Downregulated GPD1 and MAGL protein levels as potential biomarkers for the metastasis of triple-negative breast tumors to axillary lymph nodes

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Abstract. Glycerol-3-phosphate dehydrogenase (GPD1) and monoacylglycerol lipase (MAGL) levels are known to be significantly downregulated in both the tissue and serum samples of patients with triple-negative breast cancer (TNBC), compared with other BC subtypes and healthy controls. As such, the association between GPD1 and MAGL levels and lymph node metastasis was evaluated in the present study. Utilizing western blotting, lymph node protein extracts from metastasized BC subtypes were analyzed and a significant downregulation of GPD1 and MAGL protein expression levels in the lymph node metastases was demonstrated in the TNBC subtype, compared with healthy controls. This finding further highlighted the potential use of these two proteins in early BC onset and metastasis detection.

Introduction

Based on the latest data on global cancer incidence in 2020, breast cancer (BC) is ranked as the most prevalent type of cancer in women (1). In 2020 alone, >2.3 million women worldwide were newly diagnosed with BC (2). At the time of diagnosis, 3-10% of newly diagnosed patients with BC had distant metastases, and 30-40% of early-stage patients with BC progressed to an advanced stage (3). BC metastasis significantly reduces survival rates and only 29% of women with metastatic BC experience a 5-year survival time (3). Therefore, understanding the processes of BC metastasis may lead to the development of new therapeutic approaches.

BC is subtyped using specific molecular biomarkers, namely an estrogen receptor (ER), the progesterone receptor (PR) and human epidermal growth factor 2 (HER2). The Ki67 index, a proliferation indicator, is also considered during subtyping (4). Based on the expression levels of these specific biomarkers, there are five defined BC subtypes, namely Luminal A (Lum A; ER^+ and PR^+, but $HER2^-$ and Ki67 <14%), Luminal B (Lum B)-$HER2^-$ (ER^+ and PR^+, but $HER2^-$ and Ki67 >14%), Lum B-$HER2^+$ (ER^+ and PR^+, but $HER2^+$ and any Ki67 level), HER2 overexpressed [ER^- and PR^-, but high $HER2^+$ (HER2 score of >3) and any Ki67 level] and triple-negative BC (TNBC; ER^-, PR^-, $HER2^-$ and any Ki67 level) (5,6) Each subtype displays distinct behavior with regards to their metastatic potential, expression of cell surface markers, response to treatments, and their cellular and molecular characteristics (7). Therefore, understanding the metastatic differences of each subtype is critical for designing effective, subtype-specific treatments.

In the present study, changes in the expression levels of two enzymes, glycerol-3-phosphate dehydrogenase (GPD1) and monoacylglycerol lipase (MAGL), in the axillary lymph nodes of metastatic BC tissues were investigated across different subtypes. Prior research, performed by both our team and other researchers, has already reported altered levels of these enzymes in certain BC subtypes, suggesting reduced expression in cancer cells compared with controls (6,8-10). The aim of the present study was to investigate the potential significance of GPD1 and MAGL as candidate biomarkers for diagnosing and subtyping BC.

Material and methods

Patient characteristics and tissue collection. Tissues from 26 female patients diagnosed with infiltrating ductal carcinoma BC were collected at the Department of General Surgery of Kocaeli University Medical School (Kocaeli, Turkey) from March 2019 to August 2021. Written informed consent was obtained from each patient before participation in the present study. The study specifically included patients who had not undergone any form of cancer therapy. For comparison, non-metastatic lymph nodes were taken from an area separate from the metastatic lymph nodes and utilized as control samples. Control breast tissues were collected from adjacent non-tumor tissue regions. Table I presents a comprehensive list of the clinical features of the patients included in the present study.

