endocrines

MDPI

Article

Prothymosin-Alpha, a Novel and Sensitive Biomarker of the Inflammatory and Insulin-Resistant Statuses of Obese Individuals: A Pilot Study Involving Humans

Marta Greco [1,2,†], Maria Mirabelli [1,†], Vera Tocci [1,3], Yelyzaveta Mamula [4,5], Alessandro Salatino [1], Francesco S. Brunetti [1], Francesco Dragone [5], Luciana Sicilia [1], Omar Tripolino [2,5], Eusebio Chiefari [1], Daniela P. Foti [2,5,*] and Antonio Brunetti [1,3,*]

1 Department of Health Sciences, University "Magna Græcia" of Catanzaro, 88100 Catanzaro, Italy; marta.greco@unicz.it (M.G.); maria.mirabelli@unicz.it (M.M.); tocci.vera@gmail.com (V.T.)
2 Unit of Clinical Pathology, "Mater-Domini" University Hospital, 88100 Catanzaro, Italy; omar.tripolino@unicz.it
3 Unit of Endocrinology, "Mater-Domini" University Hospital, 88100 Catanzaro, Italy
4 Educational and Scientific Centre, Institute of Biology and Medicine, Taras Shevchenko National University of Kyiv, 01601 Kyiv, Ukraine
5 Department of Experimental and Clinical Medicine, University "Magna Græcia" of Catanzaro, 88100 Catanzaro, Italy
* Correspondence: foti@unicz.it (D.P.F.); brunetti@unicz.it (A.B.); Tel.: +39-0961-3694164 (D.P.F.); +39-0961-3694368 (A.B.); Fax: +39-0961-3694147 (A.B.)
† These authors contributed equally to this work.

Abstract: Background: Obesity constitutes a chronic, low-grade inflammatory status that predisposes people to the development of insulin resistance and cardiometabolic complications. Hypoxia, a main pathological feature of visceral fat in obese individuals, has been shown to affect the secretome of murine 3T3-L1 adipose cells, causing the upregulation of prothymosin-alpha (ProT-α), which is a protein with immunomodulatory functions that was originally found in the thymus. The aim of this case–control observational study was to measure the circulating levels of ProT-α in obese and lean individuals and determine whether such levels are correlated with inflammatory and metabolic parameters. Methods: Sixty-one obese patients (BMI $\geq$ 30 Kg/m^2) and fifty-one age-matched, lean controls (BMI 18.5–24.9 Kg/m^2) were recruited in the Endocrinology Unit ("Mater-Domini") of the University Hospital of Catanzaro, Italy. The exclusion criteria included affliction with acute and systemic inflammatory states (i.e., leukocytosis), recent infectious diseases or vaccinations, obesity complications (i.e., type 2 diabetes mellitus and cardiovascular diseases), hepatic or renal failure, pregnancy and lactation, cancer, use of drugs or alcohol, and smoking. Apart from routine biochemical determinations, serum samples were screened for the presence of ProT-α using an ELISA method and for the presence of a panel of inflammatory cytokines and growth factors via a multiparametric chemiluminescence micro-array. Results: Between the age-matched groups, no statistically significant differences were shown in relation to fasting glucose, HbA1c, liver function tests, lipid profiles, circulating interleukins (IL)-1α, -1β, -2, -4, -8, and -10, MCP-1, TNF-α, VEGF and EGF. Instead, significantly higher median levels were observed in obese patients vs. lean controls with respect to fasting insulin levels ($p < 0.001$), a classic insulin resistance marker, and IL-6 ($p = 0.004$). In addition, ProT-α levels were significantly and considerably higher in obese patients compared to lean controls (median ProT-α, 600.0 vs. 411.5 pg/mL, $p = 0.004$) and showed a moderate to strong positive relationship with fasting insulin levels and selected cytokines (i.e., TNF-α and IL-8). Conclusions: An increase in circulating levels of ProT-α is linked with obesity and can be detected before any clinical cardiometabolic complications develop. ProT-α may represent a novel and sensitive biomarker for inflammation and insulin resistance in obese individuals.

Citation: Greco, M.; Mirabelli, M.; Tocci, V.; Mamula, Y.; Salatino, A.; Brunetti, F.S.; Dragone, F.; Sicilia, L.; Tripolino, O.; Chiefari, E.; et al. Prothymosin-Alpha, a Novel and Sensitive Biomarker of the Inflammatory and Insulin-Resistant Statuses of Obese Individuals: A Pilot Study Involving Humans. *Endocrines* **2023**, *4*, 427–436. https://doi.org/10.3390/endocrines4020032

Academic Editors: Alessandro Genazzani and Mary Anna Venneri

Received: 29 March 2023
Revised: 5 June 2023
Accepted: 9 June 2023
Published: 15 June 2023

Keywords: prothymosin-alpha (ProT-α); obesity; inflammation; insulin resistance; cytokines; circulating biomarkers

1. Introduction

Obesity is an exponentially increasing public health problem in Western countries that predisposes individuals to several life-threatening disorders, including type 2 diabetes mellitus, atherosclerotic cardiovascular diseases, and some kinds of cancers [1,2]. The global challenge posed by the dramatic rise in obesity and its co-morbidities has produced several studies aiming to acquire a deeper understanding of the currently unexplored biological effects of adipose tissue in humans, i.e., transcending its classical role as a fat reservoir for energy storage. In the last few decades, many important investigations have attributed a novel, active role to adipose cells in the biosynthesis and secretion of several molecules, collectively named adipokines, that have different functions, roles, and impacts at the systemic level [3,4]. Among the most significant of these cells are leptin, adiponectin, interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α), which have major effects on insulin sensitivity and glucose metabolism [4,5]. Even if the identification and characterization of new biomolecules originating from adipose tissue are still ongoing, it has been widely demonstrated that in obese individuals, many adipokines are dysregulated and contribute to the development of chronic, low-grade inflammation; insulin resistance; thrombophilia; and atherogenesis, all of which being conditions underpinning the pathophysiology of obesity-related complications [1,2,6].

