Identification of Exosomal Proteins from Plasma of Saudi Males with Type II Diabetes

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Abstract
Exosomes (EXOs) are cell-derived vesicles displaying various proteins on their membrane surfaces. In addition, they are readily available in blood samples where they constitute potential biomarkers of human diseases, such as cancer. Here, we will be examining the potential of distinguishing diabetic patients from controls based on the differential levels of cellular exosomal proteins. In this study, epithelial cell adhesion molecule (EpCAM) positive EXOs were isolated from 30 diabetic males and 30 controls to quantify EXOs from the blood plasma samples. The presence of exosomal membrane bound proteins CD9 and CD63, which were analysed by flow-cytometry. The levels of +CD63 EXOs and +CD9 EXOs were found to be significantly higher in men with T2D than control (P = 0.04 and P = 0.001, respectively). The level of +CD63 EXOs was correlated positively and strongly with +CD9 EXOs in diabetics and controls (P = 0.0001). The diabetic subjects exhibited the level of +CD63 EXOs to be positively correlated with glucose (P = 0.06), whereas controls exhibited +CD63 EXOs and +CD9 EXOs levels to be correlated with high-density lipoprotein (HDL) level (P = 0.04 and P= 0.03, respectively). This study demonstrates that the levels of +CD63 EXOs and +CD9 EXOs increase in patients with T2D, possibly contributing to disease progression. In general, exosomal biomarkers are still in the early discovery stage, and their potential value in clinical diagnostics awaits to be fully explored.

Keywords: Type II diabetes, Exosome, CD9, CD63
Introduction

Type II diabetes (T2D) are increasing worldwide, reaching epidemic proportions. It is a chronic, progressive disease that is characterised by the body’s inability to properly use insulin. It is a multifactorial, multidimensional disease that affects the entire human body (Turner et al., 2016). The excess blood sugar in T2D causes complications, such as damages to eyes, kidneys and nerves and doubles the risk of heart attack and stroke (Adeshara et al., 2016). Therefore, the discovery of novel biomarkers for prognosis is essential to monitor and prevent progression of the T2D disease or for early detection of T2D. Biomarkers can be objectively measured and evaluated as an indication of normal biologic processes, pathogenic processes and pharmacologic responses to a particular treatment or condition. Biomarkers of all types provide a dynamic and powerful approach in understanding better about human diseases. Development and validation of biomarkers is as difficult as the development and approval of a new drug (Sahu et al., 2011).

In recent years, the discovery for T2D biomarkers propagated a major focus on the research of diabetes. There is also an increasing interest in research regarding the use of exosomes (EXOs) and their contents as biomarkers. Multiple experiments have investigated and established the fact that EXOs display various proteins on their membrane surface (Théry et al., 2001; Wilson et al., 2014). EXOs can carry proteins, ribonucleic acid (RNA), or microRNA (miRNA) from one cell to another through the bloodstream, protecting its content from enzymatic degradation. Therefore, these extracellular vesicles may be the cause of mediation of intercellular communications (Yellon & Davidson, 2014). Several studies have suggested that the variations in the protein and messenger ribonucleic acid (mRNA) content of EXOs represent the physiological state of the releasing cell type. Therefore, research scientists are responsible for growing interest in the scientific community. Attention has been given to EXOs as a novel and unique approach to detect diseases (De Jong et al., 2012; Yellon & Davidson, 2014).
The world health organisation (WHO) has ranked Saudi Arabia as the country the second highest rate of T2D in the Middle East and the 7th highest in the world. T2D biomarkers have gained attention regarding diabetic research. Recent research have shown that EXOs profiling could provide tailored information of individual patients and may be circular for precise prognosis. This study aims at isolating EpCAM positive EXOs from male plasma and quantify, as well as identify the presence of exosomal membrane bound proteins CD9 and CD63 as potential biomarkers for T2D.

