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Untargeted metabolomics reveals the impact of Liraglutide treatment on metabolome profiling and metabolic pathways in type-2 diabetes mellitus

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ABSTRACT

Liraglutide, a type2 diabetes mellitus (T2DM)-related treatment, improves glycemic control and reduces the risks of adverse cardiovascular events in T2DM patients. However, the underlying mechanisms of the above-mentioned beneficial effects of Liraglutide are not well understood. To have better understanding of these mechanisms, we aimed to study the metabolic impacts of Liraglutide on the metabolome and corresponding pathways in T2DM patients, especially metabolism plays a very fundamental role in health and diseases and is influenced by drugs. In this study, plasma samples collected from T2DM patients (n = 20) and taken pre- and post-Liraglutide treatment were used for untargeted metabolomics analyses, including metabolome profiling and metabolic pathway/network analyses. The metabolome profiling analyses identified 93 endogenous metabolites that were significantly affected by Liraglutide treatment where 49 and 44 metabolites were up and down regulated, respectively. Liraglutide caused metabolic alterations impacting metabolic pathways such as pentose and glucuronate interconversion and alanine, aspartate and glutamate metabolism in T2DM patients. Since the last-mentioned pathways are affected by Liraglutide, it could explain partially the overall beneficial effects of Liraglutide in T2DM, especially that glucuronate interconversion pathway is known by its important roles in eliminating toxic and undesirable substances from the human body to maintain good health status. In addition, the metabolism of amino acids induced by Liraglutide could improve the function of immune cells, strengthening the immunity of T2DM patients. Also, Liraglutide induced the level of other metabolites that help in the defense mechanism against oxidative events. Overall, the findings of this study provide a deeper understanding of the underlying mechanisms involved in the beneficial effects of Liraglutide in T2DM from the metabolic aspect.

1. Introduction

Globally, type-2 diabetes mellitus (T2DM) has persistently remained as a chronic metabolic disease characterized by elevated blood glucose levels due to insulin deficiency or resistance (American Diabetes Association 2009), and it is accompanied by multiple health complications such as obesity, cardiovascular diseases, and nonalcoholic fatty liver diseases. T2DM continuously affects many individuals, making its incidence very high over the years, impacting the health and care systems (Pearson-Stuttard et al., 2022). Due to the harmful effects of T2DM, researchers and clinicians have put efforts into the field of T2DM to overcome this disease and its related complications by understanding its

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leading factors and underlying mechanisms and give treatments and therapeutic interventions. T2DM can be developed by unhealthy habits, *e.g.* unhealthy diet and physical inactivity. The last habits can be targeted for T2DM treatment, thus, T2DM patients are advised to follow recommended healthy diets and perform physical activities in their daily lifestyles (Galaviz et al., 2018). However, these therapeutic interventions might not effectively control the increased blood glucose level for certain T2DM patients, especially those who have undergone countless weight loss failures. Therefore, in addition to the previously mentioned therapeutic intervention, those T2DM patients require further therapies/drugs that can help in the control of blood glucose level. There are many blood glucose level-controlling medications that have been prescribed for T2DM patients including glucagon-like peptide-1 (GLP-1) analogues (Bagepally et al., 2020), mimicking the natural incretin hormone GLP-1 produced by intestinal cells in response to food intake (Holst et al., 1987).

GLP-1 analogues include a drug called Liraglutide, which is a long-acting GLP-1 receptor agonist designed with 97 % sequence homology of the nature peptide hormone GLP-1. Liraglutide has its own advantages of being one of the GLP-1 analogues with less hypoglycemic effects compared to other GLP-1 analogues (Garber et al., 2011). Besides its lowering effect on the blood glucose level, Liraglutide has shown to improve other health outcomes, for instance, increased body-weight loss, reduced potential risks of cardiac diseases and fatty liver diseases (Wajcberg and Amarah 2010). Although Liraglutide has been well known by its beneficial health outcomes, the underlying mechanisms of these improved health outcomes are not fully understood and require further exploration. Thus, we performed previously a proteomic study by our research group (Ekhzaimy et al., 2022), focusing on the Liraglutide impacts on the overall health in T2DM and obesity conditions. In our previous proteomic study, we studied the effect of Liraglutide treatment on the protein profiling of T2DM patients before and after receiving a short-course of Liraglutide treatment and on finding underlying pathways and network related to those protein changes (Ekhzaimy et al., 2022). Since proteins are complementary to metabolites, we aimed in this current study to explore the impact of the short-course of Liraglutide treatment on the metabolic profiling of T2DM patients. Having comprehensive understanding of the proteomic and metabolic changes induced by Liraglutide treatment in T2DM patients could significantly improve our knowledge about the underlying biological mechanisms involved in the enhanced health outcomes observed in the diabetic patients treated with Liraglutide.

Untargeted metabolomics, the unbiased study detecting most metabolites with small molecular weight of less than 1.5 kDa in biological samples, has become an indispensable research tool in several research fields in health and diseases (Phapale 2021). For the sake of this study, we perform a comprehensive metabolomics study to examine the effects of Liraglutide on the plasma metabolome profiling of T2DM patients by utilizing mass-spectrometry-based metabolomics analyses. Also, we performed this study to uncover unappreciated metabolic pathways affected by Liraglutide treatment in a tissue-dependent manner.

