High-mobility group protein B1: a new biomarker of metabolic syndrome in obese children

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Abstract

Introduction: Obesity is associated with a chronic low-grade inflammation. High-mobility group box 1 protein (HMGB1) plays a key role in inflammation and immunostimulatory and chemotactic processes. The aim of the study was to assess the role of HMGB1 in obese children and to evaluate its diagnostic profile in identifying childhood obesity-related complications, such as the metabolic syndrome (MS).

Patients and methods: Sixty obese children were enrolled and compared with 40 healthy children (control). Homeostasis model assessment of insulin resistance (HOMA-IR), lipid profile, thyroid hormones, and pro- and anti-inflammatory peptides such as C-reactive protein (CRP), adiponectin, interleukin 6 (IL6), IL18, IL23, TNFα, resistin, and HMGB1 were evaluated. Receiver operating characteristics (ROC) analysis was employed to calculate the area under the curve (AUC) for HMGB1, IL6, and adiponectin to find the best cutoff values capable of identifying MS in obese children.

Results: HMGB1 levels were statistically higher in obese patients than in the control group (19.4 ± 6.8 vs. 3.7 ± 1.2 ng/ml; P < 0.0001). In obese patients, IL18, IL6, and resistin levels were significantly high, while adiponectin levels were low. At multivariate analysis, HMGB1 was found to be independently correlated with BMI, IL23, IL6, free triiodothyronine, HDL, and HOMA-IR. At ROC analysis, HMGB1 showed higher sensitivity and specificity (AUC, 0.992; sensitivity, 94.7%; specificity, 97.5%) than IL6 and adiponectin in identifying MS in obese children.

Conclusion: HMGB1 plays an important role in the inflammatory process associated with childhood obesity. This peptide may be an important diagnostic marker for obesity-related complications, such as MS.

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Introduction

Childhood obesity is an important risk factor for atherosclerosis and metabolic syndrome (MS), whose features include insulin resistance, dyslipidemia, and hypertension (1). Furthermore, the MS prevalence increases with the severity of obesity and reaches 50% in severely obese children (2). Increasing evidence suggests that numerous endocrine peptides, interleukins (IL), and cytokines, which are secreted mostly from adipose tissue, play a role in both short- and long-term energy balance, metabolism, and inflammatory response (3). A recent study indicates that inflammatory mechanisms linking obesity to its metabolic and cardiovascular complications are already activated in childhood obesity (4). Adiponectin, a 30 kDa protein highly expressed in adipose tissue, is decreased in obese patients, and it is characterized by insulin-sensitizing, anti-inflammatory, and anti-atherogenic properties (5). TNFα, a proinflammatory cytokine mainly produced by macrophages and lymphocytes, is involved in insulin resistance and endothelial inflammatory processes (6).

IL6, significantly enhanced by adipose tissue in obesity, is a circulating multifunctional cytokine with various functions such as inflammation, host defense, and tissue injury. The two major adverse effects of increased IL6 in obesity are insulin resistance and increased risk for cardiovascular complications (7). Resistin is another adipokine that may play a role in the development of obesity-related insulin resistance (8). In adults, it is known that obesity is associated with a chronic low-grade inflammation and inflammatory pathways could be critical in the mechanisms underlying obesity-related metabolic diseases (9).

Chronic low-grade systemic inflammation can be evaluated by circulating C-reactive protein (CRP), a nonspecific acute-phase reactant synthesized in liver, which may also have direct pro-inflammatory actions (10). Apart from adipocytes, other cells located in adipose tissue such as endothelial cells, fibroblasts, and...
immune cells may secrete inflammatory peptides and chemokines. In particular, high-mobility group box 1 protein (HMGB1), a 30 kDa nuclear and cytosolic ubiquitous protein, is actively secreted by innate immune cells and it has been shown to stimulate necrosis-induced inflammation (11). Moreover, HMGB1 induces other cytokines such as TNFα, IL1, IL6, and IL8 (12). HMGB1 has been shown to interact with toll-like receptor 2 (TLR2), TLR4, and the receptor for advanced glycation end products, inducing immunostimulatory and chemotactic responses (13). Elevated HMGB1 levels in serum have been found in acute inflammatory conditions, but there is no evidence about its role in childhood obesity (14).

