

RESEARCH ARTICLE

Omentin-regulated proteins combine a pro-inflammatory phenotype with an anti-inflammatory counterregulation in human adipocytes: A proteomics analysis

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Abstract

Aims: Experimental and epidemiological studies reported controversial data on the role of omentin in type 2 diabetes and cardiovascular diseases. This study aimed to characterise the impact of omentin on the secretome of human adipocytes to analyse the enrichment of these proteins in metabolic and cellular signalling pathways underlying its physiological function.

Material/methods: Differentiated primary human adipocytes were treated without or with 500 or 2000 ng/mL omentin for 24 hours. The secretome was analysed by liquid chromatography coupled tandem-mass spectrometry. Differences in protein secretion between untreated and omentin-treated adipocytes were compared using a paired *t*-test. Other potential upstream regulators and the overrepresentation in canonical pathways of omentin-stimulated proteins were analysed using Ingenuity Pathway Analysis.

Results: The supernatant of adipocytes contained 3493 proteins, of which 140 were differentially secreted by both concentrations of omentin compared with untreated adipocytes. Among the most strongly increased proteins, tumour necrosis factor-inducible gene 6 protein (TNFAIP6) was increased by 140-fold in the supernatant. Omentin-regulated proteins were overrepresented in seven canonical pathways including eukaryotic initiation factor 2 signalling, complement system, and inhibition of matrix metalloproteases. We further identified 25 other potential upstream activators of omentin-regulated proteins, mainly pro-inflammatory cytokines and transcription regulators including NFκB.

Conclusions: In differentiated human adipocytes, the release of the anti-inflammatory TNFAIP6 might be part of a counterregulatory response to the pro-inflammatory action of omentin. Omentin-regulated proteins were overrepresented in pathways indicating cellular stress, a pro-inflammatory environment and a crosstalk with other organs. Other potential activators of omentin-regulated proteins point towards a central role of NFκB activation in the omentin-induced secretory process.

KEYWORDS

obesity, omentin, inflammation, proteomics, adipocytes

1 | INTRODUCTION

Omentin is predominantly secreted by the stromal vascular fraction of visceral adipose tissue.¹ Currently available studies controversially discuss whether omentin exerts a protective or detrimental role in type 2 diabetes (T2D) and cardiovascular diseases (CVD).

Cross-sectional studies found decreased circulating levels of omentin in obese compared with lean individuals and lower omentin levels in patients with T2D compared with glucose tolerant people.^{2,3} In a cross-sectional analysis of the Cooperative Health Research in the Region of Augsburg (KORA) F4 study, we observed that omentin did not differ between individuals ranging from normal glucose tolerance to T2D.⁴ However, higher circulating omentin was inversely associated with multiple risk factors for T2D and CVD.²⁻⁵ These findings were supported by *in vitro* studies showing that omentin increased Akt phosphorylation and insulin-stimulated glucose uptake in human adipocytes.¹

With respect to CVD, circulating omentin was decreased in patients with coronary artery disease^{6,7} and appeared to have a protective role in patients with ischaemic stroke.^{8,9} In line, administration of omentin in mice reduced tissue damage after ischaemia/reperfusion¹⁰ and delayed the development of aortic atherosclerotic lesions with reduced vascular inflammation.¹¹

In contrast with the findings from cross-sectional and experimental studies, the prospective analysis within the KORA F4/FF4 cohort indicated that higher circulating omentin is associated with a higher risk of T2D.¹² This is in line with a prospective analysis within the European Prospective Investigation into Cancer and Nutrition-Potsdam study demonstrating an increased omentin-related diabetes risk among people with high adiponectin levels.¹³ In the same cohort, circulating omentin was associated with an increased risk of stroke,¹⁴ and another prospective study reported high circulating omentin levels as predictor of cardiovascular events.¹⁵

Data on the inflammatory properties of omentin are limited to very few biomarkers. Circulating omentin associated inversely with interleukin-6 (IL-6),^{3,16} tumour necrosis factor (TNF) α ,³ and C-reactive protein,^{5,13,16} and positively with adiponectin.^{2,4,13} On the cellular level, omentin reduced TNF α -induced inflammation in human endothelial cells and murine smooth muscle cells by inhibiting several kinases and transcription factors of inflammatory pathways.^{17,18}

Taken together, the mechanism by which omentin affects metabolic and inflammatory processes in cardiometabolic diseases is incompletely understood. Therefore, the aims of the study were (1) to investigate the impact of omentin on the secretome of proteins in differentiated human adipocytes, (2) to analyse the overrepresentation of the omentin-regulated proteins in well-characterised metabolic and cell signalling pathways, and (3) to identify other potential upstream activators of omentin-regulated proteins.