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Subtyping study groups. Molecular subtyping was performed based on ER, PR, HER2 and Ki67 protein expression levels (data not shown) (11). Molecular subtyping of tumor samples was performed by the Department of Pathology at Kocaeli University Medical School by analyzing expression levels of ER, PR, HER2 and Ki-67 proteins. The expression levels of these proteins were evaluated using routine immunohistochemical methods, as previously described (5). Based on the expression patterns, patients were divided into the following five subgroups: Lum A (ER^+ and/or PR^+, $HER2^-$ and Ki67 <14%), Lum B/$HER2^-$ (ER^+ and/or PR^+, $HER2^-$ and Ki67 >14%), Lum B/$HER2^+$ (ER^+ and/or PR^+, $HER2^+$ and any Ki67 level), HER2-OE [ER^-, PR^-, HER2 (score of >3) and any Ki67 level] and TNBC (ER^-, PR^-, $HER2^-$ and any Ki67 level) (12). The Lum A, Lum B/$HER2^+$, Lum B/$HER2^-$, HER2-OE and TNBC groups included 7, 4, 5, 4 and 6 patients, respectively. Surrogate definitions of intrinsic sub-types of breast cancer for HER2 expression were scored based on staining patterns and scored as 1, 2 and 3. Any score beyond 3 was considered as overexpressed.

Sample preparation. The collected tissues were first diced into small pieces and then washed with buffer containing 10 mM Tris (pH 7.2) and 250 mM sucrose. After centrifugation at 10,000 x g for 10 min, supernatant containing trace amounts of blood (from the tissue samples) was carefully removed. Subsequently, the tissue samples were homogenized using a Scilogex homogenizer in buffer containing 10 mM Tris-HCl containing 7 M urea, 2 M thiourea, 5 mM magnesium acetate, with 4% CHAPS (pH 8.0). To ensure thorough homogenization, further treatment with 1.4 mm stainless steel beads using a bead-beater (Bullet Blender; Next Advance, Inc.) was performed. The resulting homogenates were then subjected to centrifugation for 15 min at 15,000 x g at 4˚C to obtain cell-free extracts. Protein concentrations in these extracts were determined using a modified Bradford assay (Bio-Rad Laboratories, Inc.). Finally, the cell-free extracts were stored at -80˚C until further use.

Preparation of protein pools. Protein pools were created by mixing equal amounts of protein from the cell-free extracts of samples from the following sample types: Healthy breast (BH), tumor breast (BT), healthy lymph node (LH) and metastatic lymph node (LM). Protein concentrations in each pool were determined using the Qubit Protein Assay (Thermo Fisher Scientific, Inc.). The protein integrity of each pool was also assessed by visual examination of Coomassie stained 12% SDS-PAGE gels (data not shown). Protein (30 μg per lane) was used for qualitative and quantitative analyses. Quantity One software version 4.6.7 was used to compare protein band intensities among the pools (data not shown) (Bio-Rad Laboratories, Inc.).

Western blotting analysis. Protein pools and individual cell-free extracts were analyzed by SDS-PAGE using 12% acrylamide gels. Protein (20 μg per lane) was used for qualitative and quantitative analyses. Electrophoretic transfer of proteins onto positively charged nitrocellulose membranes was performed in a semi-dry electrophoretic transfer cell (Bio-Rad Laboratories, Inc.) for 20 min at 15 V, in a buffer containing 48 mM Tris (pH 9.2), 39 mM glycine, 20% (v/v) methanol and 0.0375 g/l SDS. The membranes were blocked in TBS-T buffer (Tris-HCl 25 mM pH 7.2, NaCl 150 mM and 0.1% Tween 20) containing 5% nonfat dry milk for 1 h at room temperature, and washed with TBS-T three times before incubation with primary antibodies diluted in TBS-T overnight at 4˚C. The membranes were then washed three times with TBS-T and incubated with goat anti-mouse HRP-labelled secondary antibody (cat. no. 170-5047; Bio-Rad) for 1 h at room temperature. Monoclonal anti-β-actin antibody (cat. no. sc81178; Santa Cruz Biotechnology, Inc.), monoclonal anti-glycerol-3-phosphate dehydrogenase NAD+, cytoplasmic antibody (anti-GPD1) (cat. no. sc-376219; Santa Cruz Biotechnology, Inc.) and monoclonal anti-monoglyceride lipase antibody (anti-MAGL) (cat. no. sc398942; Santa Cruz Biotechnology, Inc.) were used at the respective dilutions of 1:1,000, 1:750 and 1:750. Following a subsequent three washes with TBST, protein bands were visualized with an enhanced chemiluminescence detection system (Thermo Fisher Scientific, Inc.). Protein band intensities were analyzed using Image J (version 1.40 g; National Institutes of Health). B-actin was used as the internal normalization control for the band intensities. Western blotting analyses of the protein pools were performed three times.