2. Materials and methods

2.1. Ethical approval, study design and patient recruitment

The recruitment of patients and sampling are approved by the Institutional Review Board of the College of Medicine, King Saud University, Riyadh, Saudi Arabia (registration no. E-18-3075).Recruited patients were asked to sign a written informed consent form before enrolling in this study. Twenty patients (n = 20) (12 males and 8 females) who were diagnosed with T2DM (HbA1c between 8 % and 12 %) were referred to the King Khaled University Hospital's (KKUH), Obesity Research Center, where this study was conducted. Initially, blood samples of T2DM patients were taken before the Liraglutide treatment as pre-treatment samples. Next, the same T2DM patients were given an appropriate dose of Liraglutide (Victoza) for three months as described previously (Ekhzaimy et al., 2022). After the treatment course was finished, blood samples of treated T2DM patients were collected, representing post-treatment samples. Considerably, the T2DM participants were on other medications including insulin and metformin beside the Liraglutide treatment.

2.2. Clinical data collection and blood sample processing

Clinical and biochemical characteristics of the study patients were recorded before and after the Liraglutide treatment and were reported in our previously published study (Ekhzaimy et al., 2022). Blood samples were drawn from the twenty patients pre- and post-Liraglutide treatment, thus, in total, forty samples (n = 40) were collected. The blood samples were used for plasma metabolite extraction as previously described (Jaber et al., 2022) Briefly, 100 µL plasma sample were mixed with 900 µL of extraction solvent consisting of 50 % acetonitrile (ACN) and 50 % methanol (MeOH). While samples are prepared, quality control (QC) samples were also prepared by mixing equal volumes of each sample and blended well to represent the entire collection of samples included in the study and to check for system stability.

After, all the prepared samples were mixed on thermomixer at 600 rpm at room temperature for one hour (Eppendorf, CITY, Germany). Next, the samples were centrifuged at 16000 rpm at 4 °C for 10 min resulting in the formation of the supernatant that was transferred into new Eppendorf tubes. The supernatant was entirely evaporated in a SpeedVac (Christ, Germany). The SpeedVac-dried samples were reconstituted with 100 µl of 50 % mobile phase A:B (A: 0.1 % Formic acid in dH_2O, B: 0.1 % Formic acid in 50 %ACN:MeOH).

2.3. LC-MS-based metabolomics analysis

Metabolomics analysis of extracts was conducted using the Waters Acquity UPLC system coupled with an electrospray ionization source (ESI)-containing a Xevo G2-S QTOF mass spectrometer. Furthermore, the extracted metabolites were chromatographed using an ACQUITY UPLC using XSelect (100 × 2.1 mm 2.5 µm) column (Waters Ltd., Elstree, UK), the mobile phase A: B in which part A is composed of 0.1 % formic acid in dH_2O as solvent A, and B consists of 0.1 % formic acid in 50 % ACN: MeOH. The workflow of the chromatography run was done based on the gradient elution schedule as follows: 0–16 min 95–5 % A, 16–19 min 5 % A, 19–20 min 5–95 % A, 20–22 min 95–95 % A, at 300 µL/min flow rate. MS spectra were acquired under positive and negative electrospray ionization modes (ESI+, ESI-). The parameters and conditions of MS were source temperature (150 °C), the desolvation temperature [500 °C (ESI+) or 140 (ESI−)], the capillary voltage 3.20 kV (ESI+) or 3 kV (ESI−), cone voltage (40 V), desolvation gas flow (800.0 L/h), cone gas flow (50 L/h). In MSE mode, the settings of the collision energies of low and high function were at off and 10 V to 50 V respectively. Sodium formate was used for the calibration of mass spectrometer prior the MS analyses. The generated MS data were collected with Masslynx™ V4.1 workstation (Waters Inc., Milford, Massachusetts, USA) in continuum mode to be used for the downstream data processing and statistical analysis.

2.4. Data processing and statistical analyses

Profile LC–MS raw data were imported into a software called Progenesis QI v.3.0 from Waters (Waters Technologies, Milford, MA., USA) for processing. The LC-MS were processed following a standard pipeline starting from alignment based on the *m/z* value and the ion signals' retention time, peak picking, and signal filtering based on the peak quality. Features detected in at least 50 % of the samples were retained for further analyses. After that, the processed data were statically analyzed with multivariate and univariate analyses. Particularly, for the multivariate statistical analysis, the data were imported into

MetaboAnalyst version 5.0 (McGill University, Montreal, Canada) (https://www.metaboanalyst.ca) that is based on KEGG pathway (Pang et al., 2021). The proper statistical model was performed on the datasets by using the following parameters, the datasets (compounds and abundances) were mean-normalized, Pareto-scaled, and log-transformed to maintain their normal distribution. The normalized datasets were used to generate supervised analysis partial least squares-discriminant analysis (PLS-DA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) models; the last tests illustrated the difference degree between two variables and enhanced the interpretation of the metabolic differences between the pre-and post-treatment groups by excluding information that did not influence the discrimination and features. OPLS-DA models were evaluated by using two elements including the fitness of model (R2Y) and predictive ability (Q2) values that use permutation validation of 100 samples (Worley and Powers 2013) and pathway analysis based on KEGG pathway.

For univariate analysis, Mass Profiler Professional software (Agilent, Santa Clara, CA, USA). was used for dual comparison between pre- and post-Liraglutide treated T2DM patients using (*t*-test paired, no correction p value $\leq$ 0.05, FC >=1.5), which led to identify significantly altered mass features with the treatment, as described previously (Gu et al., 2020). Furthermore, heat map analysis was performed for the altered features using distance measure of according to the Pearson similarity test. Lastly, network pathway analysis was applied on the altered features by using QIAGEN Ingenuity Pathway Analysis (IPA). Next, the significantly altered features that were found with the statical analyses were used for metabolite identification analyses to explore the names of the metabolites that were impacted with Liraglutide treatment.