The aim of this study was to identify the chronic low-grade inflammatory process, characterizing obese children, using a panel of biomarkers. Moreover, we evaluated the role of HMGB1 in obesity and its relationship with inflammatory cytokines such as IL6, IL18, IL23, TNFα, adiponectin, and resistin. Furthermore, the diagnostic role of HMGB1 for MS was also assessed.

Materials and methods

Patient and control groups

A total of 100 children were enrolled in the study. In particular, 60 children or adolescents (mean age 11.1 ± 2.9 years; 33 females) who had been referred to the Section of Nutrition and Dietetics, Genetics and Pediatric Immunology Unit, Department of Pediatrics, University of Messina, between November 2011 and April 2012 were enrolled in the obese group. Subjects were eligible if they were healthy, were between 8 and 18 years of age, and had a BMI that exceeded the age- and gender-specific 90th percentile as described by McCarthy et al. (15).

Exclusion criteria included: hepatic, infectious, or endocrine diseases (other than diabetes or impaired glucose tolerance (IGT)); syndromic obesity: craniofaryngioma: and the use of medication that alters blood pressure or glucose or lipid metabolism. All subjects were Caucasian.

Forty healthy children (mean age 10.3 ± 1.6 years; 20 females) were enrolled as controls (control group). Subjects had to be normal weight. Apart from obesity, inclusion/exclusion criteria were identical to those used for the obese group.

The study protocol was approved by the Hospital’s Ethics Committee. Written informed consent was obtained from the parents and informed assent from the children and adolescents.

Procedures

Subjects were evaluated at 0800 h, after an overnight fast. A detailed medical and familial history was obtained for all subjects, and a physical examination was performed, including staging of puberty according to the criteria of Tanner & Whitehouse (16).

Waist circumference (WC) was measured midway between the lowest rib and the iliac crest at the end of gentle expiration. It was calculated according to the standards assessed by McCarthy et al. (17).

Blood pressure was measured three times with an appropriate size cuff while the subjects were seated and the lowest measurement was used for analysis (18). All measurements were made by the same pediatrician.

Definitions

The relative BMI of each patient was calculated as the BMI divided by the BMI at 50th percentile for age and gender × 100 (19). Subjects were classified as hypertensive if their systolic and/or diastolic blood pressure exceeded the 90th percentile for age and sex after adjustment for height (18). The degree of hypertension was evaluated by the difference between the systolic or diastolic blood pressure measured in one subject and the 90th percentile for age and sex after adjustment for height.

Abdominal obesity was defined using the sex- and age-specific 90th WC percentile as described by McCarthy et al. (17).

Biochemical hyperinsulinism was defined as fasting levels of insulin > 15 mU/ml, according to Ten & Maclaren (20).

Insulin resistance was determined by the homeostatic model assessment (HOMA) and calculated according to the following formula: (insulin (μU/ml) × serum glucose (mmol/l))/22.5 (21). The HOMA index was validated as a reliable measure of insulin sensitivity in obese and nonobese children and adolescents (22, 23). Impaired insulin sensitivity was defined as a HOMA of insulin resistance (HOMA-IR) of 2.5 or higher in prepubescent patients (24).

Obese children and adolescents were classified as having the MS if they met three or more of the following criteria for age and sex: a BMI above the 97th percentile; systolic or diastolic blood pressure above the 95th percentile; a triglyceride (TG) level above the 95th percentile; a HDL-cholesterol level below the 5th percentile; and IGT (25).

Collection of blood

Baseline blood samples were obtained by venipuncture (cubital vein approach with butterfly) for measurements of levels of glucose, lipid profile, insulin, thyroid hormones, and pro- and anti-inflammatory peptides (CRP, adiponectin, IL6, IL18, IL23, TNFα, resistin, and HMGB1). Serum (after allowing blood to clot at room temperature) or plasma (potassium EDTA) was rapidly obtained by refrigerated (4 °C) centrifugation at 2000 g for 15 min. Glucose, insulin, and lipids were
immediately measured; aliquots for other assays were stored at −20 °C until analysis.