Statistical analysis. Statistical analyses of the differences in MAGL and GPD1 protein expression levels between the BH and BT groups, as well as between the LH and LM groups were performed. All statistical analyses were performed using GraphPad Prism software, version 5.0 (Dotmatics). Datasets with two groups were analyzed using paired Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Protein pools within each subtype were labelled as follows: BH, BT, LH and LM. After determination and normalization of protein concentrations, the protein expression levels of GPD1 and MAGL in each BC subtype were analyzed using western blotting. Similarly to results previously observed (6,8,9), there was a significant decrease in GPD1 and MAGL protein levels in the BT pool compared with the BH pool, regardless of the BC subtype (Fig. 1). However, when comparisons were made between the LH and LM protein pools, statistically significant decreases in GPD1 and MAGL protein levels were only observed in the TNBC subtype (Fig. 1).

To assess whether the decrease in GPD1 and MAGL protein levels observed in the LM protein pool, compared with the LH protein pool, was detectable in each individual protein sample prepared from tissues from patients with TNBC, western blotting of the separate TNBC samples was performed (Fig. 2). Significantly lower mean protein expression levels of GPD1 and MAGL were detected in the LM samples compared with the LH samples, although this decrease in protein expression appeared to vary from sample to sample. Notably, the absence or low levels of GPD1 protein in LM samples suggests that it might serve as a more effective discriminator between LM and LH samples.

Table I. Clinical features of patients with breast cancer included in the present study.

Clinical feature	Luminal A	Luminal B/HER2$^-$	Luminal B/HER2$^+$	HER2-OE	TNBC
Patients, n	7	5	4	4	6
Mean (SD) age, years	41.25 (5.3)	47.3 (5.6)	59.25 (10.8)	49 (5.2)	62.75 (12.25)
Ki67 level, %	<14	≥14	≤14≥	≤14≥	≤14≥

HER2, human epidermal growth factor 2; OE, overexpression; TNBC, triple-negative breast cancer.

Discussion

BC is a metabolically complicated and heterogeneous disease and requires greater understanding at the molecular level (13). Onset, progression and metastasis are the three main steps in BC, and each step is markedly different in terms of epigenetic changes, mutations rates, secretion of certain growth factors, changes in expression of different receptor types and key cell-cell adhesion molecules (14). Metastasis is the leading cause of cancer mortality; therefore, the metastatic cascade is potentially the most important step to target for reducing the death rate. Metastasis itself is a multistep process involving local tumor cell invasion, entry into the vasculature, exiting of carcinoma cells from the circulation and colonization at distal sites (15). The local axillary lymph nodes are initially involved in metastatic BC (16). In patients with BC, a detailed and comprehensive assessment of the condition of the axillary lymph nodes is important for determining prognosis (16).

In our previous study, proteomic profiles of BC subtypes were compared and the existence of differentially regulated proteins was reported (6). Among the differentially regulated proteins, GPD1 and MAGL showed significant downregulation in tumor tissues compared with controls collected from the adjacent non-tumor tissue, which suggested that these two proteins may possess biomarker properties for the diagnosis of BC. Furthermore, GPD1 and MAGL were more notably downregulated in the TNBC subtype (an aggressive form of BC) compared with the controls, which indicated their possible role in cancer metastasis (6). Further investigation by our group indicated that these two proteins were also present at lower levels in serum samples from patients with BC compared with the serum samples from healthy controls (9). Similar to these findings, Zhou *et al* (8) independently reported GPD1 as a potential tumor suppressor protein upon investigating changes in mRNA expression levels in BC tissues. The study demonstrated that GPD1 was significantly downregulated in BC tissues compared with controls. Additionally, low GPD1 expression levels were correlated with lower survival rates.