2.5. Metabolites identification and selection

To identify the metabolite names of the significant features, the processed LC-MS dataset that was imported into Progensis IQ software were used for selecting and tagging for peak annotation. The identification was based on the chemical structures of metabolites that were identified by acquiring their accurate precursor masses, fragmentation pattern, and isotopic distribution provided by Human Metabolome Database (HMDB) (Wishart et al., 2022), and METLIN MS/MS (www.metlin.scripps.edu). Importantly, the exogenous compounds such as drugs, food additives, and environmental compounds were excluded from the final list of the identified metabolites to include only endogenous metabolites affected by Liraglutide treatment.

3. Results

3.1. Clinical and biochemical data analysis

Clinical and biochemical characteristics of the study participants pre- and post-treatment have been previously mentioned in our earlier study (Ekhzaimy et al., 2022) that is linked to our current metabolomics study. Briefly, the participants in the study were patients with T2DM who were aged 54.4 ± 9.5 years. A statistically significant change was noted in the HbA1c levels ($p \leqslant 0.006$) with a change of 1.1 % from the baseline after treatment with liraglutide for a 3-month duration. No significant changes in other parameters such as body weight, BMI, renal function markers, or markers of dyslipidemia were noted compared to the pre-treatment data.

3.2. Metabolomics profiling of plasma collected from T2DM patients received Liraglutide treatment

In order to examine the metabolic effect of Liraglutide on the metabolic profiling of T2DM patients, we used plasma samples collected from T2DM patients before and after Liraglutide treatment. These plasma samples were used for untargeted metabolomics analyses in order to identify affected metabolites, our data showed that a total of 17,255 mass ion features were detected in the 40 plasma samples collected from 20 T2DM patients (20 pre-treatment and 20 post-treatments with liraglutide) at both positive and negative ionization modes. After missing values exclusion and imputation, 13,870 features were remaining. These ions (13870 ions) were used in the separation between pre- and post-treated liraglutide diabetic groups (Fig. 1**A**). Permutation analysis with R2Y = 0.942 and Q2 = 0.517 are displayed in (Fig. 1**B**).

Univariate analysis was performed to identify statistically significant distinct features between pre- and post-treated liraglutide diabetic groups using Volcano Plot analysis (paired *t*-test, no correction p-value $\leq$ 0.05, FC 1.5), revealing 330 significantly affected metabolites, which 147 and 183 metabolites were up-and down-regulated in post-treated liraglutide diabetic groups compared with pre-treated group respectively. After excluding exogenous molecules (*i.e.*, drug metabolites, environmental exposures, *etc*.) were excluded. Thus, the remaining significant endogenous metabolites were 93 metabolites (Fig. 2). Overall, 49 metabolites were up-regulated and 44 metabolites were down-regulated in pre- Liraglutide and post-Liraglutide treated diabetic patient groups. A heatmap based on Pearson's correlation coefficient and average linkage methods representing the 93 affected metabolites. The 49 up-regulated metabolites and 44 down-regulated metabolites have been shown in (Fig. 3**A and B**).

3.3. Metabolomic pathway analysis

Pathway analyses were performed on the 93 significantly affected metabolites in order to identify the most impacted pathways in Liraglutide-treated T2DM patients. Considerably, the most highly impacted metabolic pathways targeted by Liraglutide with an impact score of $\geq$0.10 were pentose and glucuronate interconversions and alanine, aspartate and glutamate metabolism (Fig. 4). Although, there were other metabolic pathways were shown to be impacted but with less or no impact and these pathways are sphingolipid metabolism, primary bile acid biosynthesis, biosynthesis of unsaturated fatty acids, arginine biosynthesis and nitrogen metabolism. A list of pathways impacted in Liraglutide treated diabetic patients is shown in (Table 1**)**.

3.4. Network pathway analysis

Network analysis was conducted by using ingenuity pathway analysis (IPA) software to investigate the potential pathways associated with significantly altered serum metabolites related to patients with T2DM before and after receiving Liraglutide treatment. The highest-scoring network pathways identified between the two groups were related to immunological disease, inflammatory diseases, and inflammatory response (Fig. 5**A**). Moreover, the five most significantly enriched canonical pathways included the following: Stearate biosynthesis, $p = 9.56 \times 10^{-4}$ (with an overlap of 24 %, 2/84), Glutamine degradation I $p = 2.75 \times 10^{-3}$ (with an overlap of 20 %, 1/5), Glutamine biosynthesis I $p = 3.85 \times 10^{-3}$ (with an overlap of 14.3 %, 1/7), Asparagine biosynthesis I $p = 4.39 \times 10^{-3}$ (with an overlap of 12.5 %, 1/8), and L-glutamine biosynthesis II (tRNA-dependent) $p = 6.04 \times 10^{-3}$ (with an overlap of 9.1 %, 1/11) (Fig. 5**B**).