**Exosomes**

Extracellular vesicles (EVs) are small (50 nm–2 μm) vesicles that are released from the surface of many different cell types through different body fluids, including plasma, milk, saliva, sweat, tears, semen and urine (Raposo & Stoorvogel, 2013). They are classified into three main classes based on the difference in their size, biogenesis mechanism, secretin and component (Kalra *et al.*, 2012; Simpson *et al.*, 2012). They include microvesicles (MVs), apoptotic bodies (ABs) and exosomes (EXOs), which are produced by different mechanisms (Bergsmedh *et al.*, 2001; Sadallah *et al.*, 2010; Lazaro-Ibanez *et al.*, 2014). These types of EVs are released from most viable cells (Zhang *et al.*, 2015). Hematopoietic cells (B cells, T cells, dendritic cells, mast cells and platelets), intestinal epithelial cells, neuronal cells, adipocytes, fibroblasts (NIH3T3), endothelial cells, immunocytes, smooth muscle cells and tumor cells have all been shown to secrete EXOs (Wieckowski & Whiteside, 2006; van der Pol *et al.*, 2012; Liao *et al.*, 2014; Saunderson *et al.*, 2014).

All mammalian EXOs share a few common characteristics, such as structure (bilipidic layer), size (50–100 nm in diameter) and density (flotation at 1.13–1.21 g/ml on a sucrose gradient). EXOs have a particular cup-shaped morphology, membranes enriched in special lipids and a unique protein composition that characterises them as discrete organelles (Simpson *et al.*, 2009; Mathivanan *et al.*, 2010). According to the current
version of the EXOs’ contents database, Exocarta (Version 4, http://www.exocarta.org), 194 lipids, 1639 mRNAs, 764 miRNAs and 4563 proteins have been identified in EXOs of many different cell types and from multiple organisms, thus demonstrating their complexity. The EXOs that are from a different cell line were individually measured by spectra, reflecting the variation in the total exosomal protein, lipid, genetic, and cytosolic content (Smith et al., 2015).

The lipid content of circulating EXOs could be a useful biomarker of lipid-related diseases, such as T2D. The first analysis of exosomal lipid was performed ten years ago (Record et al., 2013). EXOs are enriched in cholesterol and sphingomyelin, and their accumulation in cells may modulate recipient cell homeostasis (Mulcahy et al., 2014).

Several studies have focused on exosomal nucleic acid content, which provide novel pathways for a cell–cell communication, with implications to health and disease (Huang et al., 2013; Rana et al., 2013). Purified EXOs were found to contain functional miRNAs, small RNA and mRNA (Zomer et al., 2010). The presence of functional RNA was first described in 2006 in microvesicles from murine stem cells (Ratajczak et al., 2006). EXOs was also seen to contain mitochondrial DNA (Guescini et al., 2010). Furthermore, EXOs carry DNA, non-coding RNA single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) (Balaj et al., 2011; Thakur et al., 2014).

Protein content include cytoplasmic proteins, such as tubulin, actin, actin-binding proteins, annexins and Rab proteins as well as molecules responsible for signal transduction (protein kinases and heterotrimeric G-proteins) (Thery et al., 2001; Skokos et al., 2001). The most common protein family associated with EXOs is the family of tetraspanins (transmembrane 4 superfamily). Tetraspanins are cell surface glycoproteins with four transmembrane domains that form multimeric complexes with other cell surface proteins (Charrin et al., 2009). In mammals, the tetraspanin family comprises 33
members that include CD9, CD63, CD81 and CD82 (Escola et al., 1998; Bard et al., 2004; Chaput et al., 2005). The first characterised tetraspanin was DC63 (Modderman, 1989), which is also known as lysosomal integral membrane protein LIMP-1 (Fukuda, 1991). In particular, as a cell surface glycoprotein, CD63 as well as other tetraspanins are known to complex with integrins (Berditchevski, 2001; Yunta & Lazo, 2003). CD63 exists in the cytoplasmic membranes of most cells and abundantly in the cytoplasmic compartment, such as endosomes, lysosomes and secretory vesicles (Duffield et al., 2003). The other most common member of tetraspanin is CD9, which is also known as MIC3, MRP-1, BTCC-1, DRAP-27, TSPAN29, TSPAN-29 (Erovic et al., 2003). Its functions involve differentiation, adhesion and signal transduction (Hemler, 2005). It is widely expressed in various types of tumor cells as well as normal hematopoietic, smooth muscle, endothelial and epithelial cells (Kwon et al., 2014). The expression of this CD9 gene plays a critical role in the suppression of cancer cell motility and metastasis (Fan et al., 2010).