2 | MATERIAL AND METHODS

2.1 | Cultivation and treatment of human adipocytes

Primary human preadipocytes isolated from subcutaneous adipose tissue from five donors without diabetes were purchased from

PromoCell (Heidelberg, Germany) and Lonza (Basel, Switzerland).¹⁹ Approval by an ethics committee was not required. Human preadipocytes were cultured in preadipocyte growth medium (PromoCell) containing 0.05 mL/mL fetal calf serum (FCS), 0.004 mg/mL endothelial cell growth supplement, 10 ng/mL epidermal growth factor, 1 μ g/mL hydrocortisone, and 90 μ g/mL heparin until 100% confluency. Then, preadipocytes were differentiated in preadipocyte differentiation medium (PromoCell) containing 8 μ g/mL D-biotin, 0.5 μ g/mL insulin, 400 ng/mL dexamethasone, 44 μ g/mL 3-isobutyl-1-methylxanthine, 9 ng/mL L-thyroxine, and 3 μ g/mL ciglitazone. After 3 days, differentiation medium was replaced by adipocyte nutrition medium (PromoCell) containing 0.03 mL/mL FCS, 8 μ g/mL D-biotin, 0.5 μ g/mL insulin, and 400 ng/mL dexamethasone. The adipocytes were differentiated for further 11 days. At day 14 of differentiation, adipocytes were cultivated in adipocyte nutrition medium without insulin and FCS for 4 hours and then treated with 500 or 2000 ng/mL omentin for 24 hours with untreated cells as controls. After this incubation time, adipocyte supernatant was collected and centrifuged for 10 minutes at 1800 \times g at 4°C. Then, 800 μ l were transferred in a new tube and stored at -80°C until the proteomics analysis. The aforementioned omentin concentrations were selected based on our analysis of serum samples from the KORA F4 study.¹² The concentration of 500 ng/mL is close to the median level in a non-diabetic population, whereas 2000 ng/mL is close to the highest levels found in this population, but most likely closer to local levels found in adipose tissue.

2.2 | Quantitative mass spectrometry in data-independent acquisition mode

Six hundred microlitres of each supernatant was proteolysed with a modified filter aided sample preparation procedure.^{20,21} Eluted peptides were supplied with indexed retention time hyper reaction monitoring calibration peptides (BIOGNOSYS, Schlieren, Switzerland)²² for retention time alignment and then analysed in a randomised order on a QExact high-frequency mass spectrometer (MS) (Thermo Fisher Scientific, Waltham, MA, USA) coupled online to an UltiMate 3000 nano-RSLC (Dionex, Sunnyvale, CA, USA) equipped with a customised 20-cm M-Class column (Waters, Milford, MA, USA) using a nonlinear 5% to 40% acetonitrile gradient in a 130-minute run. Spectra were recorded in data-independent acquisition mode²³⁻²⁵ with the following parameters: MS spectra from 300 to 1650 m/z were recorded at 120 000 resolution with an automatic gain control target of 3e6 and a maximum injection time of 120 ms. Data-independent acquisition windows were selected based on previous data-dependent acquisition runs, with a scan resolution of 30 000, an automatic gain control target of 3e6, and a collision energy of 28. The recorded raw files were analysed using the Spectronaut 10.0 software (BIOGNOSYS)²⁶ with an in-house human database spectral library which was generated using Proteome Discoverer 2.1, Mascot search engine (Matrix Science, London, UK), and the Swissprot Mouse database (release 2016_02). Quantification was based on cumulative MS2 area levels with the q-value percentile 0.25 setting.

2.3 | Statistical and pathway analysis

Differences in protein secretion between omentin-treated and untreated differentiated human adipocytes were assessed using the paired *t*-test on the log₂ transformed intensities. For further analyses, we used the dataset containing proteins that were differentially regulated by both 500 and 2000 ng/mL omentin treatment compared with untreated differentiated human adipocytes. Very small absolute quantification values <100 were excluded because of the risk of measuring inaccuracy. We excluded proteins (1) whose concentrations differed by a coefficient of variation of more than 100% between donors of preadipocytes and (2) those with different expression directions between low and high omentin concentrations. Statistical analyses were carried out with R version 3.3.3 (R Core Team, R Foundation for Statistical Computing, Vienna, Austria) and Python version 3.6.1 (Python Software Foundation, <https://www.python.org/>). Statistical significance was inferred at a two-tailed *P*-value <0.05.

Furthermore, we analysed the overrepresentation of proteins in canonical pathways using Ingenuity Pathway Analysis (IPA) (Qiagen, Hilden, Germany). This tool identifies pathways from the IPA library of canonical pathways that showed significant enrichment of the omentin-regulated proteins. This method enables to deduce potential physiological roles of omentin in differentiated human adipocytes from the secretome. Fisher's exact test was used to calculate the probability that the association between the proteins in the dataset and the canonical pathways were explained by chance alone. The Benjamini-Hochberg method was used to correct for multiple testing (B-H *P*-value). In addition, we analysed other potential upstream activators of the omentin-regulated proteins. Based on the characteristics of other potential upstream activators, the potential secretory mechanism for omentin-regulated proteins can be inferred from the proteins in the adipocyte supernatant. Again, Fisher's exact test was used to assess the probability that potential activators assigned to the dataset were due to chance alone. The activation *z*-score aims to infer the activation states of predicted upstream regulators. A *z*-score above 2 points towards the activation of potential upstream regulators; a *z*-score below -2 indicates the inhibition of potential upstream regulators. For all analyses in IPA, we used fold changes of omentin-regulated proteins that referred to 2000 ng/mL omentin. Data on the localisation of omentin-regulated proteins are based on the IPA library. The algorithms developed for use in IPA are described elsewhere.²⁷

3 | RESULTS

The supernatant of omentin-treated and untreated differentiated human adipocytes contained 3493 proteins. Of these proteins, 275 proteins were regulated by 500 ng/mL omentin and 317 proteins by 2000 ng/mL omentin. There was an overlap of 140 proteins between both omentin concentrations, which are listed in Suppl. Table 1. These proteins are localised either in the cytoplasm, extracellular space, plasma membrane, or nucleus (Suppl. Table 1). Suppl. Figure 1 depicts the dose-dependent effect of omentin on the secretion of these 140 proteins.