4. Discussion

In the clinical field, patients with T2DM are cured with various treatments to improve their clinical symptoms and complications. One of the prescribed treatments for T2DM is Liraglutide. The last has been used for many years due to its beneficial health outcomes observed in T2DM patients, including enhanced glucose-dependent insulin secretion, reduced glucagon secretion, improved blood glucose level, induced weight loss, decreased appetite, and lower risk factors of cardiovascular diseases (Armstrong et al., 2016, Marso et al., 2016, Bessesen and Van Gaal 2018). However, the underlying mechanisms of these beneficial

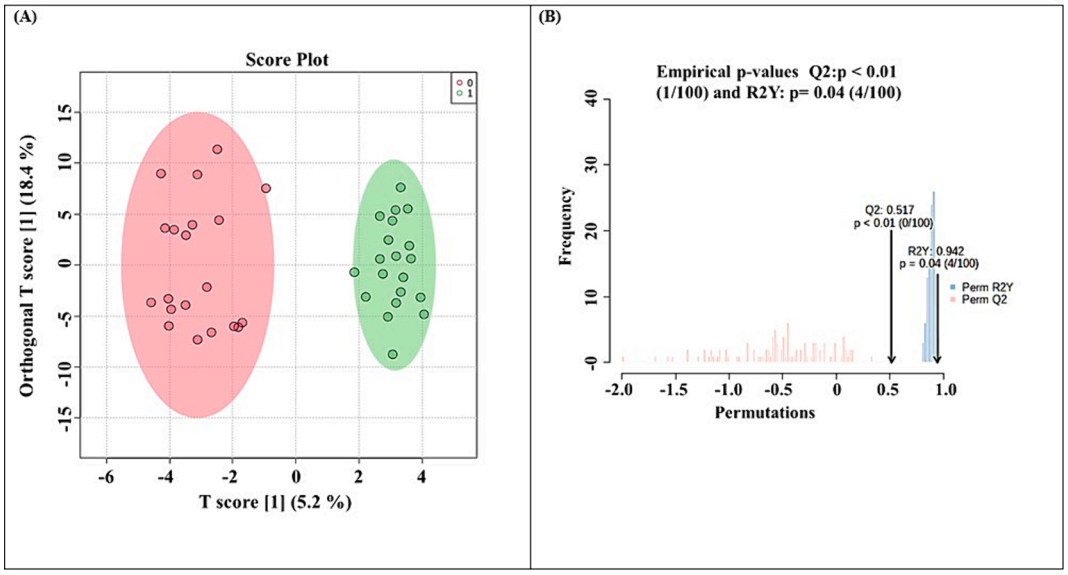

Fig. 1. **(A)** Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) shows a clear separation between the two groups (pre- and post-treated liraglutide diabetic groups). The robustness of the created models was evaluated by the fitness of the model (R2Y=0.942) and predictive ability (Q2 = 0.517) values in a larger dataset (n = 100). (**B**) Permutation analysis, showing the observed and cross-validated R2Y and Q2 coefficients.

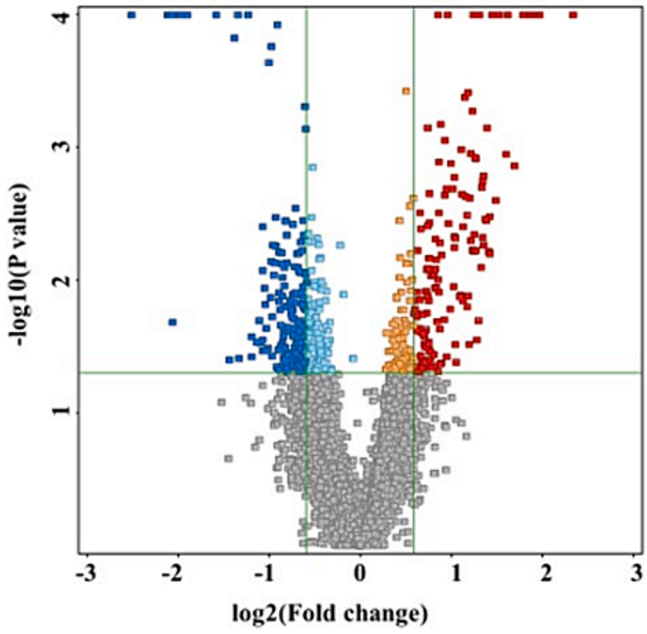

Fig. 2. Volcano plot between pre- Liraglutide and post-Liraglutide treated diabetic patients, after applying the 80 % filter on all data, A group of 330 affected exogenous and endogenous metabolites within the two groups (paired *t*-test- no correction, p-value $\leq$ 0.05, FC 1.5) were identified, 147 and 183 metabolites were up-regulated (red) and down-regulated (blue) between the two groups. 93 significant endogenous metabolites were identified 49 metabolites up-regulated and 44 metabolites down-regulated.

