>
<td>glycero kinase (GK)</td>
<td>&lt; 2500 U/l</td>
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<tr>
<td>glycero phosphate oxidase (GPO)</td>
<td>&lt; 2500 U/l</td>
</tr>
<tr>
<td>peroxidase (POD)</td>
<td>&lt; 1900 U/l</td>
</tr>
<tr>
<td>lipoprotein lipase (LPL)</td>
<td>&lt; 2000 U/l</td>
</tr>
<tr>
<td>detergents, preservatives</td>
<td></td>
</tr>
</tbody>
</table>

Warnings and notes
- Product for in vitro diagnostic use only.
- The reagents should be used by suitably qualified laboratory personnel only in accordance with intended purpose.
- The reagents contain < 0.1% sodium azide as a preservative.

Avoid contact with skin and mucous membranes.

SPECIMEN
Serum, EDTA or heparinized plasma (recommended: heparine lithium, sodium or ammonium salt) free from hemolysis.

Blood should be collected only if the patient has been fasting for minimum of 12 hours. Before blood collection patient should stay in rest position for about 30 minutes. Venous blood is recommended for triglycerides measurement.

Plasma triglycerides values have been reported to be 2% to 4% lower than serum triglycerides values.

Serum and plasma can be stored up to 3 days at 2-8°C or up to 3 months at -20°C.

Nevertheless it is recommended to perform the assay with freshly collected samples!

PROCEDURE
These reagents may be used in automatic analysers ACCENT-200 and ACCENT-200 II GEN.

1- Reagent is ready to use.

For reagent blank deionized water is recommended.

Actions required:
When performing assays with kits ACCENT-200- TG mono and LIPASE, either HDL DIRECT, LDL DIRECT, URINE PROTEINS and TG mono there is a probability of cross-contamination affecting the tests results. To avoid this effect follow the recommendations contained in the advisory note "CARRY-OVER - Preventive Actions".

APPLICATION for ACCENT-200

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<tr>
<th>Parameters</th>
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<td>Mixed Resp. Blank</td>
<td>Concentration</td>
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Calibration Rule

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APPLICATION for ACCENT-200 II GEN

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PC | Abs |

ACCENT-200 TG mono page 1
Calibration Rule

<table>
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<th>Rule</th>
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<td>Replicates</td>
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<tr>
<td>Coefficient</td>
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</tbody>
</table>

REFERENCE VALUES

serum, plasma < 150 mg/dl
<3.7 mmol/l

It is recommended for each laboratory to establish its own reference ranges for local population.

QUALITY CONTROL

For internal quality control it is recommended to use the CORMAY SERUM HN (Cat. No 5-172) and CORMAY SERUM HP (Cat. No 5-173) with each batch of samples.

For the calibration of automatic analysers systems the CORMAY MULTICALIBRATOR LEVEL 1 (Cat. No 5-174; 5-176) or LEVEL 2 (Cat. No 5-175; 5-177) is recommended.

The calibration curve should be prepared every 7 weeks (ACCENT-200, ACCENT-200 II GEN), with change of reagent lot number or as required e.g. quality control findings outside the specified range.

PERFORMANCE CHARACTERISTICS

These metrological characteristics have been obtained using automatic analysers ACCENT-200 and ACCENT-200 II GEN. Results may vary iif a different instrument or a manual procedure is used.

- Sensitivity (ACCENT-200): 1.36 mg/dl (0.0154 mmol/l).
- Sensitivity (ACCENT-200 II GEN): 1.38 mg/dl (0.0166 mmol/l).

- Linearity (ACCENT-200): up to 1300 mg/dl (14.60 mmol/l).
- Linearity (ACCENT-200 II GEN): up to 1100 mg/dl (12.43 mmol/l).

For higher triglycerides concentrations dilute the sample with 0.9% NaCl in the ratio of 1 to 4 and repeat the assay. Multiply the result by 5.

- Specificity / Interferences

Haemoglobin up to 0.31 g/dl, bilirubin up to 8.6 mg/dl and ascorbate up to 31 mg/dl do not interfere with the test.