**Biochemical analysis**

Adiponectin (R&D Systems Europe, Wiesbaden, Germany), resistin (Linco Research, Inc., Minneapolis MN, USA), HMGB1 (IBL Shino Test Corporation, Hamburg, Germany), IL18 (Bender MedSystems GmbH, Vienna, Austria), IL23 (Biovendor, Space import-export SRL, Milan, Italy), IL6, and TNFα (DRG International, Inc., Mountainside, NJ, USA) levels were determined by ELISA, according to the manufacturer’s instructions. The hs-CRP was measured by immunonephelometry (Dade-Behring, Marburg, Germany). Fasting plasma glucose was measured using a glucose oxidase method. Plasma insulin was measured by RIA (Diagnostic Product Corporation, Los Angeles, CA, USA).

Plasma total cholesterol, HDL-cholesterol, and TGs were determined by automated enzymatic methods (Boehringer Mannheim-Diagnostica). LDL-cholesterol concentrations were calculated using Friedewald’s equation, considering that serum TG values remained below 4 mmol/l in every child. Thyroid function was determined by chemiluminescence (Architect system insulin/free thyroxine (FT4)/free triiodothyronine (FT3)/TSH; Abbott Diagnostics Division).

**Statistical analyses**

Statistical analyses were performed using Medcalc 8.0 for Windows package and GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). Data were presented as mean ± s.d. for normally distributed values (at Kolmogorov–Smirnov test) and median (IQR range) for non-normally distributed values. Differences between groups were established by ANOVA followed by Bonferroni’s test for normally distributed values and by Kruskal–Wallis analysis followed by Dunn’s test for nonparametric values. Dichotomized values were compared using the χ² test. The coefficient of Pearson’s correlation was used to assess the correlations between variables. Multiple regression analysis with stepwise method was performed. Receiver operating characteristics (ROC) analysis was employed to calculate the area under the curve (AUC) for HMGB1, IL6, and adiponectin to find the best cutoff values capable of identifying MS in obese children. *P* < 0.05 was considered to be statistically significant.

**Results**

**Characteristics of the population**

The main auxological, metabolic, and biochemical data of the patients are summarized in Table 1. The study group included 33 females and 27 males with a mean chronological age (CA) of 11.1 ± 2.9 years (range 7.5–14.5 years); the control group (20 females and 20 males) was well matched for CA (10.3 ± 1.6 years; range 7.8–13.5 years). The mean BMI of obese subjects was 28.7 ± 4.3 kg/m² (range 24.3–32.5 kg/m²) and BMI SDS 2.7 ± 0.3 (range 2.4–3.7). On the basis of the threshold BMI z-score, 23 (38%) of the 60 obese patients were moderately obese and 11 (18%) severely obese.

All obese patients presented abdominal obesity. WC was 76.8 ± 10 cm (range 58.0–93 cm), above 90th percentile, and was significantly (*P* < 0.05) different compared with the control values (55.6 ± 1.8 cm; range 51.2–58.7 cm). All patients were characterized by normal fasting glucose (FG) levels. In particular, FG range was 3.8–5.7 mmol/l whereas FG levels were 4.73 ± 0.4 mmol/l. Fasting plasma insulin levels were 2.0 (2.0–6.0) μU/ml (range 2.0–21.4 μU/ml) and significantly higher (*P* < 0.001) than controls (3.0 (2.0–4.0) μU/ml; range 1.5–5.8 μU/ml). Nine of 60 patients showed fasting hyperinsulinism (15%) without clinical signs (i.e. acanthosis, skin tags). HOMA-IR was 1.5 ± 1.6 (range 0.3–3.7). Sixteen patients had impaired insulin sensitivity HOMA-IR (26%). An impaired lipid profile was also assessed in obese children, with high levels of total cholesterol, LDL-cholesterol, and TGs, whereas HDL-cholesterol values were lower than the control group (45.4 ± 8.4 vs 50.3 ± 10.7 mg/dl; *P* = 0.005). Nineteen children (31%) met the diagnostic criteria for MS.