3.1 | Impact of omentin on protein secretion

Table 1 shows the top 20 of the most upregulated and the top 20 of the most downregulated proteins by omentin (see Suppl. Table 1 for the full list of up- and downregulated proteins). Tumour necrosis factor-inducible gene 6 protein (TNFAIP6) was the by far most regulated protein in this dataset. TNFAIP6 was upregulated 57-fold by 500 ng/mL omentin ($P = 4.76 \times 10^{-3}$) (data not shown) and 140-fold by 2000 ng/mL omentin ($P = 4.44 \times 10^{-3}$) compared with untreated differentiated human adipocytes. Six other proteins were upregulated by more than 4-fold (4- to 24-fold, *P*-values between 2.88×10^{-4} and 4.65×10^{-2}) by 500 ng/mL omentin and more than 10-fold (12- to 89-fold, *P*-values between 1.49×10^{-3} and 3.03×10^{-2}) by 2000 ng/mL omentin: C-X-C motif chemokine 5 (CXCL5), complement factor B, cathepsin S (CTSS), secreted frizzled-related protein 4 (SFRP4), LIM and senescent cell antigen-like-containing domain protein 1 (LIMS1), and Lys-63-specific deubiquitinase BRCC36 (BRCC3). The remaining 13 proteins were upregulated by less than 10-fold by both omentin concentrations compared with control. In total, 18 out of the 20 proteins upregulated by 2000 ng/mL omentin (Table 1) were also among the top 20 upregulated proteins when treating adipocytes with 500 ng/mL omentin (exceptions: NEDD8 ultimate buster 1 and intercellular adhesion molecule 1 [ICAM1]).

The degree of downregulation of proteins was less pronounced for both omentin concentrations than the degree of upregulation described above. Overall, the expression of the top 20 downregulated proteins was reduced by 1.5- to 2.6-fold (*P*-values between 1.39×10^{-3} and 4.91×10^{-2}) by 2000 ng/ml omentin. Thirteen of these proteins were also in the top 20 of downregulated proteins in adipocytes treated with 500 ng/mL omentin (data not shown).

3.2 | Canonical pathways

Omentin-regulated proteins were overrepresented in seven canonical pathways (B-H $P < 0.05$). By far, the strongest association was observed for eukaryotic initiation factor (EIF)2 signalling ($P = 2.77 \times 10^{-15}$). Other pathways included complement system, inhibition of matrix metalloproteases, acute phase response signalling, regulation of eIF4 and the 70-kDa ribosomal S6 kinase (p70S6K) signalling, hepatic fibrosis/hepatic stellate cell activation, and protein ubiquitination pathway (*P*-values between 3.92×10^{-4} and 4.87×10^{-2}) (Table 2).

3.3 | Potential upstream regulators

Using the IPA analysis software, we identified 25 potential upstream activators of the omentin-regulated proteins. Most of these activators were pro-inflammatory cytokines (IL1B, TNF, IFNG, IL1A, MIF, CXCL8, IL6, OSM, IL17A, and SPP1). The second biggest group consisted of transcription regulators (STAT1 and 3, SMARCA4, SP1, and ETS1). Other groups were growth factors (TGFB1), transmembrane immune receptors (TLR3 and 4) and enzymes (PTGS2 and NOS2), protein complexes (NFkB and Cg), and kinases (IKKBK and MAPK8) (Table 3).

TABLE 1 Top 20 of the most upregulated and top 20 of the most downregulated proteins by 2000 ng/mL omentin in the supernatant of differentiated human adipocytes