effects are not extensively elucidated yet. In order to have a better understanding of these underlying mechanisms mediated by Liraglutide in T2DM, we globally studied the impacts of Liraglutide on certain biological molecules, including proteins and metabolites in T2DM patients as these biological molecules are known to be very crucial to control various cellular or systemic mechanisms in the human body. Thus, our research group performed multiple mass-spectrometry-based studies on plasma samples collected from T2DM patients before and after Liraglutide treatment and studied these plasma samples to identify Liraglutide-mediated alterations in proteins and metabolites, which could potentially contribute to the beneficial effects of Liraglutide. In our previously published work, we showed that Liraglutide treatment improves cardio-metabolic profiling in T2DM patients by impacting 72 proteins, some of which are needed to decrease the levels of acute phase responses, leading subsequently to reduced systemic inflammatory state and oxidative stress, which partially contributes to the overall cardiac health outcomes in T2DM patients (Ekhzaimy et al., 2022). In this current study, we followed up on our previous work and explored the metabolic aspect of the beneficial effects of Liraglutide seen in T2DM patients. Thus, metabolomics analyses of plasma samples taken from the same T2DM patients who were participated in our previous study were conducted to perform metabolome profiling and pathways/networks analyses. Our metabolomics study shows the following findings: Liraglutide treatment alters the metabolome profiling of T2DM patients showing that 49 and 44 endogenous metabolites were up- and down-regulated, respectively. Furthermore, Liraglutide treatment impacts metabolic pathways in the T2DM patients, most importantly, pentose and glucuronate interconversion pathway and alanine, aspartate, and glutamate metabolism. These metabolic findings may contribute to the overall clinical improvements in the Liraglutide-treated T2DM patients as discussed below.

4.1. Liraglutide may cause systemic metabolic changes impacting the overall health outcome of T2DM patients

The efficacy and impact of Liraglutide on T2DM patients could be systemic at the whole-body level, affecting multiple tissues namely pancreas, heart, brain, intestine, kidney and liver (Heppner and Perez-Tilve 2015, Mehta et al., 2017, Alruwaili et al., 2021). The impact of Liraglutide on these tissues are due to the expression of Liraglutide receptors called GLP-1 receptors (GLP-1R) in these tissues (Gupta et al., 2010, Rigato and Fadini 2014, Knudsen and Lau 2019). Pharmacologically, when Liraglutide is circulating in the bloodstream and reaching the GLP-1R expressing-tissues, it mimics the action of the native GLP-1 by binding to its receptor, initiating cellular changes and activate cellular signaling pathways including cAMP/PKA/CREB signaling and PI3K/Akt and MAPK signaling (Candeias et al., 2015). These cellular changes in turn control the systemic glucose metabolism. Also, altered

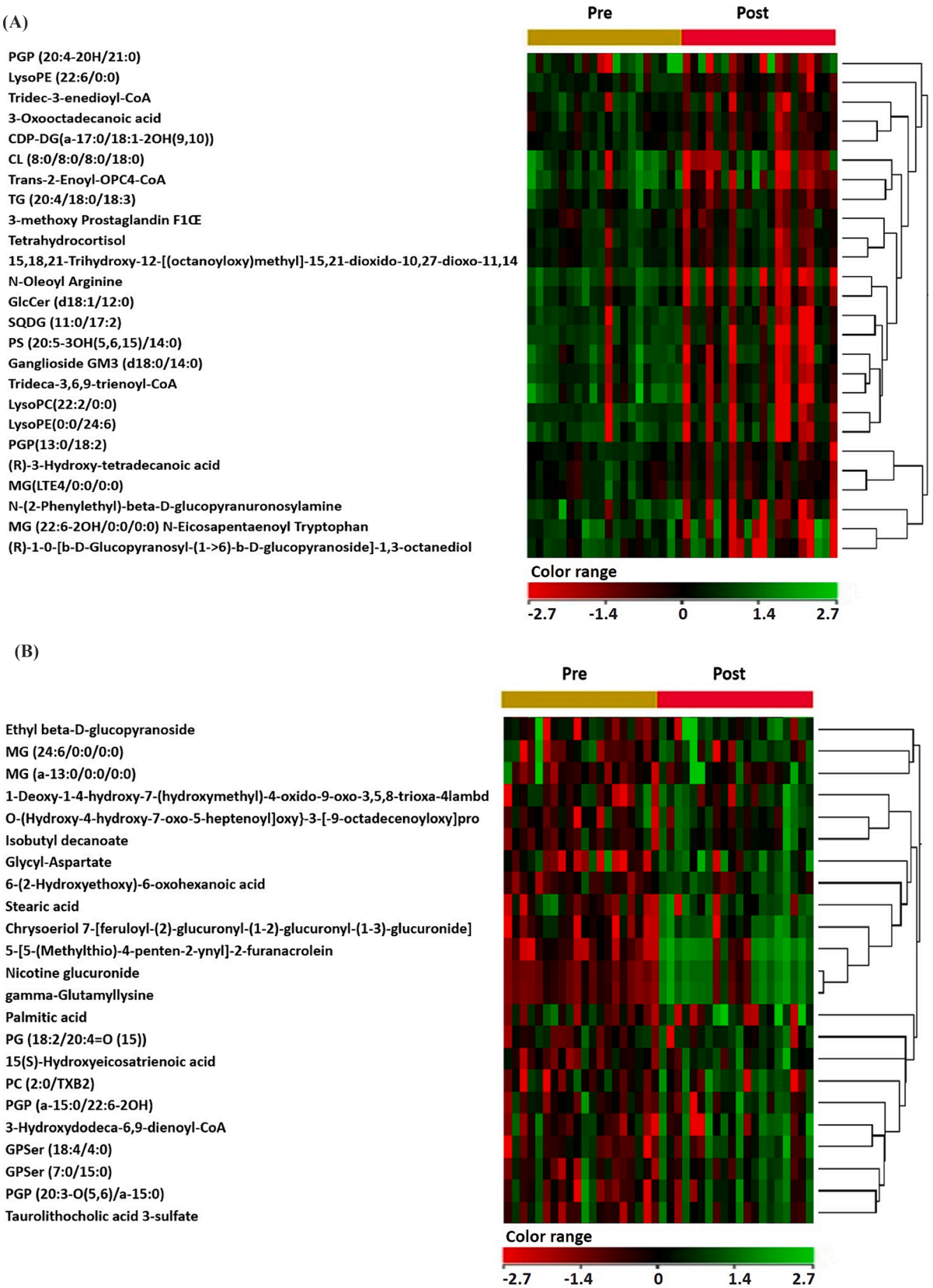

Fig. 3. Heatmap showing the changes in the 93 affected metabolites. The red color represents the trend of the downregulated metabolites and green represents the upregulation trend of metabolites. **(A)** Heatmap demonstrates 49 up-regulated metabolites and (**B**) 44 down-regulated metabolites in pre- and post-Liraglutide treated diabetic patients.