**Cytokine levels in obese and control groups**

HMGB1 levels were statistically higher in obese patients than in the control group (19.4 ± 6.8 vs 13.2 ± 2.9 pg/ml; *P* = 0.0001). Other cytokines were also higher in OB patients compared to controls (Table 1).

**Table 1** Baseline demographic, clinical, and laboratory data of the study population.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>OB patients (n=60)</th>
<th>HS (n=40)</th>
<th><em>P</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M/F)</td>
<td>27/33</td>
<td>20/20</td>
<td>0.62</td>
</tr>
<tr>
<td>Age (years)</td>
<td>11.1 ± 2.9</td>
<td>10.3 ± 1.6</td>
<td>0.10</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.7 ± 4.3</td>
<td>18.4 ± 1.5</td>
<td>0.0006</td>
</tr>
<tr>
<td>BMI (SDS)</td>
<td>2.7 ± 0.3</td>
<td>0.2 ± 0.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Systolic pressure (mmHg)</td>
<td>99.6 ± 19.2</td>
<td>98.4 ± 6.3</td>
<td>0.28</td>
</tr>
<tr>
<td>Diastolic pressure (mmHg)</td>
<td>64.8 ± 6.7</td>
<td>61.4 ± 4.3</td>
<td>0.01</td>
</tr>
<tr>
<td>Serum glucose (mmol/l)</td>
<td>4.7 ± 0.4</td>
<td>3.7 ± 0.5</td>
<td>0.22</td>
</tr>
<tr>
<td>Insulin (μU/ml)</td>
<td>2.0 (2.0–6.0)</td>
<td>3.0 (2.0–4.0)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HOMA-IR (%)</td>
<td>1.51 ± 1.6</td>
<td>0.7 ± 0.5</td>
<td>0.003</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>76.8 ± 10</td>
<td>55.6 ± 1.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>0.3 ± 0.27</td>
<td>0.2 ± 0.1</td>
<td>0.10</td>
</tr>
<tr>
<td>TNFα (pg/ml)</td>
<td>41.3 ± 13.2</td>
<td>23.5 ± 4.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL18 (pg/ml)</td>
<td>369.7 ± 142</td>
<td>236.6 ± 66.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL23 (pg/ml)</td>
<td>36.6 ± 11.1</td>
<td>36.9 ± 10.3</td>
<td>0.39</td>
</tr>
<tr>
<td>IL6 (pg/ml)</td>
<td>36.5 ± 6.4</td>
<td>15.7 ± 2.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Adiponectin (ng/ml)</td>
<td>11.5 ± 2.2</td>
<td>17.9 ± 3.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Resistin (ng/ml)</td>
<td>8.3 ± 1.2</td>
<td>2.9 ± 0.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HMGB1 (ng/ml)</td>
<td>19.4 ± 6.8</td>
<td>3.7 ± 1.2</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

OB, obese; HS, healthy subjects; HMGB1, high-mobility group protein B1; HOMA, homeostasis model assessment; IL, interleukin; CRP, C-reactive protein; TNF, tumor necrosis factor; WC, waist circumference.

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Obese patients were characterized by lower levels of adiponectin ($P<0.0001$) when compared with normal-weight children ($11.5 \pm 2.2$ vs $17.9 \pm 3.1$ ng/ml). There were no significant differences ($P=0.39$) in IL23 levels between the two groups ($38.6 \pm 11.1$ vs $36.9 \pm 10.3$ pg/ml) (Fig. 1).