**Exosomal biomarkers**

Biomarkers are powerful tools in understanding the spectrum of pathological conditions of a disease as well as in predicting drug responses (Angulo et al., 2012). EXOs have shown to have many qualities that make them excellent candidates as biomarkers. Over the past few years, numerous studies have demonstrated that the number of EXOs in body fluids may change significantly with disease progression (Skog et al., 2008; Qazi et al., 2010; Masyuk et al., 2013). Furthermore, EXOs can be used as targeted drug delivery systems; this makes them particularly attractive in the delivery of pharmaceutical proteins and short interfering RNA (siRNA). EXOs are highly stable in biological fluids; they can be isolated from blood and urine, making them very attractive targets for diagnostic application (Cheng et al., 2014; Muller et al., 2014; Mu et al., 2014).

**Methods**
Anthropometric measurement

Body weight (kg) and height (m) for all the subjects were measured to calculate the body mass index (BMI). The body weight was measured while wearing light clothes and no shoes. The precision was up to 0.1kg and the height up to 0.1cm. The BMI was calculated by dividing the weight in kilograms by the height in meters squared (kg/m²).

Preparation of plasma

Blood samples (3 ml) from participants were centrifuged at 2500Xg for 10 min at room temperature to separate the plasma from other blood content. The plasma for each sample was then aliquoted and immediately stored at -80°C until further analysis.

Metabolic characteristics analysis

The levels of glucose, total cholesterol (mg/dl), TAG (mg/dl), HDL (mg/dl) and LDL (mg/dl) were measured by using Cobas c system (Rohe, Hitachi, USA).

Preparation of 1xPBS buffer

A 1xPBS buffer was prepared by adding 10x PBS (100 ml) to deionised water (900 ml).

2.2.5 Isolation of EXOs from blood plasma

Frozen plasma samples from different donors were thawed gradually from -80°C to room temperature until samples were completely thawed. The plasma samples were centrifuged at 3000Xg for 10 min to remove any residual debris. Then, they were transferred to a fresh Eppendorf tube held on ice until use. Next, each plasma sample (200 µl) was combined with 800 µl of 1XPBS and then filtered through a 0.22 µm pore filter using 5 ml of disposable syringe (SET/SHANCHUAN). The filtrated samples were transferred into conical tubes containing 50µl of CD326 EpCAM micro-beads (EXOs-beads) and then vortexed and stored at 4°C for 24 hr.

2.2.6 Purification of EXOs

In the purification of EXOs, MACS separation columns were washed 3 times using the diluted MACS BSA stock solution (1:20) with auto-MACS rinsing solution. During the
washing process, the columns were placed in an extremely strong magnetic field (quadro MACS). Then, the EXOs-beads were pipetted into the columns and washed 3 times with 1ml of rinsing solution. The columns were removed from the solid support magnetic field (SSM), and the EXOs-beads were recovered by rinsing the columns 3 times at room temperature with 1ml of rinsing solution. The final step was conducted by adding 1ml of 1XPBS, aliquot into two tubes and stored at -20°C for further analysis.

2.2.7 Detection of EXOs
EXOs-beads were centrifuged at 10,000 rpm twice at 4°C for 10 min to precipitate EXOs-beads. The supernatants were discarded and then the precipitated EXOs bound micro-beads were suspended in 400µl of 1XPBS. Two tubes of each sample were stained. The first was stained with 5µl of Human CD63 R-PE conjugate and the other tube was stained with 10µl of Human CD9–APC. The levels of EXOs were detected by flow-cytometry (Beckman Coulter, USA).