- Precision (ACCENT-200)

Repeatability (run to run) n = 20 Mean SD CV [%]
level 1 62.77 0.47 0.75
level 2 157.62 1.28 0.81

Reproducibility (day to day) n = 80 Mean SD CV [%]
level 1 84.27 2.10 2.49
level 2 168.60 3.70 2.20

- Precision (ACCENT-200 II GEN)

Repeatability (run to run) n = 20 Mean SD CV [%]
level 1 55.30 0.57 0.66
level 2 188.95 0.86 0.48

Reproducibility (day to day) n = 80 Mean SD CV [%]
level 1 84.27 2.10 2.49
level 2 168.60 3.70 2.20

- Method comparison (ACCENT-200)

A comparison between triglycerides values determined on ACCENT-200 (y) and on CUBAS INTEGRA 400 (x) using 52 samples gave following results:
y = 0.9853 x + 4.4927 mg/dl
r = 0.997

- Method comparison (ACCENT-200 II GEN)

A comparison between triglycerides values determined on ACCENT-200 II GEN (y) and on CUBAS INTEGRA 400 (x) using 42 samples gave following results:
y = 1.0324 x + 1.4325 mg/dl
r = 0.9971

WASTE MANAGEMENT

Please refer to local legal requirements.

LITERATURE


Date of Issue: 03/2016.

ACCENT-200 TG monol page 2
Correlation of Osteocalcin Level with Blood Glucose Concentration and Insulin Level in Type II Diabetic Sudanese Patients

Ogail Yousif Dawod1*, Amar Babiker Elhussein2, Mohamed Abd El-Rahim Ahmed3, Nour Eldaim Elnoman Elhodawi4 and Omer Abdel Aziz Musa5

1 Department of Physiology, Faculty of Medicine, University of Shendi, Shendi, Sudan
2 Department of Biochemistry, Nile College for Medicine and Medical Science, Khartoum, Sudan
3 Department of Biochemistry, College of Medicine, University of Shaqra, Shaqra, Saudi Arabia
4 Department of Physiology, Faculty of Medicine, The National Ribat University, Sudan
*Corresponding e-mail: ogail76@gmail.com

ABSTRACT
Osteocalcin, a bone-derived protein, has recently been reported to affect energy metabolism, this study aimed to evaluate the effects of osteocalcin level on blood glucose concentration and insulin level in type II diabetic Sudanese patients. In this cross-sectional study, one hundred fifteen type II diabetic patients were enrolled in the study (34 males and 81 females) with ages ranging between 18 and 90 years. Total osteocalcin, undercarboxylated osteocalcin, and insulin were measured using ELISA technique. Fasting blood glucose and lipid profile were measured by a spectrophotometer. Mean serum osteocalcin concentration in diabetic patients was significantly lower than those in control subjects (p ≤ 0.001). Undercarboxylated osteocalcin in diabetic patients was significantly lower than in control subjects (p ≤ 0.001). Fasting insulin was significantly higher in diabetic subjects compared to control subjects (p ≤ 0.001). Osteocalcin was negatively correlated with insulin and FBS (r = 0.303; p ≤ 0.001), (r = 0.373; p ≤ 0.001), respectively. Undercarboxylated was negatively correlated with insulin and FBS (r = 0.534; p ≤ 0.001), (r = 0.297; p ≤ 0.001), respectively. Total osteocalcin and undercarboxylated osteocalcin levels of diabetic patients were lower than those of the healthy control subjects. Fasting serum insulin was higher in patients compared to control subjects. Total osteocalcin and undercarboxylated osteocalcin were negatively correlated with fasting serum insulin and FBS.

Keywords: Diabetes, Total osteocalcin, Undercarboxylated osteocalcin and Insulin

INTRODUCTION
Diabetes mellitus is a chronic disease which occurs either when the pancreas did not produce enough insulin or when the body tissues could not efficiently use the insulin [1]. The prevalence of diabetes worldwide in the age between 20 to 70 years was estimated to be 8.3% in 2013 and 10.1% in 2035 [2]. Type II diabetes is a syndrome characterized by metabolic disorders and hyperglycaemia resulting from low levels of insulin with or without insulin resistance [3].

Organ damage is one of the long-term complications of diabetes mellitus; it is expected as a consequence of chronic hyperglycaemia. These complications result from atherosclerosis caused by the long-standing diabetes to the coronary arteries, cerebrovascular circulation, retinal and renal micro vessels and the vas nerves [4].

Animal studies and cell-based assays revealed that the skeleton acts as an endocrine tissue by secreting osteocalcin, which plays a role in the metabolism of glucose and lipids [5].