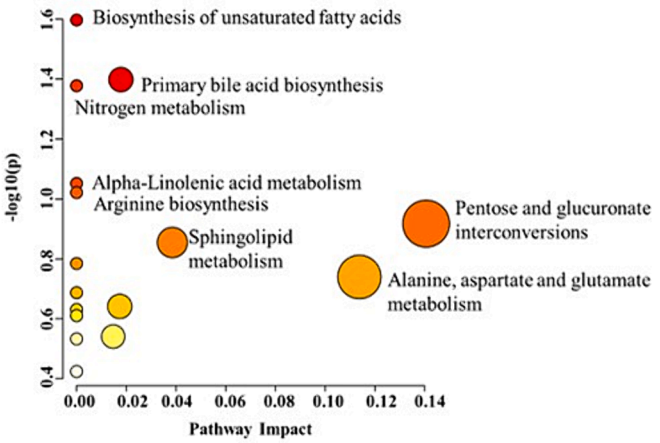

Fig. 4. Pathway analysis of significantly affected metabolites in pre- and post-Liraglutide treated diabetic patients.

metabolites identified in our study were involved in regulating signaling pathway such as insulin, MAPK1 and ERK1/2 pathways as demonstrated by IPA analyses. In addition to the systemic beneficial impact of Liraglutide at the whole-body level, Liraglutide may also improve the function of particular tissues and systems through certain metabolites and/or their related pathways as explained below.

4.2. Liraglutide may improve the cardiac health outcomes through regulating the lipid metabolism in T2DM patients

Regarding Liraglutide-mediated cardiac health benefits in the Liraglutide-treated T2DM patients, we showed in our previously published proteomics work and mentioned above that Liraglutide treatment improved cardiac-metabolic profile in T2DM patients, partially, by regulating important 72 proteins involved in the acute phase, the systemic chronic and inflammatory state and oxidative stress, which may improve the health outcomes of the heart in T2DM patients treated with Liraglutide (Ekhzaimy et al., 2022). In support with our previous findings, our current metabolomics study showed that certain lipid species, in particular triacylglycerol (TG), were decreased in the plasma samples taken from T2DM patients treated with Liraglutide in comparison with the plasma samples taken before the treatment. In line with our findings, work done by Taskinen, M-R. *et. al* showed that liraglutide treatment in T2D patients impacts chylomicron and VLDL kinetics and leads to decreased the generation of TG and their lipoproteins in the treated T2DM patients (Taskinen et al., 2021). In addition, mass-spectrometry-based untargeted lipidomics analyses of plasma samples collected from T2DM participant ($n = 102$) treated either with Liraglutide or placebo revealed that Liraglutide decreased the levels of ceramides, phospholipids and triglycerides (Zobel et al., 2021). Interestingly, our metabolomics data indicates that there are variations in the levels of phospholipids in response to the Liraglutide treatment which is controversial to the previously published findings (Zobel et al., 2021), and one reason could explain this contradictory findings is that the period of Liraglutide treatment used in our study was for 3 months, which is less-time than used in the other published work. Moreover, Liraglutide is reported to affect the metabolism and digestion of lipids. In our metabolomics study we showed that the primary bile acid and unsaturated fatty acids biosynthesis were impacted although the score of impact is low, but it could be explained by the acute duration of the treatment. The regulation and synthesis of bile acids are dependent on the proteins involved in the LXR/RXR and FXR/RXR pathways (Chiang 2009, Kemper 2011, Schulman 2017). In agreement with our findings and literature, our previous proteomics showed that LXR/RXR and FXR/RXR pathways were enriched and affected by the treatment of Liraglutide in the T2DM patients. However, further studies are required to fully understand how Liraglutide improves the lipid metabolism in the levels of tissues or whole-body system. Taken together, these findings are supportive to the notion that Liraglutide treatment could potentially reduce the risk of cardiovascular diseases in T2DM patients through lowering TG species and improving the metabolism of lipids, which are known to play important roles in the cardiovascular system health.

4.3. Liraglutide may potentially impact hepatic glucuronidation in T2DM patients

Notably, the treatment of Liraglutide also influences the hepatic processes related to the glucuronidation which is a very important process for the detoxification and elimination of undesired or toxic elements from T2DM patients (Yang et al., 2017), which may contribute to the overall beneficial systemic effects of Liraglutide. In details, our metabolome profiling identified an increased level of a metabolite called 6-Hydroxy-5-methoxyindole glucuronide in T2DM patients treated with Liraglutide. Furthermore, it is known that 6-Hydroxy-5-methoxyindole glucuronide is a metabolite produced in the liver by the action of enzyme called UDP glucuonyltransferase (Luukkanen et al., 2005), the

Table 1
List of pathways impacted in Liraglutide treated T2DM patients.