2.2.8 Statistical analysis
The Statistical Package for Social Sciences (SPSS, Chicago, IL, USA) 20 program was used for the statistical analysis. Comparison of the measured parameters between controls and diabetics was calculated using an unpaired sample t-test. Correlations between the measured parameters were performed using the Pearson correlation. The results were presented as the mean ± standard deviation (Mean ± SD). Statistical significance was set at a P-value of < 0.05 for all the analysis.

Results
Detection of EXOs
EXOs that are positive for EpCAM were stained by CD9 and CD63 fluorescent antibodies in separate staining process to detect their levels by flow-cytometry. The results show that the level of +CD9 EXOs detected by dot plot from diabetics and
controls is lower than the level of +CD63 EXOs (Figures 1 and 2). The results shown in Figure 3 suggest that +CD9 EXOs on the surface of epithelial derived EXOs do not always share the same EXOs with +CD63 EXOs.
Figure 1. Detection of EXOs. (A) Level of +CD9 EXOs in T2D male patients. D1: for stained +CD9 EXOs; D2: for stained +CD9 EXOs and CD63 EXOs; D3: for un-stained EXOs; D4: for stained +CD63 EXOs. (B) Level of +CD63 EXOs in T2D male patients. D1: for stained +CD9 EXOs; D2: for stained +CD9 EXOs and CD63 EXOs; D3: for un-stained EXOs; D4: for stained +CD63 EXOs.

Figure 2. Detection of EXOs. (A) Level of +CD9 EXOs in male controls. D1: for stained +CD9 EXOs; D2: for stained +CD9 EXOs and CD63 EXOs; D3: for un-stained EXOs; D4: for stained +CD63 EXOs. (B) Level of +CD63 EXOs in male controls. D1: for stained +CD9 EXOs; D2: for stained +CD9 EXOs and CD63 EXOs; D3: for un-stained EXOs; D4: for stained +CD63 EXOs.
Figure 3 Detection of EXOs. Mixed stain of +CD9 EXOs and +CD63 EXOs. D1: for stained +CD9 EXOs; D2: for stained +CD9 EXOs and CD63 EXOs; D3: for un-stained EXOs; D4: for stained +CD63 EXOs.

The levels of +CD63 EXOs and +CD9 EXOs in the diabetics and the controls
The levels of +CD63 EXOs and +CD9 EXOs in diabetics and controls were analysed, and the result showed no significant difference (P = 0.882 and P = 0.207, respectively) between the two groups. Therefore, the groups were divided into sub-groups based on their smoking status. The levels of +CD63 EXOs and +CD9 EXOs of the smokers and the non-smokers in diabetics and controls are shown in Table 1. The results show that the smokers have significantly higher +CD63 EXOs and +CD9 EXOs levels than the non-smokers in the control group (P=0.001, P=0.001; respectively). In the diabetic group, the smokers had a significantly higher +CD63 EXOs level than the non-smokers (P=0.05). Through the comparison of the levels of +CD63 EXOs and +CD9 EXOs between controls and diabetics in the smokers and non-smokers, the results showed significant higher (P= 0.04) levels of +CD63 EXOs and +CD9 EXOs (P=0.001) in the non-smokers (Table 2).

Correlation between different characteristics in the control group
The correlation between the characteristics of controls showed a significant correlation between +CD9 EXOs and +CD63 EXOs levels \((P = 0.0001)\), positive correlation between +CD63 EXOs level and HDL level \((P=0.04)\) and a positive correlation between +CD9 EXOs level and HDL level. The results are presented in Table 3, Figure 4.

Table 1. The levels of +CD9 EXOs and +CD63 EXOs of smokers and non-smokers in controls and diabetics.