Osteocalcin (OC) is a type of non-collagenous protein which is produced and secreted by osteoblasts [6]. It is composed of undercarboxylated osteocalcin (ucOC) and carboxylated osteocalcin (cOC) [7]. cOC is synthesized after the glutamic acid residues of OC on the 17th, 21th, and 24th sites are carboxylated by vitamin K-dependent carboxylase [8]. Osteocalcin with non-carboxylated glutamic acid residues is known as ucOC. OC play a crucial role in maintaining normal bone mineralization, inhibition of abnormal hydroxypatite formation, and reduction of growth cartilage mineralization [7].
Previous studies had concluded that ucOC, but not eOC, is able to promote adiponectin and insulin secretion in mice [5,9]; other studies had shown independent associations between serum OC and metabolic traits in adults [10-11]. However, the carboxylated form of OC which is associated with metabolism in humans is considered a neglected area of research [13-14].

Several studies showed that circulating osteocalcin level was associated with glucose/lipid metabolism in humans [12,15]. Zhou, et al., showed that serum osteocalcin was inversely correlated with blood sugar and positively correlated with insulin secretion in the Chinese population [15]. Other researchers have also found a correlation between osteocalcin and glucose metabolism in humans [10,16]. Previous clinical studies demonstrated that uncontrolled Diabetes could reduce serum OC level, while serum OC increased after blood glucose was well controlled [17]. These data indicated that changes of glucose metabolism could influence OC levels [18].

The current study aimed to evaluate the effects of osteocalcin level on blood glucose concentration and insulin levels in Type II diabetic Sudanese patients.

**Methods**

One hundred fifteen adults with type II diabetes mellitus patients were enrolled in this cross-sectional hospital based case-control study (34 males and 81 females) with ages ranging between 18 and 90 years who visited the diabetic centers in Khartoum (Sudan). Type II diabetes was confirmed according to WHO criteria and 65 healthy adults from Khartoum town were randomly selected as control group.

All patients were newly diagnosed with diabetes. They visited Khartoum diabetic centers during the period of March 2015 up to June 2015. Brief clinical history of present and past illness and medical therapy was recorded from all participants.

Patients using the following treatments or suffering from one of the following complications were excluded from the study:

1. Insulin treatment.
2. Using agents such as Warfarin, Heparin, Vitamin D3, Glucocorticoids, Anticonvulsants.
3. Drugs and drugs of osteoporosis.
4. Bone diseases such as multiple myeloma, osteomalacia, Paget’s disease, and fracture up to one year.
5. Malignancy, cardiovascular disease, hypertension, and hyperthyroidism.

Venous blood was taken after overnight fasting and the level of fasting blood glucose was measured by a spectrophotometer, fasting insulin, lipid profile, and both total and undercarboxylated osteocalcin were measured by ELISA Method. Height and weight were measured and body mass indices were calculated.

**Data analysis**

Results of this study were statistically analysed using statistical package for social science (SPSS) program 64 bits for Windows 8. Independent t-test, one-way analysis of variance (ANOVA) and Person correlation coefficients were used; significance levels were set at (P<0.05).

**Results**

Total osteocalcin (TOC) level was significantly lower (p ≤ 0.001) in diabetic subjects compared to controls, the levels were (2.4 ± 1.4 ng/ml) and (14.8 ± 2.1 ng/ml) respectively. The mean of ucOC level was significantly (p ≤ 0.001) lower in diabetic subjects than in control subjects, the levels were (1.1 ± 0.7 ng/ml) and (2.6 ± 0.5 ng/ml) respectively. The mean insulin level was significantly (p ≤ 0.001) higher in diabetic subjects than in control subjects, the levels were (19.7 ± 6.2 ml U/L) in diabetic subjects and (9.5 ± 2.4 ml U/L) in control subjects.

Table 1 shows highly significant (p ≤ 0.001) difference in mean concentrations of FBS between diabetics and controls, the concentration was (195.4 ± 59.0 mg/dl) in diabetics and (85.4 ± 24.9 mg/dl) in controls.

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<th>Parameter</th>
<th>Diabetic patients Mean ± SD</th>
<th>Healthy control Mean ± SD</th>
<th>p-value</th>
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<td>ucOC ng/ml</td>
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</tbody>
</table>
Mean of TOC showed highly significant negative correlation with insulin level in diabetic subjects \( (r = -0.530; p < 0.001) \), it also showed a highly significant negative correlation with FBS \( (r = -0.373; p < 0.001) \). Whereas ucOC levels was negatively highly significant correlated with insulin \( (r = -0.634; p < 0.001) \), it showed the same trend of correlation with FBS \( (r = -0.297; p < 0.001) \) (Table 2).