The differential regulation of GPD1 is not unique to BC as GPD1 is also downregulated in other cancer types, including bladder cancer, ovarian cancer and renal clear cell carcinoma (17-19). However, to the best of our knowledge, there has not yet been a detailed study investigating the involvement of GPD1 in cancer metastasis. Therefore, in the present study, changes in GPD1 and MAGL expression levels in BC metastasized to axillary lymph nodes were investigated. Both proteins were significantly downregulated in the axillary lymph nodes of patients from all BC subtypes compared with the controls, which indicated the potential involvement of these two proteins in metastasis. However, the cause of this downregulation in BC and how much contribution GPD1 and MAGL make to the metastatic process is currently unknown.

Glycerol is a key metabolite likely involved in the cross talk between MAGL and GPD1 (Fig. 3). MAGL catalyzes the hydrolysis of monoacylglycerols in the production of free fatty acids and glycerol (20). The free fatty acids are then used in numerous metabolic processes, including the production of fatty acyl-CoA, which then undergoes fatty acid oxidation for energy release. The glycerol produced by MAGL can be converted to glycerol-3-phosphate (G-3-P) by glycerol kinase, which then serves as a substrate for certain enzymes such as glycerol-3-phosphate dehydrogenase, glycerol-3-phosphate acyltransferase and glycerol-3-phosphate deacylase (21). G-3-P can be converted to dihydroxy acetone phosphate by GPD1, which is used as a source in carbohydrate metabolism. Depending on the cellular energy requirement, G-3-P can be used in glycolysis, gluconeogenesis or the pentose phosphate pathway. G-3-P is also the substrate for the enzyme, glycerol-3-phosphate acyl transferase (GPAT) (22). GPAT produces the simplest form of phospholipids, phosphatidic acid, which is then used for the production of triacylglycerols using fatty acyl-CoA. It is known that triacylglycerols are good for storing free fatty acids for later use (23). Looking at this metabolic scheme, it is clear that there is a complex intertwined chain of events occurring within cells. A decrease in MAGL levels may result in a decrease in GPD1 levels since MAGL produces the precursor molecule, glycerol, for the production of G-3-P, which in turn is a substrate of GPD1. The decrease in GPD1 levels could direct the synthesis of triacylglycerols via the GPAT-associated pathway. However, whether GPAT levels in BC tissues are associated with a downregulation in GPD1 remains to be determined. A functional proteomics study comparing the activities of MAGL, GPD1 and other metabolically relevant enzymes would also indicate how the activities of these enzymes change in parallel to the changes observed at the protein expression levels.

A limitation of the present study was the low number of enrolled cases. However, it was challenging to identify and include suitable cases for inclusion in the study, as not all patients wanted to participate and samples could only be used from Kocaeli University Medical School according to Ethics Committee requirements. Additionally, certain potential participants who met the inclusion criteria declined to take part. As a result, the process of collecting samples extended beyond 2 years. Despite these challenges, the preliminary