Protein	Entrez Gene Name	Expression Fold	Expression P-Value
Upregulation			
TNFAIP6	Tumour necrosis factor-inducible gene 6 protein	+140.39	4.44×10^{-3}
CXCL5	C-X-C motif chemokine 5	+89.21	1.49×10^{-3}
CFB	Complement factor B	+35.23	7.98×10^{-3}
CTSS	Cathepsin 5	+12.92	1.89×10^{-2}
SFRP4	Secreted frizzled-related protein 4	+12.03	3.03×10^{-2}
LIMS1	LIM and senescent cell antigen-like-containing domain protein 1	+11.95	8.99×10^{-3}
BRCC3	Lys-63-specific deubiquitinase BRCC36	+11.90	8.62×10^{-3}
PTX3	Pentraxin-related protein PTX3	+8.04	6.13×10^{-3}
PROCR	Endothelial protein C receptor	+5.11	4.03×10^{-3}
PLA2G2A	Phospholipase A2, membrane associated	+5.05	3.35×10^{-2}
RPL22L1	60S ribosomal protein L22-like 1	+4.50	3.74×10^{-2}
VCAM1	Vascular cell adhesion molecule 1	+4.13	1.15×10^{-2}
ALDH1L2	Mitochondrial 10-formyltetrahydrofolate dehydrogenase	+3.91	3.48×10^{-3}
CORO2B	Coronin-2B	+3.91	4.59×10^{-2}
TFPI2	Tissue factor pathway inhibitor 2	+3.67	2.58×10^{-2}
RPN2	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2	+3.64	2.09×10^{-2}
NUB1	NEDD8 ultimate buster 1	+3.56	1.05×10^{-2}
NVL	Nuclear valosin-containing protein-like	+3.27	2.66×10^{-2}
ICAM1	Intercellular adhesion molecule 1	+3.22	9.13×10^{-4}
NKAP	NF-kappa-B-activating protein	+3.21	8.02×10^{-3}
Downregulation			
NUCKS1	Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1	-2.62	1.73×10^{-3}
TTK	Dual specificity protein kinase TTK	-2.60	5.34×10^{-3}
PLCG2	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-2	-2.29	2.65×10^{-2}
MAP1S	Microtubule-associated protein 1S	-2.09	8.47×10^{-3}
NCL	Nucleolin	-2.00	2.04×10^{-3}
CASQ1	Calsequestrin 1	-1.89	1.31×10^{-2}
EIF3D	Eukaryotic translation initiation factor 3 subunit D	-1.76	3.53×10^{-2}
RALGAP1	Ral GTPase-activating protein subunit alpha-1	-1.75	1.39×10^{-3}
EXOC2	Exocyst complex component 2	-1.65	4.91×10^{-2}
TPD52L2	Tumour protein D54	-1.64	3.27×10^{-2}
GCN1	eIF-2-alpha kinase activator GCN1	-1.63	2.14×10^{-2}
FAM120A	Constitutive coactivator of PPAR-gamma-like protein 1	-1.63	3.26×10^{-2}
DNMBP	Dynamin-binding protein	-1.61	2.93×10^{-2}
SEPP1	Selenoprotein P	-1.56	7.24×10^{-3}
RPL24	60S ribosomal protein L24	-1.55	4.00×10^{-2}
FAHD1	Acylpyruvase FAHD1, mitochondrial	-1.53	3.85×10^{-3}
ROR2	Tyrosine-protein kinase transmembrane receptor ROR2	-1.52	1.24×10^{-2}
PTMA	Prothymosin alpha	-1.51	4.88×10^{-2}
PLXDC2	Plexin domain-containing protein 2	-1.50	3.21×10^{-2}
TSPAN3	Tetraspanin-3	-1.48	3.05×10^{-2}

The fold-change in expression for the listed proteins refers to adipocytes treated with 2000 ng/mL omentin for 24 h compared with untreated adipocytes. The expression *P*-value was calculated by a paired *t*-test.

4 | DISCUSSION

The main findings of this study were (1) that the most strongly regulated secreted protein upon omentin treatment of differentiated human adipocytes supernatant was the anti-inflammatory protein TNFAIP6, (2) that omentin-regulated proteins were

overrepresented in pathways which might promote cellular stress, a pro-inflammatory environment, and a crosstalk with other organs, and (3) that the pro-inflammatory phenotype of other potential upstream activators might point to the induction of inflammatory pathways, predominantly of NFκB, for the omentin-induced secretory process.

TABLE 2 Enrichment of the omentin-regulated proteins in differentiated human adipocyte supernatant in canonical pathways

Canonical Pathway	B-H P-Value	Omentin-Regulated Proteins
EIF2 signalling	2.77×10^{-15}	RPL11, RPL24, RPL4, RPL22, RPL22L1, RPL35A, RPL12, RPL10A, RPL27, RPL27A, RPL18A, RPS13, EIF3D, RPL23A, RPS27L, RPL5, RPS15A, RPL36, RPS3, RPS24
Complement system	3.92×10^{-4}	C1R, C3, C1S, CFB, CFH
Inhibition of matrix metalloproteases	5.99×10^{-3}	HSPG2, TIMP1, MMP2, TFPI2
Acute phase response signalling	5.99×10^{-3}	C1R, C3, C1S, CFB, SERPINA3, CP, HNRNPK
Regulation of eIF4 and p70S6K signalling	2.23×10^{-2}	EIF3D, RPS13, RPS27L, RPS15A, RPS3, RPS24
Hepatic fibrosis/hepatic stellate cell activation	4.63×10^{-2}	VCAM1, LY96, ICAM1, TIMP1, HGF, MMP2
Protein ubiquitination pathway	4.87×10^{-2}	PSMB3, USP32, PSMA7, UBE3B, HSPA13, PSMA1, UBE2I

The analysis was based on proteins which were differentially secreted upon treatment with 2000 ng/mL omentin. *P*-values for the canonical pathways were calculated by Fisher's exact test and corrected for multiple testing using the Benjamini-Hochberg method (B-H *P*-value).

EIF, eukaryotic initiation factor; p70S6K, 70-kDa ribosomal S6 kinase.

Abbreviations of omentin-regulated proteins are explained in Suppl. Table 1.