In the context of obesity, hypoxia has been implicated as a crucial, early trigger in the switch to a less favorable adipokine profile in animal models and humans [7,8], wherein hypoxia-inducible factor-1 (HIF-1) acts as the master regulator, at the transcriptional level, of the molecular effects linked to hypoxia [9,10]. Molecular changes due to hypoxia sustain inflammation and insulin resistance, which are two important features commonly observed in obesity [7,11].

In an attempt to contribute to the identification of new molecules of interest with respect to obesity, our group previously analyzed differentially expressed proteins in the secretome of hypoxic vs. normoxic cultured murine differentiated 3T3-L1 adipose cells and identified prothymosin-alpha (ProT-α) as numbering among the upregulated proteins in cases of hypoxia [12].

ProT-α is a small acidic protein encoded in humans by the *PTMA* gene that was initially identified in the thymus but is also expressed in many mammalian cells [13,14]. Among its multifaceted activities, it plays a role in inflammation by enhancing T-helper type 1 (Th1) adaptive immune responses and stimulating the differentiation of monocytes into dendritic cells and their production of pro-inflammatory cytokines in vitro. [15]. Moreover, it has been proven to stimulate cell proliferation and inhibit apoptosis and oxidative stress [13,16]. The peculiar pleiotropic effects of ProT-α are evidenced by the variety of its cellular action sites, being the main localization within the nucleus, in which ProT-α plays a role in the compaction of chromatin by interacting with the histone H1, but also within the cytoplasm and even the extracellular compartment. In the latter case, ProT-α, which lacks a signal peptide, functions like many molecules termed "alarmins"; it is released after degranulation, cell death, injury, or following immune stimulation to help orchestrate the immune response [17]. One of its mechanisms of action is related, through toll-like receptor 4 (TLR4), to the activation of NF-kB, which is the main transcriptional mediator of inflammation [18].

To the best of our knowledge, even if adipose tissue has been previously described as a source of ProT-α [14] and an increase in ProT-α has been reported in a surrogate in vitro model of obesity (i.e., hypoxic 3T3-L1 adipose cells) [12], little is known about the circulating levels of this biomolecule in obese patients, in which ProT-α could theoretically promote the low-grade, chronic inflammation that typically characterizes obesity.

The aim of this study is to investigate ProT-α levels in patients with obesity and to evaluate this protein in terms of its role as a potential biomarker in obesity-related inflammatory states.

2. Materials and Methods

2.1. Study Participants

For this case–control observational study, 61 obese patients and 51 lean, age-matched controls of both genders were consecutively recruited at the Endocrinology Unit outpatient service of the "Mater-Domini" University Hospital of Catanzaro, Italy, from June 2022 to February 2023. Inclusion criteria were as follows: age of 18–55 and body mass index (BMI) $\geq$ 30 Kg/m^2 (obese patient group) or between 18.5 and 24.9 Kg/m^2 (lean control group). Exclusion criteria were as follows: known chronic or acute inflammatory states, including autoimmune disorders, erythrocyte sedimentation rate (ESR) > 30 mm/h, leukocytosis (white blood cell count (WBC) > 10.0 $\times$ 10^6/mL), diagnosis of type 2 diabetes mellitus (according to American Diabetes Association criteria [19]), hepatic or renal failure, cancer, hypertension, history of cardiovascular events or instrumental evidence of atherosclerotic vascular lesions, pharmacological treatments (only hormonal substitution therapy, such as L-thyroxine for managing non-autoimmune subclinical hypothyroidism, was admitted), smoking, and consumption of alcohol. Potential participants who had received vaccinations or were aware of having contracted systemic infection diseases, including the novel coronavirus disease COVID-19 [20], within the last 30 days were excluded from the study. In addition, to mitigate the possible impact of circulating estrogens and other sex hormones on the cellular immune responses and production of pro-inflammatory molecules [21], including ProT-α [22], women who were either pregnant, lactating, or experiencing menopause were prevented from participating in the study.

On the morning of enrollment, after fasting for at least 8 h, patients and controls underwent a clinical examination, which included the recording of anthropometric parameters and blood pressure measurement, phlebotomy for blood collection, and laboratory assessments, as detailed in the next section.

2.2. Laboratory Assessments

Venous blood of fasting participants was collected and immediately processed to determine WBC count via the blood analyzer ADVIA 2120i (Siemens Healthcare Diagnostics, Berlin, Germany) and for routine biochemical analysis, for which parameters such as glycemia, insulin, glycated hemoglobin (HbA1c), liver function tests (AST, ALT, and γGT), total cholesterol, HDL-cholesterol, triglycerides, ESR, and creatinine were investigated. Serum aliquots, obtained following centrifugation, were frozen and stored at −80 °C for further determination of ProT-α and cytokines/growth factors. HbA1c analysis was carried out using an affinity boronate chromatographic method performed using Hb9210, (Menarini Diagnostics, Florence, Italy), insulin analysis was performed using an immunochemiluminescent method carried out on ADVIA Centaur XP (Siemens, Germany), and glycemia, AST, ALT, γGT, creatinine, ESR, and lipid profile analyses were performed using a Cobas 8000 (Roche Diagnostics, Switzerland). ProT-α levels were determined using an ELISA method with the human Prothymosin-alpha kit from MyBiosource (MyBioSource Inc., San Diego, California, United Stated) on the Triturus analyzer (Diagnostics Grifols, S.A., Barcelona, Spain). Intra-assay and inter-assay precision were <8% and <10%, respectively, while the reported sensitivity was 3.9 pg/mL. Cytokines and growth factors (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, monocyte chemoattractant-1 (MCP-1), TNF-α, epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF)) were determined via a multiparametric chemiluminescent method using Cytokine Array I (Randox Labs, Dublin, Ireland) and the Evidence Investigator analyzer, as previously described [23]. Concentrations below the limit of quantification (LOQ) are indicated with zero. All assays were performed using the recommended reagents and according to the manufacturers' instructions.