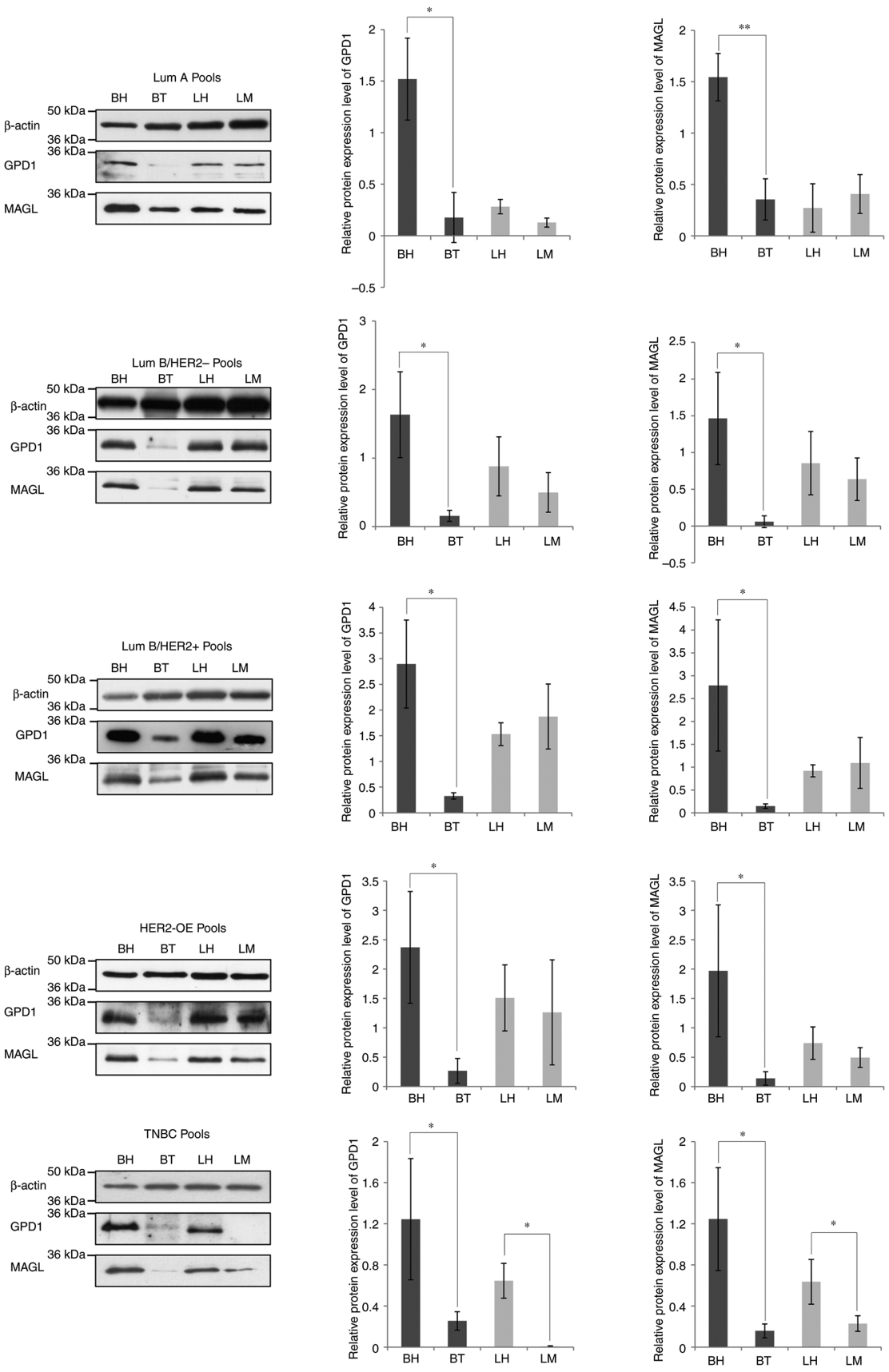

Figure 1. Western blot analysis of protein pools prepared from Lum A, Lum B/HER2^{-}, Lum B/HER2^{+}, HER2-OE and TNBC tissues for the assessment of GPD1 and MAGL levels. The western blot was re-probed with an anti-β-actin antibody for the normalization of protein expression levels. The band intensities were semi-quantified using ImageJ software and statistical significance was calculated using GraphPad Prism software. *P<0.05, **P<0.001. BH, healthy breast; BT, breast tumor; GPD1, glycerol-3-phosphate dehydrogenase; HER2, human epidermal growth factor 2; LH, healthy lymph node; LM, metastatic lymph node; Lum, Luminal; MAGL, monoacylglycerol lipase; OE, overexpressed; TNBC, triple-negative breast cancer.