Table 2: Correlations of TOC and ucOC with plasma Insulin and Fasting Blood Sugar

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control group</th>
<th>Diabetic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TOC ng/ml</td>
<td>ucOC ng/ml</td>
</tr>
<tr>
<td>Insulin ml/l</td>
<td>r value</td>
<td>-0.307</td>
</tr>
<tr>
<td>p value</td>
<td>0.006</td>
<td>0.00</td>
</tr>
<tr>
<td>FBS mg/dl</td>
<td>r value</td>
<td>-0.117</td>
</tr>
<tr>
<td>p value</td>
<td>0.177</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Results presented in Table 3 shows a highly significant difference in mean TOC level \( (p < 0.001) \) in underweight group between diabetic individuals and healthy control subjects, the higher levels were in control subjects \( (13.8 \pm 1.1 \text{ng/ml}) \) compared to diabetic subjects \( (12.7 \pm 0.7 \text{ng/ml}) \). The ucOC in underweight group showed significant differences \( (p < 0.01) \) between studied groups, the higher levels were shown in control subjects \( (2.3 \pm 0.5 \text{ng/ml}) \) compared to diabetic patients \( (1.2 \pm 0.5 \text{ng/ml}) \). In contrast, mean insulin was highly significant \( (p < 0.01) \) higher in diabetic subjects \( (14.3 \pm 0.5 \text{ng/ml}) \) than in control subjects \( (10.1 \pm 2.0 \text{ng/ml}) \).

Table 3: Mean concentrations of TOC, ucOC and plasma insulin according to BMI in diabetics and controls

<table>
<thead>
<tr>
<th>Variables</th>
<th>Underweight</th>
<th>Diabetic</th>
<th>Normal weight</th>
<th>Overweight</th>
<th>Obese</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOC ng/ml</td>
<td>2.7 \pm 0.7</td>
<td>13.8 \pm 1.1</td>
<td>2.4 \pm 1.4</td>
<td>15.1 \pm 2.5</td>
<td>2.4 \pm 1.7</td>
</tr>
<tr>
<td>p value</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>ucOC ng/ml</td>
<td>1.2 \pm 0.5</td>
<td>2.3 \pm 0.5</td>
<td>1.1 \pm 0.3</td>
<td>2.7 \pm 0.5</td>
<td>1.1 \pm 0.6</td>
</tr>
<tr>
<td>p value</td>
<td>0.013</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Insulin ml/l</td>
<td>14.3 \pm 0.5</td>
<td>10.1 \pm 2.0</td>
<td>20.0 \pm 0.4</td>
<td>9.7 \pm 0.7</td>
<td>19.3 \pm 5.6</td>
</tr>
<tr>
<td>p value</td>
<td>0.008</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

TOC in normal weight group showed a highly significant difference \( (p < 0.001) \) the higher level was \( (15.1 \pm 2.5 \text{ng/ml}) \) in control subjects compared to \( (2.4 \pm 1.4 \text{ng/ml}) \) in diabetic subjects. ucOC in the same group also showed a highly significant differences \( (p < 0.001) \) in mean level, the higher level was in control subjects \( (2.7 \pm 0.5 \text{ng/ml}) \) followed by diabetic patients \( (1.1 \pm 0.6 \text{ng/ml}) \). In this group insulin showed highly significant level \( (p < 0.001) \) in diabetic subjects \( (20.0 \pm 0.4 \text{ng/ml}) \) compared to \( (9.7 \pm 0.7 \text{ng/ml}) \) control subjects.

In overweight BMI group, TOC was significantly higher \( (p < 0.001) \) in control subjects \( (14.8 \pm 1.7 \text{ng/ml}) \) compared to \( (2.4 \pm 1.4 \text{ng/ml}) \) diabetic subjects. ucOC was also significantly higher \( (p < 0.001) \) in control subjects \( (1.1 \pm 0.6 \text{ng/ml}) \) compared to diabetic subjects \( (2.6 \pm 0.4 \text{ng/ml}) \). On the other hand, insulin level showed highly significant difference \( (p < 0.001) \) in both groups the higher level was seen in diabetic subjects \( (19.3 \pm 5.6 \text{ng/ml}) \) compared to control subjects \( (9.0 \pm 1.8 \text{ng/ml}) \).