2.3. Statistical Analysis

Continuous traits were expressed as medians and interquartile ranges (IQR), while categorical traits were expressed as numbers and percentages. The Mann–Whitney U test was used to determine if there was a significant difference in the continuous variables between obese patients and lean controls. The Chi-square test was used to compare proportions. To investigate the relationship between ProT-α and obesity and its related known inflammatory clinical and biochemical parameters that suggest a high cardiometabolic risk [10], a series of Pearson's correlation analyses were performed. To confirm that ProT-α is associated with increased serum fasting insulin levels [24], linear regression analysis was performed, adjusting for potential confounding factors. The dependent and independent variables were log-transformed, and the assumption of residuals' normality was checked by visually inspecting the Q-Q plot distribution. A significance level of 0.05 was set for all analyses. Data were analyzed using JASP Graphical Statistical Software Version 0.17.1.0 (University of Amsterdam, Amsterdam, Netherlands) based on R Stats packages.

3. Results

3.1. Clinical and Biochemical Features of the Study Participants

Even though patients with complicated obesity were prevented from participating in this study (as shown in Table 1), the case and control groups differed with respect to a number of clinical and biochemical variables that negatively affect cardiometabolic risk. With the exception of weight and BMI, which were indicative of the inclusion criteria of the study and the appropriate selection of patient groups, obese individuals had, on average, higher systolic blood pressure levels (median systolic pressure: 128 vs. 110 mmHg, $p = 0.016$) and a tendency for higher diastolic blood pressure compared to lean individuals of the same age. These findings are consistent with the idea that obesity may lead to a more rapid progression to hypertension and cardiovascular damage [1,2,6]. In addition, obese patients had higher fasting insulin levels (median insulin, 20.0 vs. 8.0 μU/mL, $p < 0.001$) and higher HOMA-IR indices (median HOMA-IR, 4.5 vs. 1.6, $p < 0.001$), suggesting that they were efficiently compensating for insulin resistance by producing more insulin in order to maintain glucose homeostasis. In fact, the fasting glucose levels and HbA1c fractions were similar between the two patient groups and well below the diagnostic thresholds for type 2 diabetes mellitus [19]. Additionally, there were no significant differences in the liver function tests or lipid profiles (Table 1). However, obese patients tended to have lower HDL cholesterol and higher triglyceride levels, which may indicate an initial metabolic impairment that could lead to the development of metabolic syndrome over time.

The WBC counts did not indicate that there were any significant differences between the two groups. However, the obese patients displayed marginally elevated rates of erythrocyte sedimentation, despite the fact that the values fell within the normal range (median ESR, 8 vs. 2, $p < 0.001$).

3.2. Cytokines, Growth Factors, and ProT-α as Markers of Inflammation and Insulin Resistance in Obese Individuals

While searching for novel biomarkers of inflammation in obese individuals, we measured the circulating ProT-α levels together with selected cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, MCP-1, and TNF-α) and growth factors (EGF, VEGF) that originate, among other tissues, from visceral fat and are potentially linked with insulin resistance [10]. As shown in Table 2, no significant differences in the levels of serum cytokines and growth factors could be observed between the obese patients and lean controls, with the exception of the levels of IL-6 (median IL-6, 3.1 vs. 1.3 pg/mL, $p = 0.004$) and a trend for higher levels of VEGF in the obese group. Remarkably, ProT-α levels were significantly and considerably higher in the cases compared to the controls (median ProT-α, 600.0 vs. 411.5 pg/mL, $p = 0.004$), thus supporting the original hypothesis that increased secretion of this protein from visceral adipocytes might occur under the hypoxic conditions of obesity [12].

Table 1. Clinical and biochemical characteristics of study participants grouped into obese patients and lean controls.

	Lean Controls (n = 51)	Obese Patients (n = 61)	*p* Value
Female gender, N	27 (52.9%)	45 (75.0%)	**0.015**
Age, years	33 (30–40)	33 (28–41)	0.690
Weight, Kg	60.0 (53.0–67.5)	99.0 (89.3–117.8)	**<0.001**
BMI, Kg/m^2	21.9 (20.0–23.3)	37.5 (33.3–42.0)	**<0.001**
Systolic pressure, mmHg	110 (110–122)	128 (120–134)	**0.016**
Diastolic pressure, mmHg	80 (75–80)	80 (80–88)	0.080
Glucose, mg/dL	88.0 (82.5–91.0)	90.0 (85.5–98.0)	0.087
Insulin, µU/mL	8.0 (5.3–10.0)	20.0 (12.0–26.0)	**<0.001**
HOMA-IR	1.6 (1.0–2.2)	4.5 (2.7–6.5)	**<0.001**
HbA1c, %	5.3 (5.2–5.4)	5.3 (5.1–5.5)	0.684
AST, U/L	15 (15–16)	17 (14–23)	0.455
ALT, U/L	19 (17–20)	19 (14–27)	0.949
γGT, U/L	29 (22–35)	18 (13–29)	0.486
Total Cholesterol, mg/dL	184.0 (153.7–215.3)	166.0 (152.0–186.0)	0.692
HDL Cholesterol, mg/dL	59.0 (52.0–68.0)	45.0 (38.0–49.0)	0.072
Triglycerides, mg/dL	81.0 (67.8–98.8)	106.0 (82.0–145.0)	0.210
Creatinine, mg/dL	0.74 (0.63–0.82)	0.69 (0.61–0.77)	0.761
WBC, 10^6/mL	5.5 (4.6–7.0)	5.1 (4.1–5.8)	0.133
ESR, mm/h	2 (2–5)	8 (5–16)	**<0.001**

Data are expressed as medians (IQR) or as N (%). Differences between patient groups were compared using the Mann–Whitney U test or the Chi-square test as appropriate. Bold values denote statistical significance at $p < 0.05$. HOMA-IR, the homeostasis model assessment of insulin resistance, was calculated using the following formula: glucose (mg/dL) × insulin (µU/mL)/405 [25].