4.1 | Anti-inflammatory TNFAIP-6 as most regulated protein upon omentin treatment

Tumour necrosis factor-inducible gene 6 protein is only produced upon exposure to inflammatory factors and has been described as protein with anti-inflammatory and tissue-protective properties.²⁸ It acts as an endogenous inhibitor of inflammatory processes and represents a component of a negative feedback loop.²⁸ Interestingly, one anti-inflammatory action of TNFAIP6 is the regulation of chemokine function.²⁸ TNFAIP6 binds to multiple chemokines and interacts with them through their glycosaminoglycan-binding site, which leads to the inhibition of their binding to glycosaminoglycans and endothelial cell surfaces.²⁸ In this comprehensive proteomics study, the chemokine CXCL5 was the second most upregulated protein in the supernatant of omentin-treated adipocytes. We hypothesise that TNFAIP6 might counterregulate the pro-inflammatory action of omentin in the acute setting by neutralising the potentially detrimental effects of chemokines such as CXCL5 secreted in response to omentin exposure on other cells such as adipocytes and endothelial cells. Such counterregulation would be in line with findings from cross-sectional studies showing an inverse association between omentin and cardiometabolic risk factors. The contrasting data from prospective studies linking omentin to higher cardiometabolic risk during follow-up would be consistent with persistently elevated omentin and loss or attenuation of this endogenous negative feedback loop.

In addition, our study revealed pentraxin-related protein (PTX3) as further binding partner of TNFAIP6, which was upregulated by omentin. PTX3, TNFAIP6, and hyaluronic acid are able to build multi-molecular PTX3/TNFAIP6/hyaluronic acid complexes.²⁹ PTX3 belongs to the pentraxin family of acute-phase proteins and is expressed after exposure to inflammatory signals such as IL-1 and TNF α .³⁰ Moreover, PTX3 is associated with endothelial function and cardiovascular events.³¹

Taken together, our study identified TNFAIP6 and two potential binding partners (CXCL5 and PTX3) as upregulated proteins upon acute omentin treatment. These proteins represent novel candidates for further studies that might reinforce or attenuate omentin activity as separate proteins or bound to TNFAIP6 and thereby affect cardiometabolic risk.

4.2 | Omentin-regulated proteins associated with inflammation, T2D, and comorbidities

It is noteworthy that further proteins that are among the most omentin-regulated proteins in adipocyte supernatant have been implicated in the pathogenesis of T2D. One protein is the adipokine SFRP4 which belongs to the Sfrp family, the largest family of wingless (Wnt) inhibitors.³² Higher levels of SFRP4 were associated with a higher risk of T2D and associated with higher fasting glucose, and lower insulin sensitivity index and disposition index.³³

CTSS is another protein which has been upregulated by omentin. This protein has been reported to be higher in diabetic rats compared to healthy ones and correlated with blood glucose levels.³⁴ In addition, CTSS is upregulated in adipose tissue from obese patients, and its inhibition led to a decrease of lipid content and adipocyte marker expression³⁴ indicating that it might affect both glucose metabolism and adipocyte function.

Two other proteins are known to be involved in endothelial dysfunction: ICAM-1 and vascular cell adhesion protein 1 (VCAM-1) belong to the Ig superfamily of cell membrane proteins.³⁵ The release of the soluble form of ICAM-1 (sICAM-1) and VCAM-1 (sVCAM-1) is related to inflammatory and infectious diseases.³⁵ Higher levels of both proteins are associated with increased risk for cardiovascular events.³⁶ Interestingly, we found that sICAM-1 and sVCAM-1 are also released by adipocytes in response to omentin. Although it is not known why adipocytes produce the soluble forms of these proteins and if they represent mere risk markers of CVD or rather contribute to its development, it should be noted that this finding agrees with the aforementioned positive association between omentin and cardiovascular risk in prospective studies.

LIMS1 has been described to be upregulated by the pro-inflammatory TNF α .³⁷ We found that also omentin is able to upregulate LIMS1 in adipocytes. One important role of LIMS1 is the regulation of the function of renal tubules³⁷ suggesting a potential omentin-mediated crosstalk with kidneys and diabetes-associated nephropathy.

With respect to potential omentin-induced signalling pathways, we identified an omentin-stimulated upregulation of the NF κ B activating protein (NKAP), which activates NF κ B. It has been proposed that

TABLE 3 Top 25 of other potential upstream activators of omentin-regulated proteins