<table>
<thead>
<tr>
<th>Levels of the exosomal proteins</th>
<th>Controls ((n = 30)) Mean ± SD</th>
<th>Diabetics ((n = 30)) Mean ± SD</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Smokers ((n = 17))</td>
<td>Non Smokers ((n = 13))</td>
<td>(P)-value</td>
<td>Smokers ((n = 7))</td>
</tr>
<tr>
<td>Level of +CD9 EXOs</td>
<td>25.7 ± 19.3</td>
<td>5.8 ± 1.8</td>
<td>0.001**</td>
</tr>
<tr>
<td>Level of +CD63 EXOs</td>
<td>65.6 ± 7.1</td>
<td>56.4 ± 6</td>
<td>0.001**</td>
</tr>
</tbody>
</table>

Abbreviations: standard deviation, SD; \(n\), no. of subjects. Data are expressed as mean ± SD. Comparison between the levels of +CD9 EXOs and +CD63 EXOs of smokers and non-smokers in controls and diabetics were calculated using Unpaired Student’s t-test and Mann-Whitney test. *significant; **highly significant.

Table 2. Comparison of +CD63 EXOs and +CD9 EXOs levels between controls and diabetics in smokers and non-smokers.

<table>
<thead>
<tr>
<th>Levels of the</th>
<th>Smokers Mean ± SD</th>
<th>Non-smokers Mean ± SD</th>
</tr>
</thead>
</table>
Abbreviations: SD, standard deviation; n, no. of subjects. Data are expressed as mean ± SD. Comparison between diabetics and controls were calculated using Unpaired Student’s t-test. *significant;**highly significant

Table 3. Correlation between the characteristics of the control group.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Glucose (mg/dl)</th>
<th>TAG (mg/dl)</th>
<th>Cholesterol (mg/dl)</th>
<th>HDL (mg/dl)</th>
<th>LDL (mg/dl)</th>
<th>+CD63 EXOs</th>
<th>+CD9 EXOs</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>0.2 (0.4)</td>
<td>0.05 (0.8)</td>
<td>0.1(0.8)</td>
<td>-0.04 (0.9)</td>
<td>0.04(0.8)</td>
<td>-0.13 (0.5)</td>
<td>-0.02 (0.9)</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>0.2 (0.3)</td>
<td>-0.04 (0.83)</td>
<td>-0.4 (0.03)</td>
<td>-0.06 (0.7)</td>
<td>-0.14 (0.46)</td>
<td>-0.3 (0.1)</td>
<td></td>
</tr>
<tr>
<td>TAG (mg/dl)</td>
<td>0.6 (0.001)</td>
<td>-0.5 (0.002)</td>
<td>0.08 (0.7)</td>
<td>-0.29 (0.12)</td>
<td>-0.2 (0.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>0.01 (0.95)</td>
<td>0.8 (0.0001)**</td>
<td>-0.07 (0.7)</td>
<td>-0.07 (0.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>0.01 (0.6)</td>
<td>0.04 (0.04)*</td>
<td>0.4 (0.03)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Abbreviations: BMI, body mass index; TAG, triacylglycerol; HDL, high density lipid; LDL, low density lipid; r, spearman rank order correlation; P, P-value. Correlations between the measured parameters were calculated using the Pearson Correlation. *significant; **highly significant.

<table>
<thead>
<tr>
<th></th>
<th>LDL (mg/dl)</th>
<th>+CD63 EXOs</th>
<th>+CD9 EXOs</th>
<th>+CD63 EXOs and HDL</th>
<th>+CD9 EXOs and +CD63 EXOs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-0.09</td>
<td>0.7</td>
<td></td>
<td>0.38, P = 0.04</td>
<td>0.7, P = 0.0001**</td>
</tr>
<tr>
<td></td>
<td>(0.63)</td>
<td>(0.0001)**</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 4. Correlation between different characteristics in the control group.** (A) Correlation between +CD63 EXOs level and HDL level in the control group. Weak positive correlation (r = + 0.38, P = 0.04) between +CD63 EXOs and HDL levels. (B) Correlation between +CD9 EXOs and +CD63 EXOs levels in the control group. Strong positive correlation (r = + 0.7, P = 0.0001) between +CD9 EXOs and +CD63 EXOs levels.