Table 2. Serum levels of cytokines, growth factors, and prothymosin alpha (ProT-α).

	Lean Controls (n = 51)	Obese Patients (n = 61)	*p* Value
IL-1α, pg/mL	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.828
IL-1β, pg/mL	0.0 (0.0–0.9)	0.0 (0.0–0.0)	0.433
IL-2, pg/mL	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.457
IL-4, pg/mL	0.0 (0.0–1.5)	1.4 (0.3–1.7)	0.269
IL-6, pg/mL	1.3 (1.1–1.9)	3.1 (2.3–4.5)	**0.004**
IL-8, pg/mL	19.6 (6.4–38.1)	18.8 (10.0–55.9)	0.565
IL-10, pg/mL	0.0 (0.0–1.2)	0.0 (0.0–1.2)	0.906
MCP-1, pg/mL	263.8 (228.1–290.1)	259.0 (199.5–303.5)	0.838
TNF-α, pg/mL	8.0 (0.0–11.9)	3.7 (3.0–5.4)	0.239
EGF, pg/mL	172.7 (94.3–179.4)	140.7 (103.3–151.5)	0.838
VEGF, pg/mL	101.1 (69.1–193.9)	203.9 (166.9–305.0)	0.061
ProT-α, pg/mL	411.5 (267.3–663.0)	600.0 (395.8–915.8)	**0.004**

Data are expressed as medians (IQR). Differences between patient groups were compared using the Mann–Whitney U test. Bold values denote statistical significance at $p < 0.05$.

Considering the immunomodulatory function of ProT-α and its theoretical potential to promote the low-grade, chronic inflammatory status that predisposes obese individuals to cardiometabolic risk, we performed a series of Pearson's correlation analyses of this protein and the clinical and biochemical markers relevant to inflammation and/or insulin resistance. While the obese patients showed a slight but significant increase in levels of cytokine IL-6 compared to the lean controls, there was no correlation between ProT-α levels and this inflammatory marker. This suggests that ProT-α might have a modulatory influence on inflammatory pathways different from those related to IL-6. The data shown in Table 3 indicate that there is a moderate to strong positive relationship between ProT-α and TNF-α (r = 0.561, $p < 0.01$), between ProT-α and IL-8 (r = 0.491, $p < 0.01$), and between ProT-α and fasting insulin levels (r = 0.351, $p < 0.01$). The significant negative association between ProT-α and age was lost when only female participants were examined in the correlation analysis, thus correcting the imbalance in sex distribution that existed between

cases and controls. In this regard, it is important to note that before their menopausal transition, women are typically less likely to experience cardiovascular morbidity as a result of obesity. When compared to men, the appearance of atherosclerotic vascular lesions can be delayed by approximately ten years in women [26]. Gender-related factors affecting the incidence and severity of cardiometabolic diseases may have contributed to the higher enrollment rate of eligible female participants in the (uncomplicated) obese patient group compared to the lean control group (75.0% vs. 52.9%, $p = 0.015$) (Table 1). However, the possibility that female gender may influence cardiometabolic risk in obesity through a distinct modulation of immune functions, possibly related to estrogens and other sex hormones, cannot be excluded [21,27], as only women presented evidence of a significant positive moderate association between ProT-α and IL-10 and between ProT-α and BMI (r = 0.340 for both, $p < 0.05$) (Table 3).

Table 3. Correlation analysis concerning ProT-α and clinical and biochemical parameters of inflammation and insulin resistance.

	BMI	Age	Insulin	IL-1α	IL-1β	IL-2	IL-4	IL-6	IL-8	IL-10	MCP-1	TNF-α	EGF	VEGF
ProT-α	0.214	−0.327 **	0.351 **	0.208	−0.137	0.171	−0.268	−0.090	0.492 **	0.149	0.010	0.561 **	0.240	0.259
ProT-α §	0.340 *	−0.220	0.285 *	0.199	−0.173	0.159	−0.385	−0.153	0.513 *	0.340 *	0.124	0.603 **	0.303	0.184

Pearson's coefficients are shown. § only female participants were included in correlation analysis. * indicates $p < 0.05$; ** indicates $p < 0.01$.

Finally, to confirm the relationship between ProT-α and insulin resistance, linear regression analysis was conducted, adjusting for age and gender as the potential confounding factors and using fasting insulin levels as the dependent variable (Table 4). Increments in circulating ProT-α were again found to relate, possibly with causality, with increments in fasting insulin levels (standardized β = 0.443, $p = 0.002$). Hyperinsulinism is a known compensatory mechanism for insulin resistance during the progression from obesity to the early onset of type 2 diabetes mellitus. Additionally, it is a major factor contributing to the increased risk of cancer and cardiovascular complications in obese individuals [28]. By understanding the pro-inflammatory biomolecules that contribute to the initial disruption of insulin signaling, we may be able to diagnose and manage insulin resistance in obese individuals more effectively.

Table 4. Linear regression analysis elucidating the positive association between ProT-α and fasting insulin levels.

	Standardized β	t Value	*p* Value
ProT-α	0.407	3.650	**<0.001**
ProT-α *	0.443	3.263	**0.002**

* Age and gender were added as covariates in the adjusted regression model. Bold values denote statistical significance at $p < 0.05$.