Pathway	Total	Expected	Hits	Raw p	FDR	Impact
Biosynthesis of unsaturated fatty acids	36	0.37161	2	0.051323	1	0
D-Glutamine and D-glutamate metabolism	6	0.061935	1	0.060454	1	0.01782
Nitrogen metabolism	6	0.061935	1	0.060454	1	0
Primary bile acid biosynthesis	46	0.47484	2	0.079427	1	0
alpha-Linolenic acid metabolism	13	0.13419	1	0.12665	1	0
Arginine biosynthesis	14	0.14452	1	0.13574	1	0
Pentose and glucuronate interconversions	18	0.18581	1	0.17123	1	0.14062
Sphingolipid metabolism	21	0.21677	1	0.19694	1	0.03854
Lysine degradation	25	0.25806	1	0.23006	1	0
Alanine, aspartate and glutamate metabolism	28	0.28903	1	0.25406	1	0.11378
Glyoxylate and dicarboxylate metabolism	32	0.33032	1	0.28496	1	0
Glycerophospholipid metabolism	36	0.37161	1	0.31467	1	0.01736
Amino sugar and nucleotide sugar metabolism	37	0.38194	1	0.32191	1	0
Fatty acid elongation	39	0.40258	1	0.33618	1	0
Fatty acid degradation	39	0.40258	1	0.33618	1	0
Pyrimidine metabolism	39	0.40258	1	0.33618	1	0
Fatty acid biosynthesis	47	0.48516	1	0.39049	1	0.01473
Aminoacyl-tRNA biosynthesis	48	0.49548	1	0.39698	1	0
Purine metabolism	65	0.67097	1	0.49785	1	0

The above table shows the metabolic pathways were impacted by the treatment of Liraglutide; higher impact values represent the relative importance of the pathway that may be of interest to the metabolite perturbations observed in T2DM after treatment.

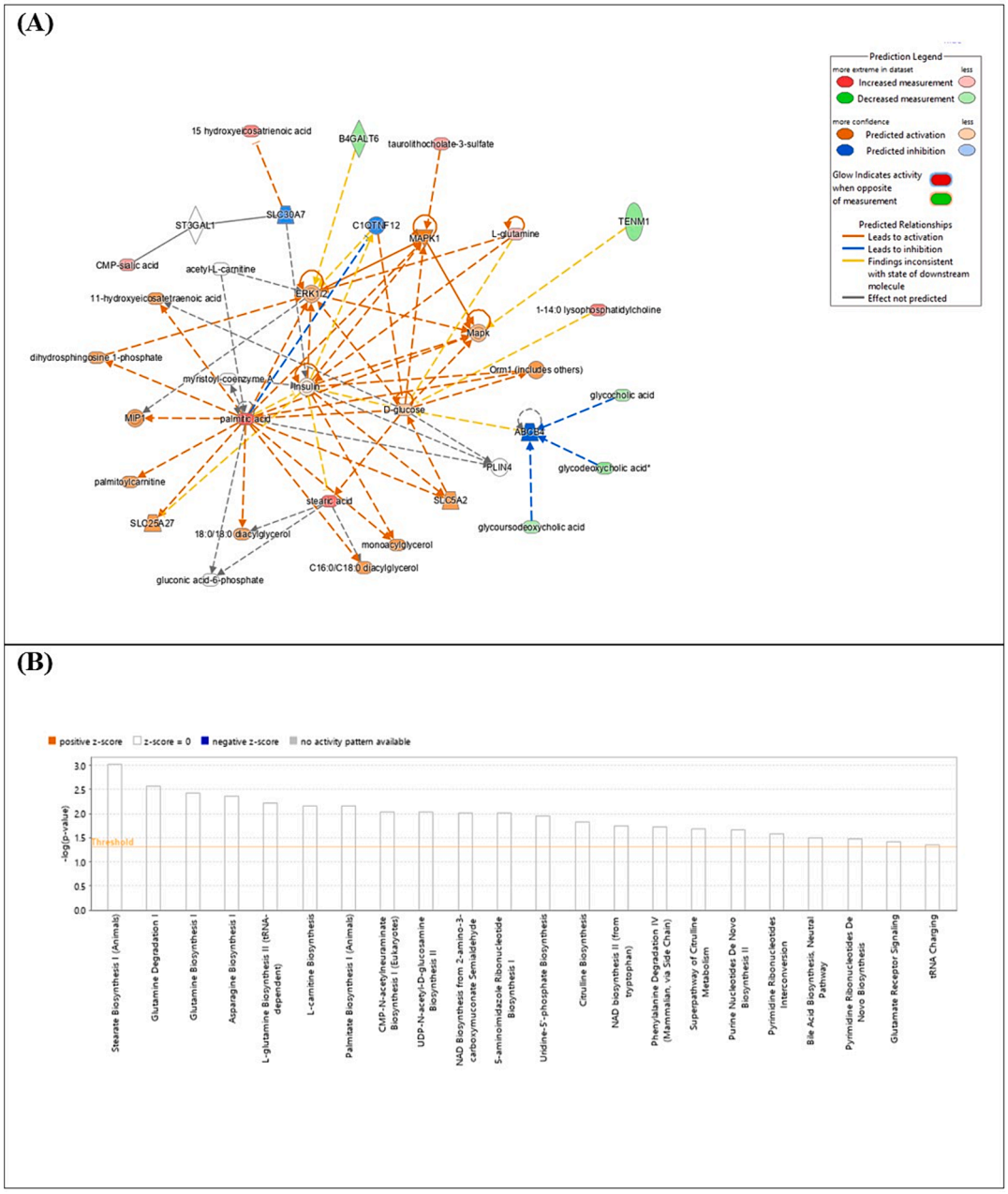

Fig. 5. Network analysis and biological pathways related to the significantly identified metabolomics in the study population. **(A)** Network pathway analysis of the significantly affected metabolomics identified in the pre- and post-Liraglutide treated diabetic patients revealed. The identified metabolites were related to the immunological disease, inflammatory diseases, inflammatory response. (**B)** The top canonical pathways related to the significantly impacted metabolites identified in the plasma samples collected from diabetic patients before and after receiving Liraglutide treatment.