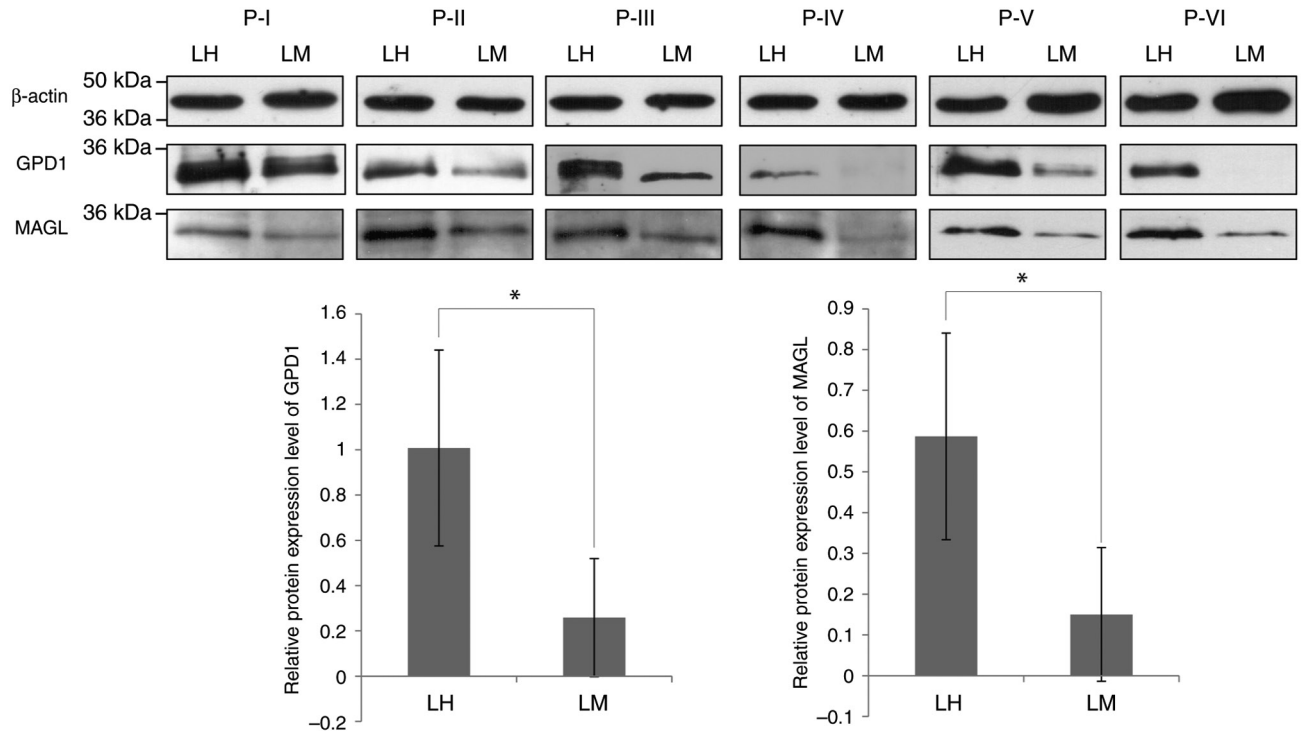

Figure 2. Western blot analysis of GPD1 and MAGL protein expression levels in each triple-negative breast cancer sample from the LH and LM groups. The western blot was re-probed with an anti-β-actin antibody for the normalization of protein expression levels. The band intensities were semi-quantified using ImageJ software and statistical significance was calculated using GraphPad Prism Software. *P<0.05. GPD1, glycerol-3-phosphate dehydrogenase; LH, healthy lymph node; LM, metastatic lymph node; MAGL, monoacylglycerol lipase; P, patient (number).