Results in Table 3 revealed that in the obese group, the TOC showed significantly higher level \( (p < 0.001) \) in control subjects \( (14.6 \pm 0.8 \text{ng/ml}) \) compared to \( (2.4 \pm 1.5 \text{ng/ml}) \) in diabetic patients. In the same group ucOC level was significantly increased \( (p < 0.001) \) in control subjects \( (2.6 \pm 0.55 \text{ng/ml}) \) compared to diabetic subjects \( (1.1 \pm 0.6 \text{ng/ml}) \). In the obese insulin level showed a significant difference \( (p < 0.05) \) in mean level between diabetic subjects and control subjects, the higher level was in diabetic subjects \( (20.4 \pm 6.8 \text{ng/ml}) \) compared to control subjects \( (10.6 \pm 2.8 \text{ng/ml}) \).

**DISCUSSION**

The current cross-sectional hospital based case-control study revealed that serum TOC level was significantly low in diabetic patients compared to healthy control individuals. Several clinical studies have shown that serum TOC concentration was associated with glucose metabolism in humans.[12] Rozato, et al., reported decreased levels of TOC in type II diabetic patients, [19] Zhou, et al., observed decreased TOC levels in diabetic patients compared to those...
Our results showed a negative correlation of TOC, ucOC with insulin and FBS, some studies carried on Caucasian and Asian populations showed that TOC level was independently correlated to FBS and fasting insulin [13, 14, 15].

In our study, the ucOC was low in diabetic subjects compared to control subjects and negatively correlated with insulin. This result is consistent with results of Sanchez-Enriquez, et al., who found lower levels of ucOC in diabetic patients than in control subjects [23]. Decreased TOC levels and the index ucOC/OC are associated with increased FBS and insulin resistance and with risk for developing type II diabetes [21, 22]. Both in vitro and in vivo previous studies demonstrated that it was probably the ucOC played a major role in decreasing blood sugars, enhancing insulin synthesis, and improving insulin resistance [5, 9]. Takashi, et al., revealed that there is a stronger relationship between diabetes and skeletal metabolism through ucOC [24].

Diabetes is well known to affect bone integrity, because mature osteoblastic cells become weakened by abnormal glucose metabolism [25, 26]. Thus, it is speculated that some humoral factors derived from bones, including ucOC, might stimulate β-cells for improving abnormal glucose metabolism. It is possible to consider that ucOC plays a crucial role in protecting bone degradation in disturbance of glucose metabolism by normalizing glucose metabolism, which is achieved by ucOC induced insulin secretion [24].

Certain studies have demonstrated that in hyperglycaemic, the osteoblast mass and function are decreased which suppress osteocalcin synthesis and secretion [27, 28]. Osteocalcin also has an effect on Blood sugar regulation. In line with results from experimental animal research, which showed that recombinant osteocalcin can enhance insulin secretion and β-cell proliferation [5, 9]. Mice lacking osteocalcin gene develops a group of abnormalities such as reduced β-cell proliferation, low insulin secretion, insulin resistance and hyperglycaemia than wild-type mice [5]. Furthermore, administration of recombinant osteocalcin into wild-type mice increase pancreatic β-cell proliferation, insulin secretion and conserve them from weight gain and developing type II diabetes mellitus [5]. These results suggest that osteoblastic insulin signalling through osteocalcin can affect systemic glucose homeostasis [29].

Therefore, the association between TOC and glycemic variability may be linked, in part, to the improvement in insulin secretion. Contrary, the improvement in insulin resistance may participate in the positive effect of osteocalcin on glycemic control [30].

CONCLUSION

By estimating the circulating levels of TOC, ucOC, insulin and FBS in patients with type II diabetes, we found that the TOC and ucOC levels of patients were lower than those of the healthy control subjects. Fasting serum insulin was higher in patients compared to healthy control subjects. TOC and ucOC negatively correlate with Fasting serum insulin and FBS, therefore, TOC and ucOC may play an important role in regulating blood glucose and improving insulin secretion and insulin sensitivity. So, bones are not just a hard-calcified structure, but it may play an important role in controlling obesity, energy, and sugar metabolism.

DECLARATIONS

Ethical consideration

Written informed consent was obtained from diabetic patients and control subjects before entry into the study according to the guidelines of the Animal and Human Ethical Committee of Shendi University.

Conflict of interest

The authors and planners have disclosed no potential conflicts of interest, financial or otherwise.

REFERENCES


[22] Sarkar, P., et al. “Relationships between serum osteocalcin levels versus blood glucose, insu-