Upstream Regulator	Activation z-Score	P-Value	Omentin-Regulated Proteins
Cytokines			
IL1B	4.38	4.20×10^{-10}	C1R, C3, CD44, CFB, CP, CTSS, CXCL5, HGF, HPRT1, HSPG2, ICAM1, LY96, MMP2, MYLK, PAPP, PLA2G2A, PLXDC2, PTX3, SELENOP, SERPINA3, TFPI2, TIMP1, TNFAIP6, TPD52L2, VCAM1
TNF	4.65	5.47×10^{-9}	ALAD, B4GALT1, C3, CA12, CALR, CD44, CFB, CP, CTSS, CXCL5, EXT1, HGF, HPRT1, HSPG2, ICAM1, LY96, MMP2, MYLK, OGN, PAPP, PDIA4, PLA2G2A, PLXDC2, PTX3, RPS13, RPS3, SELENOP, SERPINA3, SMPD1, TFPI2, TIMP1, TNFAIP6, TPD52L2, VCAM1
IFNG	2.10	5.08×10^{-5}	AHCY, C1R, C3, CD44, CFB, CP, CTSS, CXCL5, HSPG2, HTRA1, ICAM1, KCTD7, LY96, MMP2, PAPP, PLA2G2A, PTX3, RPL23A, SELENOP, TIMP1, TNFAIP6, VCAM1
IL1A	2.81	1.13×10^{-4}	CD44, CXCL5, HSPG2, ICAM1, MMP2, PTX3, SERPINA3, VCAM1
MIF	2.07	1.13×10^{-4}	CD44, HGF, ICAM1, MMP2, TIMP1, VCAM1
CXCL8	2.18	1.39×10^{-4}	CD44, HSPG2, ICAM1, MMP2, VCAM1
IL6	2.89	9.49×10^{-4}	AHNAK, C3, CD44, CP, CXCL5, HGF, ICAM1, MMP2, PLA2G2A, SERPINA3, TIMP1, TTK, VCAM1
OSM	2.55	2.65×10^{-3}	C1R, C1S, CXCL5, HGF, ICAM1, MMP2, SERPINA3, SMPD1, TIMP1, VCAM1
IL17A	2.39	3.69×10^{-3}	C3, CXCL5, ICAM1, MMP2, TIMP1, VCAM1
SPP1	2.18	6.39×10^{-3}	CD44, CXCL5, ICAM1, MMP2, TIMP1
CSF2	2.41	7.75×10^{-2}	C3, CFH, HGF, ICAM1, LY96, MMP2
Growth factors			
TGFB1	2.06	8.50×10^{-7}	AHNAK, C1R, C1S, C3, CD44, CFB, CFH, CRMP1, CTSS, DAAM1, EXT1, EXT2, FBLN2, HGF, HSPG2, HTRA1, ICAM1, LASP1, LIMS1, LOXL2, MMP2, MYLK, MYO1C, PAPP, PTX3, RPN2, SERPINA3, TIMP1, TNFAIP6, VCAM1
Transmembrane receptors			
TLR4	2.19	3.03×10^{-3}	C3, CD44, CFB, HTRA1, ICAM1, PTX3, SMPD1, VCAM1
TLR3	2.43	6.34×10^{-3}	C3, CFB, ICAM1, PROCR, PTX3, TIMP1
Enzymes			
PTGS2	2.22	1.02×10^{-4}	CD44, CXCL5, ICAM1, MMP2, NOP2, PTMA, TNFAIP6
NOS2	2.00	3.41×10^{-2}	CD44, CP, SERPINA3, TIMP1
Transcription regulators			
STAT1	2.37	4.51×10^{-4}	C1R, C1S, C3, CFB, CTSS, HTRA1, ICAM1, LY96, SERPINA3
SMARCA4	2.56	6.30×10^{-4}	AHNAK, CD44, CP, CTSS, ICAM1, LMNA, LOXL2, LUM, MMP2, MYLK, PTX3, SELENOP
SP1	2.58	1.01×10^{-3}	B4GALT5, CXCL5, HGF, ICAM1, MAT2B, MMP2, MYLK, PROCR, SMPD1, TFPI2, TIMP1
STAT3	2.24	1.04×10^{-2}	CFB, HGF, ICAM1, MMP2, PDIA4, PLA2G2A, SERPINA3, TFPI2, TIMP1
ETS1	2.18	1.39×10^{-2}	B4GALT5, CD44, HGF, ICAM1, MMP2
Complexes			
NFkB (complex)	3.63	6.73×10^{-7}	B4GALT1, C1R, C3, CD44, CFB, CGREF1, CXCL5, ICAM1, MMP2, MYLK, PLA2G2A, PTX3, SERPINA3, TFPI2, TIMP1, TNFAIP6, VCAM1
Cg	2.79	2.48×10^{-3}	ICAM1, MMP2, PAPP, PTX3, SFRP4, TFPI2, TIMP1, TNFAIP6
Kinases			
IKBKB	2.01	1.46×10^{-5}	C3, CD44, CP, CXCL5, HGF, ICAM1, MMP2, OGN, PTX3, VCAM1
MAPK8	2.19	4.21×10^{-3}	B4GALT1, B4GALT5, BDP1, MMP2, PTX3

The identification of potential upstream activators was based on differentially regulated proteins by omentin treatment with 2000 ng/mL in differentiated human adipocytes supernatant. The *P*-values were calculated by Fisher's exact test. The molecule type was filtered according to the following categories: complex, cytokine, enzyme, growth factor, kinase, phosphatase, transcription regulator, translation regulator, transmembrane receptor, and transporter.

Abbreviations: Cg, choriogonadotropin; CXCL8, C-X-C motif chemokine ligand 8; ETS1, ETS proto-oncogene 1; IFNG, interferon gamma; IKBKB, inhibitor of kappaB kinase beta; IL1A, interleukin-1 alpha; IL1B, interleukin-1 beta; IL6, interleukin-6; IL17A, interleukin-17 alpha; MAPK8, mitogen-activated protein kinase 8; MIF, macrophage migration inhibitory factor; NFkB (complex), nuclear factor kappa B; NOS2, nitric oxide synthase 2; OSM, oncostatin M; PTGS2, prostaglandin-endoperoxide synthase 2; SMARCA4, SMI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily 5, member 4; SP1, Sp1 transcription factor; SPP1, secreted phosphoprotein 1; STAT1, signal transducer and activator of transcription 1; STAT3, signal transducer and activator of transcription 3; TGFB1, transforming growth factor beta 1; TNF, tumour necrosis factor; TLR3, toll-like receptor 3; TLR4, toll-like receptor 4.