**Correlation between different characteristics in the diabetic group**
The correlation between the characteristics of the diabetic group showed a significant correlation between +CD9 EXOs and +CD63 EXOs levels (P = 0.0001) and a positive correlation between +CD63 EXOs and glucose level (P = 0.06). The results are presented
in Table 4 and Figure 5.

Table 4. Correlation between the characteristics of the diabetic group.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Glucose (mg/dl)</th>
<th>TAG (mg/dl)</th>
<th>Cholesterol (mg/dl)</th>
<th>HDL (mg/dl)</th>
<th>LDL (mg/dl)</th>
<th>+CD63 EXOs</th>
<th>+CD9 EXOs</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>-0.15 (0.42)</td>
<td>0.27 (0.14)</td>
<td>0.18 (0.3)</td>
<td>0.02 (0.9)</td>
<td>0.004 (0.1)</td>
<td>0.2 (0.4)</td>
<td>-0.1 (0.6)</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>-0.06 (0.85)</td>
<td>0.20 (0.3)</td>
<td>0.04 (0.8)</td>
<td>0.3 (0.2)</td>
<td>0.4 (0.06)*</td>
<td>0.1 (0.6)</td>
<td></td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>0.42 (0.014)*</td>
<td>-0.5 (0.003)*</td>
<td>0.10 (0.6)</td>
<td></td>
<td>0.1 (0.7)</td>
<td>-0.1 (0.5)</td>
<td></td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td></td>
<td>0.10 (0.6)</td>
<td>0.88 (0.0001)**</td>
<td>0.2 (0.4)</td>
<td>-0.1 (0.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td></td>
<td></td>
<td>0.03 (0.9)</td>
<td>-0.1 (0.5)</td>
<td>-0.1 (0.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td></td>
<td></td>
<td></td>
<td>0.2 (0.3)</td>
<td>0.005 (0.98)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+CD63 EXOs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.6 (0.0001)**</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: BMI, body mass index; TAG, triacylglycerol; HDL, high density lipid; LDL, low density lipid; r, spearman rank order correlation; P, P-value. Correlations between measured parameters were calculated using the Pearson Correlation. *significant; **highly significant
Discussion

The current literature demonstrates that chronic diseases with an inflammatory component are characterised by an increase in the release of exosomes (EXOs) into plasma. T2D is the most common type of diabetes in humans. Efforts are underway to find T2D biomarkers that can be analysed in a single blood sample. Circulating EXOs are now warranted to advance upon finding these markers. Recently, an increasing number of exosomal membrane proteins have been found to be potential biomarkers in the clinical diagnostics for a variety of diseases, including cancer and liver and kidney diseases. Although there is limited data on the clinical applicability of exosomal biomarkers for prediction of T2D progression, quantification and comparison of EXOs in blood of controls and T2D patients may exhibit the potential as a screening biomarker for T2D.

The primary aim of this study was to isolate, characterise and demonstrate that epithelial derived EXOs reflect the physiological status of the T2D patients. Our key findings are as
follows: 1) +CD63 EXOs and +CD9 EXOs percentage was significantly higher in men with T2D and were primarily derived from epithelial cells. +CD63 EXOs percentage was shown to be correlated strongly and positively with +CD9 EXOs percentage in T2D patients and control individuals. 2) +CD63 EXOs percentage was shown to be correlated strongly and positively with +CD9 EXOs percentage in diabetics and controls. 3) The percentage of +CD63 EXOs was shown to be correlated positively with glucose in diabetic subjects. 4) Men belonging to the control group exhibited +CD63 EXOs and +CD9 EXOs percentage correlated with HDL.