4. Discussion

The discovery of molecules that could represent useful clinical tools and/or contribute to our knowledge of the pathogenetic mechanisms of diseases is an important issue in biomedical research [29,30]. Since obesity predisposes individuals to metabolic and cardiovascular risks without showing prodromal symptoms, investigations on potentially relevant biomarkers predicting the its evolution into obesity-associated comorbidities could eventually represent a big challenge in terms to improve surveillance and clinical care. In this context, using proteomic strategies, our group previously identified ProT-α to number among the upregulated proteins in a surrogate in vitro model of obesity [12]. In this observational study, we tested serum ProT-α protein levels in obese vs. lean patients and demonstrated significantly higher circulating levels of ProT-α in the obese individuals.

It has been demonstrated that ProT-α is a pleiotropic protein, with a role in the modulation of immune responses, proliferation/apoptosis, oxidative stress, neuroprotection, and

cancer [16,31–33]. The molecular mechanisms linking ProT-α to these processes are known to be mediated by a variety of protein–protein interactions [31,33–35].

Transgenic mice overexpressing ProT-α have been reported to develop pulmonary emphysema [36] and polycystic kidney disease [37], while the administration of either ProT-α or a mimetic hexapeptide has been shown to protect against brain damage in a mouse model of cerebral ischemia [38]. In addition, of relevance to this work, a study evidenced that transgenic mice overexpressing ProT-α become insulin-resistant and that the inhibition of ProT α using a lentiviral vector in mice fed a high fat-diet can prevent this condition [39]. In the same report, higher levels of serum ProT-α compared to control subjects were found in patients with type 2 diabetes mellitus, even after adjustment for BMI [39]. However, even if we found a correlation between fasting insulin levels and ProT-α, thus confirming the association between ProT-α and insulin resistance, unlike the cited study, our major focus was on obesity, as we enrolled obese individuals who had not yet developed glucose homeostasis issues. Furthermore, our data indicate that the circulating levels of ProT-α may be affected by gender, which could help explain why the clinical manifestations of obesity differ between men and women [40]. This result parallels what is seen in chronic cardiovascular inflammatory lesions unrelated to insulin resistance (i.e., rheumatic heart valve disease), where ProT-α plays a role in orchestrating autoimmune responses associated with estrogen receptor alpha activity [41].

TLR-4 is an important mediator of both insulin resistance and inflammation [42]. Previous studies have proven its crucial molecular role with regard to adipose cells/tissue in vitro in cultured 3T3-L1 adipocytes and in vivo in TLR-4 knock-out animal models [42,43]. Although several mechanisms may contribute to these strictly associated conditions, a major consideration is that ProT-α, a known ligand of TLR-4 [18], may induce inflammation and insulin resistance through TLR-4 and a downstream NF-kB-dependent pathway. In addition, while adipose tissue hypoxia precociously induces both NF-kB and ProT-α expression [44], ProT-α has been shown to functionally cooperate with this factor and to enhance its pro-inflammatory role by promoting NF-kB translocation to the nucleus [39]. Triggering NF-kB activation has important implications for both metabolic disorders and cancer [28]. High abundance of circulating ProT-α may potentially contribute to the development of cancer among individuals with insulin resistance, although more research is needed to fully understand the causal mechanisms linking insulin resistance and cancer, both of which obese patients are particularly predisposed to develop [28].

Another crucial way to contribute to inflammation is that ProT-α (or its immunoactive carboxyl-terminal peptide) modulates the immune response via the maturation of monocytes into monocyte-derived dendritic cells, with the production of cytokines, whose favorable milieu may ultimately promote lymphocyte activation [17,32]. In this regard, since the adipose tissue of the obese compared to the adipose tissue of the lean is more infiltrated by macrophages (with the prevalent M1 phenotype), dendritic cells, and Th1 and Th17 lymphocytes [45], it is plausible that ProT-α, which is secreted by hypoxic adipocytes [12], may also influence adipose tissue inflammation by contributing to the abundance and phenotypic and functional properties of resident immune cells. In this sense, the positive correlation between ProT-α, TNF-α, and IL-8 presented in our study suggests the potential for the polarization of lymphocytes toward a pro-inflammatory Th1 response [46] and the infiltration of M1 phenotype macrophages [47].

Interestingly, when we determined some of the relevant biochemical markers of obesity-related inflammation among obese and lean groups, with the exception of IL-6, we did not find significant differences in circulating cytokines and growth factors but only an increasing trend in obese individuals with regard to VEGF, whose expression in the adipose tissue is controlled by hypoxia via HIF-1 activation [9,10]. This finding can be explained, at least in part, by the limited sample size of the study cohorts, but it is predominantly explained by the relatively young age of both the cases and controls and the strict selection criteria, whose aim was to exclude overt inflammatory conditions and obesity-related cardiometabolic comorbidities. In fact, while we previously reported that

obese, hypertensive, and dyslipidemic patients who undergo bariatric surgery have higher levels of circulating inflammatory cytokines and growth factors (which negatively affect insulin signaling and glucose metabolism) than lean controls [10], these results were not confirmed in other studies of our group addressing obesity, in which the limited number of cases, and their mild clinical manifestations of cardiometabolic impairment, may have affected significance [48,49]. These data, however, further emphasize our findings on ProT-α, which can be seen as a potentially more sensitive marker of obesity-related inflammation and insulin resistance compared to more conventional parameters. In this regard, it is worth noting that even in the absence of systemic autoimmune and rheumatic disorders, obesity may skew the traditional indicators of inflammation, such as ESR, and this phenomenon is more pronounced in women [50]. However, factors other than pro-inflammatory cytokines, such as paraproteins and erythrocyte morphology, may contribute to the increase in ESR and adiposity may not be the primary cause per se [51].