**Univariate correlations and multiple regression of HMGB1**

Correlations between HMGB1 values and clinical parameters were analyzed in all obese patients. HMGB1 was found to be directly correlated with BMI ($r=0.65; P<0.0001$), IL23 ($r=0.30; P=0.02$), IL6 ($r=0.53; P<0.0001$), CRP ($r=0.44; P=0.001$), FT$_3$ ($-0.31; P=0.008$), HDL ($r=0.52; P=0.001$), and HOMA-IR ($r=0.42; P=0.0008$), whereas an inverse correlation was found with diastolic blood pressure (DBP) values ($r=-0.30; P=0.01$). The variables found to be significantly correlated at univariate analysis were introduced in a multivariate model using HMGB1 as a dependent variable. Significance was only maintained for the correlation between HMGB1 and BMI, IL23, IL6, FT$_3$, HDL, and HOMA-IR (Fig. 2). By contrast, the correlations with CRP and diastolic blood pressure values found at univariate analysis were lost. The obtained data are summarized in Table 2.

**Characteristics of HMGB1 as diagnostic marker of MS in obese children**

ROC analyses were performed in order to define the diagnostic profile of HMGB1, IL6, and adiponectin in identifying the MS among obese children. The area under the ROC curve for HMGB1 was 0.992. When the cutoff values of HMGB1 were set at 18.6 ng/ml, sensitivity and specificity of the marker used for the diagnosis were 94.7 and 97.5% respectively.

ROC analysis showed an AUC for IL6 of 0.74 with a best cutoff level of 36.9 ng/ml (sensitivity 73.7% and specificity 78%), while the AUC for adiponectin was 0.72 with a best cutoff level of 10.9 mg/dl (sensitivity 68.4% and specificity 75.6%).

HMGB1 area was statistically different compared with that of IL6 and adiponectin ($P=0.002$ and $P<0.001$ respectively). On the contrary, the difference between the IL6 and adiponectin areas was not significant ($P=0.75$; Fig. 3).

**Discussion**

This study demonstrated that childhood obesity is characterized by a chronic low-grade inflammation process, detected through a panel of inflammatory markers. In particular, we underlined the role of HMGB1, which was extremely high in obese children and strictly connected with other cytokines, such as IL6,
TNFα, IL18, resistin, and adiponectin. The direct correlation observed between HMGB1 and BMI is evidence of the link between obesity and inflammation.

Several studies have highlighted the strong relationship between obesity, BMI, and inflammation (26, 27). Garanty-Bogacka et al. (28) revealed that, after a program of physical activity and low-calorie diet, there was a reduction in the levels of inflammatory cytokines associated with a consequential improvement in obesity markers, such as BMI. Furthermore, we demonstrated a direct correlation between HMGB1, CRP, and IL6.

The liver is known to be a major source of CRP, but its secretion is strongly dependent on IL6 levels (29). This could be a physiopathological explanation for the loss of correlation, after multivariate analysis, between HMGB1 and CRP. The results concerning the independent correlation between IL6 and HMGB1 are a further confirmation that HMGB1 plays a key role in the inflammatory process characterizing the obese child.

In addition, Scaffidi et al. (30) and O’Connor et al. (31) have shown that, during the inflammatory process, HMGB1 is released by monocytes and macrophages actively and passively from necrotic cells, inducing the production of inflammatory cytokines, such as IL6.

We also found, after multivariate analysis, an inverse correlation between HMGB1 and FT3 levels. Elevated TSH concentrations in association with normal or slightly elevated FT4 and/or FT3 levels have been consistently found in obese subjects (32), but the mechanisms underlying these thyroid hormonal changes are still unclear. Whether higher TSH in childhood obesity is adaptive, or an increasing metabolic rate is an attempt to reduce further weight gain, or indicates subclinical hypothyroidism or resistance, remains controversial (33). D’Adamo et al. (34) demonstrated that an increased oxidative stress might represent one of the key regulators of thyroid hormones levels. HMGB1 may be a marker of thyroid dysfunction, probably due to the chronic inflammatory process associated with obesity. While we did not find a statistically significant difference of IL23 levels between obese children and the control group, we have noted, however, a direct and strict correlation between IL23 and HMGB1.