The present study demonstrates that the percentage of +CD63 EXOs and +CD9 EXOs increase in patients with T2D, where they can contribute to disease progression. Our result upon comparison with the published results confirms the findings of Alberti et al. and Shareef et al., who investigated the effects of T2D on oral epithelial cells (Alberti et al., 2003 and Shareef et al., 2008). Observations from previous studies have shown that hyperglycaemia induces an increase in cell cycle progression and DNA synthesis in breast cancer cells (Yamamoto et al., 1999 and Masur et al., 2011). Another study demonstrated that hyperglycaemia clearly increases proliferation of both non-tumorigenic and malignant mammary epithelial cells (Lopez et al., 2013). Our result revealed a promising biomarker (+CD9 EXOs) for use in diagnosis, assessment and prognosis. The relation between EXOs and progression of the disease makes EXOs and their membrane proteins very valuable biomarkers in a clinical setting. It is clear that a deeper understanding of their molecular mechanisms that lead to diseases will contribute to the development of more effective therapies.

In this study, the smoking status were found to effect the levels of +CD63 EXOs and +CD9 EXOs. Male smokers were found to exhibit an increase in the levels of +CD63 EXOs and +CD9 EXOs compared to non-smoking males. Therefore, that they can serve as potential biomarkers in smoking-related disease. Furthermore, the results have shown
that the level of +CD63 EXOs was correlated with the level of sugar in the diabetic males. It is well known that EXOs are usually secreted in response to local environmental factors, such as sugars. It can be concluded that +CD63 EXOs may accompany or even induce the development of diabetes and obesity-linked diabetes.

In summary, EXOs possess unique molecular characteristics that are promising in the development of biomarkers for early detection of T2D. Further characterisation of EXOs, their mechanisms of action and their relation with different stages of diseases will undoubtedly provide the basis for a deeper understanding of the role of EXOs in the T2D progress. Further studies are needed in order to validate an optimal set of markers that could provide the best diagnostic performance. The future challenge will be to identify the EXOs signatures that are specific to cells and tissues of origin involved in the pathogenesis of T2D: pancreatic beta cells, liver, skeletal and smooth muscle, adipose tissues, endothelial cells and macrophages.

**Conclusion**

This study is the first to investigate the presence of exosomal membrane bound proteins CD9 and CD63 as potential biomarkers for T2D. The levels of +CD63 EXOs and +CD9 EXOs in T2D, originating from epithelial cells, show an increase in comparison to controls. This result concludes that +CD9 EXOs and +CD63 EXOs can help predict the onset of T2D, which have the potential to contribute as biomarkers for T2D. Although the initial results are promising, further investigations are required to assess the clinical value of these EXOs in T2D.

**Abbreviations**

BMI: Body mass index; DNA: Deoxyribonucleic acid; EpCAM: Epithelial cell adhesion molecule; EVs: Extracellular vesicles; EXOs: Exosomes; HDL: High density lipid; LDL: Low density lipid; MACS BSA: Magnetic-activated cell sorting- bovine serum albumin; miRNAs: Micro RNA; mRNA: Messenger ribonucleic acid; PBS: Phosphate buffer saline; RNA: Ribonucleic acid; SD: Standard deviation; ssDNA: Single-strand
DNA; SSM: Solid support magnetic field; T2D: Type 2 diabetes; TAG: Triacylglycerol; USA: United States of America; WHO: World Health Organization.

Acknowledgements
Completing this project has been an absolute pleasure, and would not have happened without God blessing. I would like to thank Dr. Ahmad Al-shaal for his guidance concerning flow-cytometry. I also like to thank my parents for allowing me to realize my own potential. All the support they have provide me over the years was the greatest gift anyone has ever given me. In addition, a special thanks to my brother Diab, for his continuous support. Without all, I may never gotten to where I am today. Finally, I would like to thank King Abdul-Aziz city for science and technology for financial support to the project (AT-63-752).

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