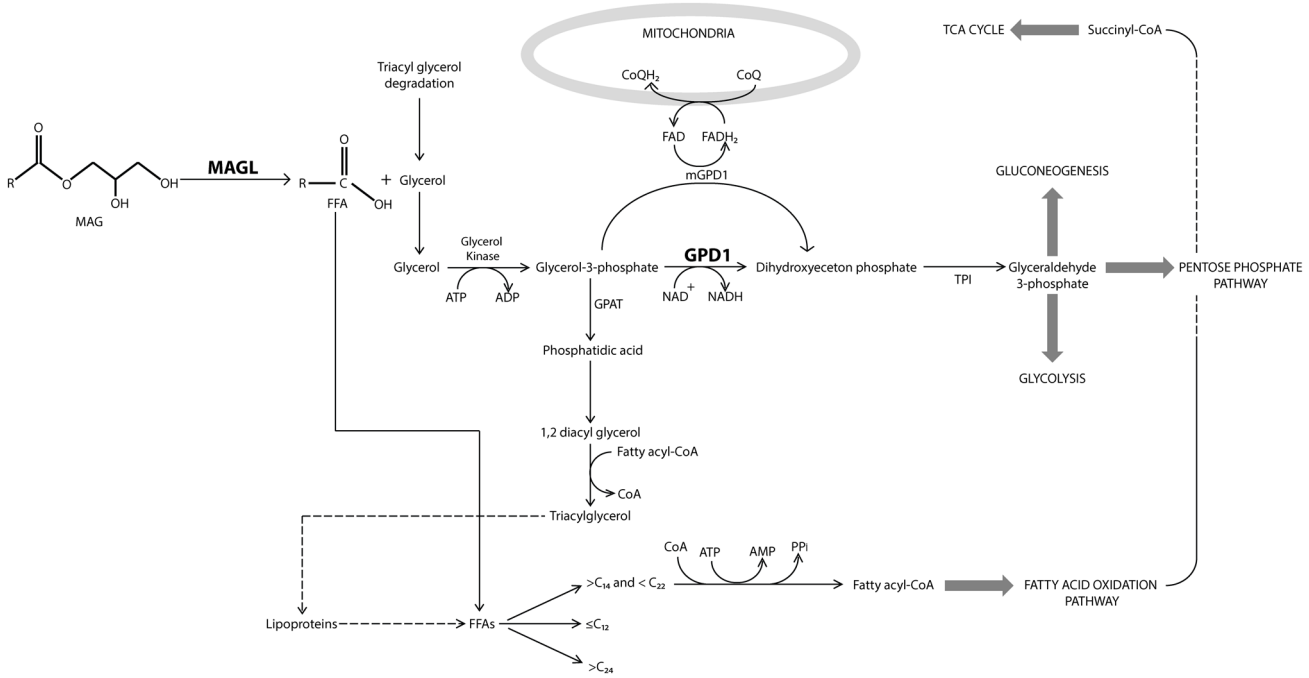

Figure 3. Metabolic map of reactions associated with MAGL and GPD1. Co, coenzyme; FFA, free fatty acid; GPD1, glycerol-3-phosphate dehydrogenase; GPAT, glycerol-3-phosphate acyltransferase; MAG(L), monoacylglycerol (lipase); TCA, tricarboxylic acid cycle; TPI, triose phosphate isomerase.

results of the present study may pave the way for future studies with a larger number of enrolled cases.

The present study was a follow-up study, specifically aimed at addressing a particular question: The association between GPD1 and MAGL protein levels and lymph node metastasis. To further establish this association, western blotting, ELISA, flow cytometry analysis and targeted mass spectrometry analysis could be performed. In combination, these approaches may provide a more comprehensive understanding of the protein levels in lymph node metastasis. The western blotting

analysis performed in the present study indicated that GPD1 and MAGL proteins were downregulated in lymph node metastasis, particularly in the TNBC subtype when compared with the control samples. Additional methods, such as ELISA, flow cytometry and targeted mass spectrometry analysis, could be employed in future studies to establish reference values for tumor diagnosis and monitoring. Performing such studies would necessitate more extensive sample collection and substantial financial resources, and future work will need to meet the requirements in this regard.

In conclusion, an accelerated lipid metabolism is crucial to support cellular proliferation and biosynthetic activities in cancer cells (24). Evidence suggests that cell proliferation can be suppressed through a reduction in the availability of fatty acids to the cell, making free fatty acids play a central and important role in the process of the suppression of cell proliferation (25). In addition, aggressive cancer cells have been shown to have a higher lipid content and elevated lipogenic and lipolytic switching. MAGL and GPD1 are important for the modulation of lipid synthesis, along with GPAT, which has previously been determined to be an effective target for cancer treatment. Future research should therefore address the possible utility of these three proteins as tools in diagnostic, prognostic or therapeutic strategies.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TS, MGBA, GA, ZC and MK confirm the authenticity of all the raw data. Study conception and experimental design were performed by MK and GA. Tissue collection during surgeries and storage were performed by TS and NZC. Sample preparation and protein isolations were performed by MGBA and TS. Western blotting analyses were performed by TS, MGBA, MK, and GA. Data analyses were performed by MGBA and GA. All authors read and approved the final version of the manuscript.

Ethical approval and consent to participate

This study was approved by The Ethics Committee of Kocaeli University (Kocaeli, Turkey; approval no. KOU GOKAEK-2019/16.04 2019/139). Written informed consent, approved by the ethics committee, was obtained for each patient before participation in the study. The Declaration of Helsinki was complied with to safeguard human subjects and uphold the highest ethical standards.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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