last assists in the process of hepatic glucuronidation, which is physiologically induced to eliminate predominantly drugs, dietary substances, toxins and endogenous substances (Yang et al., 2017). Expectedly, it could be that by some means, Liraglutide induces the uptake of glucose in the liver to be used for the formation of the glucuronide metabolites, potentially to help eliminate the undesirable and toxic metabolites from the body of T2DM patients after receiving Liraglutide, which in turn improves the overall health. In support with our findings, our pathway analyses revealed that pentose and glucuronate interconversions pathway, mainly taking place in the liver, was significantly impacted in T2DM patients after receiving Liraglutide, suggesting a new hepatic impact of Liraglutide in T2DM. Further validation experiments are required to investigate the metabolic roles of 6-Hydroxy-5-methoxyindole glucuronide and other glucuronide metabolites in T2DM and to examine their excretion routes from these T2DM patients.

4.4. Liraglutide could improve the systemic immunity and oxidative event in T2DM patients

In our previous proteomics study, we reported that Liraglutide treatment could improve the systemic inflammatory state and oxidative stress of T2DM patients ((Ekhzaimy et al., 2022)). In the same line, our current metabolomics study, notably, showed certain metabolic findings that could contribute to the overall improved immunity status. One of our findings is that the metabolism of amino acids particularly, alanine, aspartate and glutamate, was impacted. It is well known that amino acids are fundamental metabolites needed for the function of the immune cells (Kelly and Pearce 2020), potentially Liraglutide may improve

the immunity of T2DM by targeting the metabolism of amino acids. In further details, Liraglutide may induce the uptake of alanine and glutamine in the immune cells, resulting in the release of important cytokines required for the immune responses of the host, impacting the overall systemic health. Also, it could be that Liraglutide treatment in T2DM patients targets the immune cells indirectly by certain means, which merits investigations. In addition to that, Liraglutide treatment may contribute to the potential antioxidant events-mediated by metabolic alterations in T2DM patients, shown in our data. To explain, our data showed that QH (2) ubiquinol was reduced in the treated group, potentially ubiquinol metabolites could be utilized in the oxidative events resulted from the degradation of the lipids and their related derived as a defense mechanism (Nohl et al., 1998). Overall, Liraglutide impacts fundamental metabolites that could be involved in the overall improved systemic immunity or oxidative states of T2DM patients. Taken together, our study has provided important insights into the metabolic perturbations caused by Liraglutide in T2DM patients. Also, our findings provide a data resource for further studies with similar interests and focuses in the field of T2DM and its therapies. However, several limitations do exist and need to be addressed in the future. One of these limitations is that the current analyses do not consider all types of metabolites due to the lack accuracy of KEGG annotation of metabolites; thus, further 'omics' approaches are suggested to be done for the same study to cover most of the metabolite types in T2DM condition with Liraglutide treatment. Second, the sample size of the study needs to be larger for validation purposes. Moreover, performing targeted metabolomics analyses for the identified metabolic biomarkers are suggested in the future work.

5. Conclusion

To conclude, our study shed light on the metabolic impacts and beneficial effects of Liraglutide in T2DM patients and on the underlying mechanisms of these metabolic impacts and benefits. Liraglutide treatments induces systemic and tissue-specific metabolic changes, impacting the overall health of T2DM patients. Interestingly, for the first time, we proposed that the hepatic pentose and glucuronate interconversions is impacted by Liraglutide which could be potentially used as a target for developing treatments for T2DM. We provided new avenues for other research groups to follow up on research focusing on T2DM and its related prescribed treatments to enrich our knowledge from various scientific aspects.

Data availability statement

Metabolomics data were deposited to Metabolomics Workbench database with the identifier number ST002735. The complete data set can be accessed at https://www.metabolomicsworkbench.org/data/DRCCMetadata.php?Mode=Study&StudyID=ST002735 (2023-07-01).

Author contributions

AAA, AAR and HB, conceived and designed the study. AAA, and AM were involved in patient recruitment. HB, RS prepared the samples for the metabolomic work. RS and AAR performed the metabolomics analyses. RS, RA, AM and HB conducted the data analyses. RS wrote the original manuscript draft. RS, AM, AAR, HB and AAA review and edit the writing of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Hicham Benabdelkamel: Writing – review & editing, Methodology, Formal analysis, Conceptualization. **Rajaa Sebaa:** Writing – original draft, Formal analysis. **Reem H. AlMalki:** Writing – review & editing, Software, Methodology. **Afshan Masood:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Assim A. Alfadda:** Writing – review & editing, Resources, Project administration, Formal analysis, Conceptualization. **Anas M. Abdel Rahman:** Writing – original draft, Software, Methodology, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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