IL23 is a cytokine that regulates the chronic inflammation and plays a key role during bacterial infections, regulating T lymphocyte actions (35). Similarly, HMGB1 has been shown to have a key role

![Figure 2 Univariate correlations of HMGB1. Number of obese patients: 60.](image)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Partial R</th>
<th>β</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>0.62</td>
<td>0.60</td>
<td>0.003</td>
</tr>
<tr>
<td>FT3</td>
<td>−0.31</td>
<td>−0.32</td>
<td>0.0004</td>
</tr>
<tr>
<td>HDL</td>
<td>0.40</td>
<td>0.39</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL23</td>
<td>0.33</td>
<td>0.61</td>
<td>0.02</td>
</tr>
<tr>
<td>IL6</td>
<td>0.30</td>
<td>0.32</td>
<td>0.02</td>
</tr>
<tr>
<td>Diastolic BP</td>
<td>−0.34</td>
<td>−0.26</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>CRP</td>
<td>0.50</td>
<td>0.09</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.48</td>
<td>0.47</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Dependent variable: HMGB1; β: standardized coefficient of correlation. BP, blood pressure; HOMA-IR, homeostasis model assessment of insulin resistance; IL, interleukin; CRP, C-reactive protein; HMGB1, high-mobility group protein B1.
in different infectious diseases. Wang has in fact demonstrated that HMGB1 is a late mediator in animal models of sepsis (36), able to promote the bacterial products identification by immune system cells mediating the beginning of the immune reaction (37). Moreover, anti-HMGB1 antibodies have been demonstrated to confer protection in animal models of sepsis and endotoxemia (38). Recently, Liu et al. (39) demonstrated that IL23 is strongly dependent on HMGB1, which is able to induce IL23 synthesis through the pathway of TLR4. To our knowledge, these are the first data evaluating the roles of HMGB1 in childhood obesity, demonstrating that it may be also an important diagnostic marker for MS.

MS is a cluster of cardiovascular disease risk factors that include glucose intolerance, hypertension, elevated TG, low HDL-cholesterol, and obesity, characterized by low prevalence (40). The analysis of our data revealed a close correlation between HMGB1 and all parameters included in the diagnostic criteria of MS.

As shown by ROC curves, HMGB1 is an excellent diagnostic marker for this condition. In fact, we have demonstrated that HMGB1, when compared with adiponectin and IL6, had the highest sensitivity and specificity to identify MS among obese children.

This study has some limitations that should be mentioned. It was a single-center study, and the cohort of patients was relatively small. A larger study population could further reduce this bias and make the results more reliable. At the same time, the lack of adult confounding factors (smoking, advanced vascular disease, chronic conditions, etc.) in our young participants confers a certain advantage in clarifying the interrelationships found. In fact, HMGB1, as a critical mediator of inflammation, could be altered by acute infectious processes.

Additional research should determine whether inflammation incites a cascade that over many years leads to cardiovascular damage and subsequent cardiovascular events and whether earlier exposure to inflammation causes cumulative damage. Knowledge about early markers, such as HMGB1, may facilitate to refine prevention strategies and to provide a useful tool for the diagnosis and treatment of childhood obesity and its complications, such as MS.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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**Author contribution statement**

Dr T Arrigo carried out the initial analyses, reviewed and revised the manuscript, and approved the final manuscript as submitted. Dr V Chirico conceptualized and designed the study, drafted the initial manuscript, and approved the final manuscript as submitted. Dr C Munafò designed the data collection instruments, coordinated and supervised data collection, critically reviewed the manuscript, and approved the final manuscript as submitted. Dr V Salpietro carried out the initial analyses, reviewed and revised the manuscript, and approved the final manuscript as submitted. Dr C Salpietro designed the data collection instruments, coordinated and supervised data collection, critically reviewed the manuscript, and approved the final manuscript as submitted. Dr E Gitto carried out the initial analyses, reviewed and revised the manuscript, and approved the final manuscript as submitted. Dr A Lacquaniti conceptualized and designed the study, drafted the initial manuscript, and supervised data collection, critically reviewed the manuscript, and approved the final manuscript as submitted. Dr V Chirico conceptualized and designed the study, drafted the initial manuscript, and approved the final manuscript as submitted.

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