Abbreviations of omentin-regulated proteins are explained by Suppl. Table 1.

NKAP mediates TNF- and IL-1-induced NF κ B activation.³⁸ Our data show that NKAP is also released into the supernatant of omentin-treated adipocytes. Again, we can exclude that NKAP is released because of cell death as we performed cell viability tests that did not indicate any differences between untreated and omentin-treated adipocytes (data not shown).

Activated NF κ B signalling is associated with the action of the inflammasome. Interestingly, Lys-63-specific deubiquitinase BRCC36 (BRCC3), which also belongs to the strongest upregulated proteins, has been identified as deubiquitinating enzyme of NLR family pyrin domain containing 3 (NLRP3) and thereby regulates the activity of the inflammasome.³⁹ This in turn supports the pro-inflammatory action of omentin.

4.3 | Omentin-regulated proteins associated with general cellular functions

Most of the top 20 of the most-downregulated proteins by omentin also play a role in the regulation of cellular processes. Several proteins are involved in protein synthesis such as the eukaryotic translation initiation factor 3 subunit D (EIF3D), and the eIF2 alpha kinase activator homologue GCN1.^{40,41} In obesity, stress kinases are able to inhibit the general protein synthesis and thereby affect endoplasmic reticulum (ER) function, which in turn has a harmful impact on inflammation and metabolism.⁴² This finding indicates that omentin might cause cellular stress in differentiated human adipocytes.

The most interesting protein in this group was selenoprotein P (SEPP1) which has anti-oxidant activity. Secreted SEPP1 is suggested to bind to proteoglycans on the vascular endothelial surface and thereby protects against oxidative damage.⁴³ However, several studies reported a detrimental effect of SEPP1 on insulin sensitivity.^{43,44} Nevertheless, this finding might be explained by a dysregulated carbohydrate metabolism in T2D because SEPP1 is regulated by insulin and glucose levels.⁴⁴ Further studies are necessary to evaluate the role of adipocyte-derived selenoprotein on adipocytes and/or other cell types relevant for cardiometabolic diseases.

4.4 | Omentin-regulated secretory proteins were overrepresented in pathways relevant for cellular stress, a pro-inflammatory environment, and crosstalk between organs

Figure 1 shows a hypothetical model for the potential physiological role of omentin in differentiated human adipocytes which will be explained in detail in the following:

The *EIF2 signalling* pathway was the most significant pathway across the omentin-regulated proteins. Nineteen out of 20 affected proteins are ribosomal proteins. Interestingly, apart from one protein, all proteins were downregulated in our dataset indicating that the EIF2 signalling pathway is inhibited. In the pathway named *Regulation of eIF4 and p70S6K Signalling*, five out of six regulated proteins were also ribosomal proteins mostly overlapping with those from the *EIF2 signalling* pathway. The protein machinery can be downregulated by phosphorylation of EIF2 by various stress kinases which are activated by metabolic overload leading to inhibition of general protein synthesis and ER stress.⁴² This again indicates that omentin-treated adipocytes might suffer from cellular stress associated with reduced protein synthesis.

The pathways named *Complement System* and *Acute Phase Response Signalling* are additional canonical pathways in which omentin-regulated proteins are significantly overrepresented. The acute-phase response leads to a higher release of pro-inflammatory factors which are able to activate the complement system.⁴⁵ Apart from immune defence, the complement system is also involved in metabolic and inflammatory processes in adipose tissue.⁴⁶ The omentin-regulated complement components C3, complement factors B and H have pro-inflammatory properties and are associated with insulin resistance and T2D.^{46,47} Based on our data, we conclude that omentin directly induced the acute-phase response and components of the complement system which in turn might contribute to the observed pro-inflammatory condition in adipocytes and thus promote the development of insulin resistance and T2D.

The pathway designated *Inhibition of the Matrix Metalloproteases* contains matrix metalloproteases (MMPs), as the omentin-regulated MMP2, which are able to regulate the bioavailability and activity of

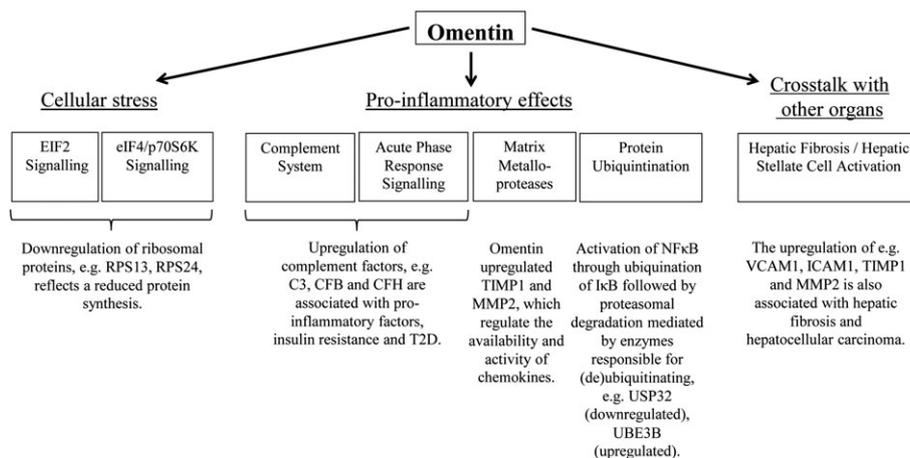


FIGURE 1 Proposed physiological role of omentin in differentiated human adipocytes

chemokines.⁴⁸ The tissue inhibitors of metalloproteases (TIMP) tightly control the synthesis and degradation of MMPs.⁴⁹ The omentin-regulated TIMP1 is known as an adipocyte-secreted protein upregulated by pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF α mediated by NF κ B.^{50,51} Our data suggest that omentin-regulated MMP2 might contribute to the pro-inflammatory environment and its regulator TIMP1 might be regulated by omentin through activation of NF κ B in adipocytes.