The major limitations of this work include the relatively small sample size of our cohorts and the study's cross-sectional design. Moreover, the inability to detect certain cytokines in the bloodstream of these asymptomatic individuals, even with the utilization of a highly sensitive chemiluminescent method, might have inflated the statistical power of the correlation analysis. However, this represents a pilot study, and as such, it is designed to obtain proof-of-concept that may lead to further research. In contrast, a strength of the study is the fact that—to the best of our knowledge—it represents the first report concerning the measurement of ProT-α in obese individuals. The fact that this parameter increases in obese individuals, whereas many relevant parameters—including metabolic indices—do not, encourages further studies aiming to validate this molecule as a novel biomarker that could be potentially useful for following up and properly treating obese patients.

5. Conclusions

Our data indicate that ProT-α is a novel, sensitive biomarker of inflammation and insulin resistance associated with obesity. Further studies are necessary to validate these findings in a larger population and in appropriate clinical settings to better evaluate their potential clinical use.

Author Contributions: Conceptualization, D.P.F. and A.B.; investigation, M.G., V.T., Y.M., F.S.B., A.S., F.D., L.S. and O.T.; data curation, M.G., M.M. and E.C.; writing—original draft preparation, M.G. and M.M.; writing—review and editing, D.P.F. and A.B.; project administration, D.P.F. and A.B. All authors have read and agreed to the published version of the manuscript.

Funding: We thank the Department of Experimental and Clinical Medicine of the University of Catanzaro for providing the Prothymosin-alpha ELISA kits. The funder played no role in the research.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Regione Calabria, Sezione Area Centro, Catanzaro, Italy (protocol code n. 116, 14 May 2015).

Informed Consent Statement: Written informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data supporting the reported results are available from the corresponding author upon reasonable request.

Acknowledgments: Abstract of this study has been accepted for presentation at the 25th IFCC-EFLM European Congress of Clinical Chemistry and Laboratory Medicine (EuroMedLab) 2023 (Rome, 21–25 May 2023).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Blüher, M. Obesity: Global epidemiology and pathogenesis. *Nat. Rev. Endocrinol.* **2019**, *15*, 288–298. [CrossRef]
2. Malnick, S.D.H.; Knobler, H. The medical complications of obesity. *Qjm Int. J. Med.* **2006**, *99*, 565–579. [CrossRef] [PubMed]
3. Kershaw, E.E.; Flier, J.K. Adipose tissue as an endocrine organ. *J. Clin. Endocrinol. Metab.* **2004**, *89*, 2548–2556. [CrossRef]

4. Ouchi, N.; Parker, J.L.; Lugus, J.J.; Walsh, K. Adipokines in inflammation and metabolic disease. *Nat. Rev. Immunol.* **2011**, *11*, 86–97. [CrossRef] [PubMed]
5. Kwon, H.; Pessin, J.E. Adipokines mediate inflammation and insulin resistance. *Front. Endocrinol.* **2013**, *4*, 11. [CrossRef] [PubMed]
6. Henning, R.J. Obesity and obesity-induced inflammatory disease contribute to atherosclerosis: A review of the pathophysiology and treatment of obesity. *Am. J. Cardiovasc. Dis.* **2021**, *11*, 504–529.
7. Trayhurn, P. Hypoxia and adipose tissue function and dysfunction in obesity. *Physiol. Rev.* **2013**, *93*, 1–21. [CrossRef]
8. Foti, D.P.; Brunetti, A. Editorial: "Linking hypoxia to obesity". *Front. Endocrinol.* **2017**, *8*, 34. [CrossRef]
9. Messineo, S.; Laria, A.E.; Arcidiacono, B.; Chiefari, E.; Luque Huertas, R.M.; Foti, D.P.; Brunetti, A. Cooperation between HMGA1 And HIF-1 contributes to hypoxia-induced VEGF and visfatin gene espression in 3T3-L1 adipocytes. *Front. Endocrinol.* **2016**, *7*, 73. [CrossRef]
10. Arcidiacono, B.; Chiefari, E.; Foryst-Ludwig, A.; Currò, G.; Navarra, G.; Brunetti, F.S.; Mirabelli, M.; Corigliano, D.M.; Kintscher, U.; Britti, D.; et al. Obesity-related hypoxia via miR-128 decreases insulin-receptor expression in human and mouse adipose tissue promoting systemic insulin resistance. *EBioMedicine* **2020**, *59*, 102912. [CrossRef]
11. Eltzschig, H.K.; Carmeliet, P. Hypoxia and inflammation. *N. Engl. J. Med.* **2011**, *364*, 656–665. [CrossRef]
12. Laria, A.E.; Messineo, S.; Arcidiacono, B.; Varano, M.; Chiefari, E.; Semple, R.K.; Rocha, N.; Russo, D.; Cuda, G.; Gaspari, M.; et al. Secretome analysis of hypoxia-induced 3T3-L1 adipcytes uncovers proteins potentially involved in obesity. *Proteomics* **2018**, *18*, e1700260. [CrossRef] [PubMed]
13. Segade, F.; Gómez-Márquez, J. Prothymosin alpha. *Int. J. Biochem. Cell. Biol.* **1999**, *31*, 1243–1248. [CrossRef] [PubMed]
14. Eschenfeldt, W.H.; Berger, S.L. The human prothymosin α gene is polymorphic and induced upon growth stimulation: Evidence using a cloned cDNA. *Proc. Natl. Acad. Sci. USA* **1986**, *83*, 9403–9407. [CrossRef] [PubMed]
15. Freire, M.; Barbeito, P.; Sarandeses, C.S.; Díaz-Jullien, C.; Muras, J.; Covelo, G.; Moreira, D.; Freire-Cobo, C. Prothymosin α, a protein implicated in the proliferation and survival of lymphocytes. *J. Immunol. Sci.* **2018**, *2*, 19–25. [CrossRef]
16. Karapetian, R.N.; Evstafieva, A.G.; Abaeva, I.S.; Chichkova, N.V.; Filonov, G.S.; Rubtsov, Y.P.; Suchacheva, E.A.; Melnikov, S.V.; Schneider, U.; Wanker, E.E.; et al. Nuclear oncoprotein prothymosin alpha is a partner of Keap1: Implications for expression of oxidative stress-protecting genes. *Mol. Cell. Biol.* **2005**, *25*, 1089–1099. [CrossRef]
17. Samara, P.; Karachaliou, C.E.; Ioannou, K.; Papaioannou, N.E.; Voutsas, I.F.; Zikos, C.; Pirmettis, I.; Papadopoulos, M.; Kalbacher, H.; Livaniou, E.; et al. Prothymosin alpha: An alarmin and more. *Curr. Med. Chem.* **2017**, *24*, 1747–1760. [CrossRef]
18. Mosoian, A.; Teixeira, A.; Burns, C.S.; Klotman, M.E. Prothymosin-α inhibitsHIV-1 via Toll-like receptor 4-mediated type I interferon induction. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 10178–10183. [CrossRef]
19. American Diabetes Association Professional Practice Committee. 2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2022. *Diabetes Care* **2022**, *45* (Suppl. S1), S17–S38. [CrossRef]
20. Mirabelli, M.; Chiefari, E.; Puccio, L.; Foti, D.P.; Brunetti, A. Potential Benefits and Harms of Novel Antidiabetic Drugs During COVID-19 Crisis. *Int. J. Environ. Res. Public Health* **2020**, *17*, 3664. [CrossRef]
21. Murgia, F.; Giagnoni, F.; Lorefice, L.; Caria, P.; Dettori, T.; D'Alterio, M.N.; Angioni, S.; Hendren, A.J.; Caboni, P.; Pibiri, M.; et al. Sex Hormones as Key Modulators of the Immune Response in Multiple Sclerosis: A Review. *Biomedicines* **2022**, *10*, 3107. [CrossRef]
22. Passos, L.S.A.; Jha, P.K.; Becker-Greene, D.; Blaser, M.C.; Romero, D.; Lupieri, A.; Sukhova, G.K.; Libby, P.; Singh, S.A.; Dutra, W.O.; et al. Prothymosin Alpha: A Novel Contributor to Estradiol Receptor Alpha-Mediated CD8+ T-Cell Pathogenic Responses and Recognition of Type 1 Collagen in Rheumatic Heart Valve Disease. *Circulation* **2022**, *145*, 531–548. [CrossRef] [PubMed]
23. Caroleo, M.; Carbone, E.A.; Greco, M.; Corigliano, D.M.; Arcidiacono, B.; Fazia, G.; Rania, M.; Aloi, M.; Gallelli, L.; Segura-Garcia, C.; et al. Brain-behavior-immune interaction: Serum cytokines and growth factors in patients with eating disorders at extremes of the body mass index (BMI) spectrum. *Nutrients* **2019**, *11*, 1995. [CrossRef]
24. Ter Horst, K.W.; Gilijamse, P.W.; Koopman, K.E.; de Weijer, B.A.; Brands, M.; Kootte, R.S.; Romijn, J.A.; Ackermans, M.T.; Nieuwdorp, M.; Soeters, M.R.; et al. Insulin resistance in obesity can be reliably identified from fasting plasma insulin. *Int. J. Obes.* **2015**, *39*, 1703–1709. [CrossRef] [PubMed]
25. Karne, R.J.; Chen, H.; Quon, M.J. Diagnosing insulin resistance by simple quantitative methods in subjects with normal glucose metabolism. *Diabetes Care* **2004**, *27*, 1247–1249. [CrossRef] [PubMed]
26. Garcia, M.; Mulvagh, S.L.; Merz, C.N.; Buring, J.E.; Manson, J.E. Cardiovascular Disease in Women: Clinical Perspectives. *Circ. Res.* **2016**, *118*, 1273–1293. [CrossRef] [PubMed]
27. Gillis, E.E.; Sullivan, J.C. Sex Differences in Hypertension: Recent Advances. *Hypertension* **2016**, *68*, 1322–1327. [CrossRef]
28. Chiefari, E.; Mirabelli, M.; La Vignera, S.; Tanyolaç, S.; Foti, D.P.; Aversa, A.; Brunetti, A. Insulin Resistance and Cancer: In Search for a Causal Link. *Int. J. Mol. Sci.* **2021**, *22*, 11137. [CrossRef]
29. Califf, R.M. Biomarker definitions and their applications. *Exp. Biol. Med.* **2018**, *243*, 213–221. [CrossRef]
30. Giltnane, J.M.; Rimm, D.L. Technology Insight: Identification of biomarkers with tissue microarray technology. *Nat. Clin. Pract. Oncol.* **2004**, *1*, 104–111. [CrossRef]
31. Ueda, H.; Matsunaga, H.; Halder, S.K. Prothymosin alpha plays multifunctional cell robustness roles in genomic, epigenetic, and nongenomic mechanisms. *Ann. N. Y. Acad. Sci.* **2012**, *1269*, 34–43. [CrossRef] [PubMed]