Another inflammation-related canonical pathway is the *Protein Ubiquitination Pathway*. The ubiquitin-proteasome system is known as activator of NF κ B because its activity depends on the ubiquitination of I κ B followed by proteasomal degradation.⁵² Therefore, these data again support the potential importance of NF κ B in omentin action in adipocytes.

Our study also revealed *Hepatic Fibrosis/Hepatic Stellate Cell Activation* as pathway in which omentin-affected proteins are overrepresented. It is known that proteins that are released from adipocytes are also able to control hepatic inflammation and fibrosis.⁵³ In our dataset, predominantly VCAM-1 and ICAM-1 as well as MMP2 and TIMP1 were upregulated by omentin and involved in this pathway. Serum levels of VCAM-1 and ICAM-1 are predictors of hepatic fibrosis and hepatocellular carcinoma risk, respectively.^{54,55} Interestingly, the transcriptional activation of TIMP-1 and MMP-2 is important for the pathogenesis of liver fibrosis.⁵³ The potential link between these proteins and pathways should be investigated by omentin treatment of hepatocytes or co-culture experiments of adipose tissue and hepatocytes to characterise the potential role of omentin in the cross-talk between both tissues.

4.5 | Potential upstream regulators of omentin-regulated proteins are predominantly NF κ B activators

The in silico characterisation of potential upstream regulators of the omentin-regulated secretion pattern identified several molecular mechanisms by which omentin might regulate protein secretion and thereby affect the development of T2D and CVD in human studies. Most of the potential upstream activators of the proteins that are differentially regulated by omentin are pro-inflammatory cytokines. Interestingly, the most activated upstream cytokines IL-1 β and TNF α regulate the activity of NF κ B, and all three classes of MAPK (JNK, ERK1/2, and p38 MAPK).^{56,57} The cytokine IFN- γ is known to activate the transcription factor signal transducer and activator of transcription (STAT) 1 via the receptor-associated Janus kinases JAK 1 and 2 leading to the regulation of alternative cytokine signalling pathways. In addition, IFN- γ is also described to activate ERK1/2 and has been implicated in the vascular proliferative responses in atherosclerosis.⁵⁸ The cytokine IL-6 activates STAT3 through JAKs and thereby induces the transcription of target genes and regulates lipid and glucose metabolism as well as immune responses.⁵⁹ The transmembrane receptors toll-like receptors (TLR)3 and 4 also regulate NF κ B activity,⁵⁶ and Sp1 transcription factor (SP1) regulates transcription factor p65 (RelA) which is involved in NF κ B heterodimer formation.⁶⁰ In addition, NF κ B and its inhibitor of nuclear factor kappa-B kinase subunit beta (IKKB) were themselves identified as potential upstream regulators. Therefore, this study argues for a central role of NF κ B in the pro-inflammatory action of omentin.

4.6 | Strengths and limitations

One important strength of our study is the use of primary human differentiated adipocytes. In addition, we used physiological concentrations of omentin and performed a comprehensive analysis of the almost 3500 secreted proteins induced by omentin using proteomics analysis.

Regarding limitations, it should be noted that we used commercially available preadipocytes which we differentiated into mature adipocytes. Although the preadipocytes had been purified by magnetic beads or cultivation in medium which impeded the adherence of other cell types, a low level of contamination with, eg, immune or endothelial cells cannot be excluded as in all in vitro assays using primary cells isolated from adipose tissue. Additionally, differentiation into mature adipocytes never reaches 100%, so that our cultures may also have contained a low percentage of preadipocytes, which however was the same for each treatment group for any given cell donor. We found that omentin-regulated proteins are localised either in the cytoplasm, extracellular space, plasma membrane, or nucleus. According to a colorimetric cell viability test (WST-8), no differences following omentin treatment were observed compared with control (data are not shown), so that it is unlikely that cell death due to omentin treatment contributed to the effects described here. However, we cannot exclude that a low level of cell death in all treatment groups led to the release of proteins that are usually localised within the cell.

5 | CONCLUSION

Omentin increased the secretion of a series of proteins, most importantly TNFAIP6, from differentiated human adipocytes. This protein is only expressed in response to inflammatory stimuli and has anti-inflammatory activities which may suggest an inhibitory feedback loop to omentin release. However, the majority of omentin-regulated proteins and their potential upstream regulators pointed towards pro-inflammatory effects of omentin that may be mediated primarily by NF κ B. Pathway analysis supported a role of omentin in inflammation, cellular stress, and inter-organ cross-talk, which may explain the positive associations between omentin and cardiometabolic risk in prospective studies in humans.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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