32. Skopeliti, M.; Iconomidou, V.A.; Derhovanessian, E.; Pawelec, G.; Voelter, W.; Kalbacher, H.; Hamodrakas, S.J.; Tsitsilonis, O.E. Prothymosin alpha immunoactive carboxyl-terminal peptide TKKQKTDEDD stimulates lymphocyte reactions, induces dendritic cell maturation and adopts a beta-sheet conformation in a sequence-specific manner. *Mol. Immunol.* **2009**, *46*, 784–792. [CrossRef] [PubMed]
33. Ueda, H. Prothymosin α plays a role as a brain guardian through Ecto-F_1 ATPase-$P2Y_{12}$ complex and TLR4/MD2. *Cells* **2023**, *12*, 496. [CrossRef]
34. Ha, S.Y.; Song, D.H.; Hwang, S.H.; Cho, S.Y.; Park, C.K. Expression of prothymosin alpha predicts early recurrence and poor prognosis of hepatocellular carcinoma. *Hepatobiliary Pancreat. Dis. Int.* **2015**, *14*, 171–177. [CrossRef] [PubMed]
35. Karetsou, Z.; Kretsovali, A.; Murphy, C.; Tsolas, O.; Papamarcaki, T. Prothymosin α interacts with the CREB-binding protein and potentiates transcription. *EMBO Rep.* **2002**, *3*, 361–366. [CrossRef]
36. Su, B.H.; Tseng, Y.L.; Shieh, G.S.; Chen, Y.C.; Shiang, Y.C.; Wu, P.; Li, K.J.; Yen, T.H.; Shiau, A.L.; Wu, C.L. Prothymosin alpha overexpression contributes to the development of pulmonary emphysema. *Nat. Commun.* **2013**, *4*, 1906. [CrossRef]
37. Li, K.J.; Shiau, A.L.; Chiou, Y.Y.; Yo, Y.T.; Wu, C.L. Transgenic overexpression of prothymosin α induces development of polycystic kidney disease. *Kidney Int.* **2005**, *67*, 1710–1722. [CrossRef]
38. Halder, S.K.; Matsunaga, H.; Ueda, H. Prothymosin alpha and its mimetic hexapeptide improve delayed tissue plasminogen activator-induced brain damage follwing cerebral ischemia. *J. Neurochem.* **2020**, *153*, 772–789. [CrossRef]
39. Su, Y.C.; Ou, H.Y.; Wu, H.T.; Wu, P.; Chen, Y.C.; Su, B.H.; Shiau, A.L.; Chang, C.J.; Wu, C.L. Prothymosin-α overexpression contributes to the development of insulin resistance. *J. Clin. Endocrinol. Metab.* **2015**, *100*, 4114–4123. [CrossRef]
40. Cooper, A.J.; Gupta, S.R.; Moustafa, A.F.; Chao, A.M. Sex/Gender Differences in Obesity Prevalence, Comorbidities, and Treatment. *Curr. Obes. Rep.* **2021**, *10*, 458–466. [CrossRef]
41. Shi, H.; Kokoeva, M.V.; Inouye, K.; Tzameli, I.; Yin, H.; Flier, J.S. TLR4 links innate immunity and fatty acid-induced insulin resistance. *J. Clin. Investig.* **2006**, *116*, 3015–3025. [CrossRef] [PubMed]
42. Song, M.J.; Kim, K.H.; Yoon, J.M.; Kim, J.B. Activation of Toll-like receptor 4 is associated with insulin resistance in adipocytes. *Biochem. Biophys. Res. Commun.* **2006**, *346*, 739–745. [CrossRef] [PubMed]
43. Ghosh, A.K.; O'Brien, M.; Mau, T.; Yung, R. Toll-like receptor 4 (TLR4) deficient mice are protected from adipose tissue inflammation in aging. *Aging* **2017**, *9*, 1971–1982. [CrossRef] [PubMed]
44. Roland, I.; Minet, E.; Ernest, I.; Pascal, P.; Michel, G.; Remacle, R.; Michiels, C. Identification of hypoxia-responsive messengers expressed in human microvascular endothelial cells using differential display RT-PCR. *Eur. J. Biochem.* **2000**, *267*, 356–3574. [CrossRef]
45. Lu, J.; Zhao, J.; Meng, H.; Zhang, X. Adipose tissue-resident immune cells in obesity and type 2 diabetes. *Front. Immunol.* **2019**, *10*, 1173. [CrossRef]
46. Ioannou, K.; Derhovanessian, E.; Tsakiri, E.; Samara, P.; Kalbacher, H.; Voelter, W.; Trougakos, I.P.; Pawelec, G.; Tsitsilonis, O.E. Prothymosin α and a prothymosin α-derived peptide enhance T(H)1-type immune responses against defined HER-2/neu epitopes. *BMC Immunol.* **2013**, *14*, 43. [CrossRef]
47. Murray, P.J. Macrophage Polarization. *Annu. Rev. Physiol.* **2017**, *79*, 541–566. [CrossRef]
48. Greco, M.; Chiefari, E.; Montalcini, T.; Accattato, F.; Costanzo, F.S.; Pujia, A.; Foti, D.; Brunetti, A.; Gulletta, E. Early effects of a hypocaloric, mediterranean diet on laboratory parameters in obese individuals. *Mediat. Inflamm.* **2014**, *2014*, 750660. [CrossRef]
49. Caroleo, M.; Carbone, E.A.; Arcidiacono, B.; Greco, M.; Primerano, A.; Mirabelli, M.; Fazia, G.; Rania, M.; Hribal, M.L.; Gallelli, L.; et al. Does NUCB/Nesfatin-1 influence eating behavior in obese patients with binge eating disorders? Toward a neurobiological pathway. *Nutrients* **2023**, *15*, 348. [CrossRef]
50. Cohen, E.; Margalit, I.; Shochat, T.; Goldberg, E.; Krause, I. Markers of Chronic Inflammation in Overweight and Obese Individuals and the Role of Gender: A Cross-Sectional Study of a Large Cohort. *J. Inflamm. Res.* **2021**, *14*, 567–573. [CrossRef]
51. George, M.D.; Giles, J.T.; Katz, P.P.; England, B.R.; Mikuls, T.R.; Michaud, K.; Ogdie-Beatty, A.R.; Ibrahim, S.; Cannon, G.W.; Caplan, L.; et al. Impact of Obesity and Adiposity on Inflammatory Markers in Patients With Rheumatoid Arthritis. *Arthritis Care Res.* **2017**, *69*, 1789–1798. [CrossRef] [PubMed]