Original Paper

High Circulating Alarin Levels Are Associated with Presence of Metabolic Syndrome

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Key Words

Alarin • Metabolic syndrome • Insulin resistance • Cytokine

Abstract

Background/Aims: Alarin has been reported to be related with increased food intake and body weight. The relationship of circulating Alarin with insulin resistance or metabolic syndrome (MetS), however, is unknown. This study aimed to investigate the physiological role of Alarin and its association with MetS in humans. **Methods:** Newly diagnosed MetS patients (n=237) and age-matched healthy subjects (n=192) were recruited for this study. Oral glucose tolerance test, treadmill exercise, lipid infusions and euglycemic-hyperinsulinemic clamp (EHCs) were performed. Circulating Alarin and TNF α levels were measured by ELISA. **Results:** Circulating Alarin levels were significantly higher in MetS patients compared with healthy subjects (0.46 ± 0.22 vs. 0.41 ± 0.14 $\mu\text{g/L}$, $P < 0.01$). In all studied subjects, circulating Alarin levels were positively correlated with WC, blood pressure, FBG, triglyceride, HbA1c, HOMA-IR, AUC_{glucose} and TNF α ($P < 0.05$ or $P < 0.01$). Multivariate logistic regression analyses revealed that circulating Alarin levels were correlated with MetS and insulin resistance. There was no significant change of circulating Alarin levels in the subjects with treadmill exercise for 45 min. In healthy individuals, however, glucose challenge, acute hyperglycemia and lipid infusions

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resulted in increased circulating Alarin levels, while acute hyperinsulinaemia transiently decreased circulating Alarin levels. **Conclusion:** The present study provides the evidence that circulating Alarin levels are associated with MetS and insulin resistance.

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Introduction

Obesity and metabolic syndrome (MetS) are major health and socioeconomic problems that have reached epidemic proportions in the world [1]. Cumulative evidence indicates that obesity and MetS are two important risk factors for metabolic-related diseases, such as type 2 diabetes (T2D), coronary artery disease (CAD) and hypertension [2]. A number of studies have performed to address the connection between MetS and metabolic-related diseases in rodent models and humans. However, the mechanism underlying these links is unclear, partly because of the lack of appropriate animal models and the difficulty related human studies. Recently, a large number of adipokines and cytokines have been reported to be related to insulin resistance and MetS, such as betatrophin, zinc- α 2-glycoprotein (ZAG) and myonectin [3-6]. Therefore, investigation of the relationship between new cytokines and MetS has an important clinical significance.

Alarin is a 25 amino acid cytokine, a member of the galanin peptide family isolated from the gangliocytes of human neuroblastic tumors, and is named after its N-terminal alanine and its C-terminal serine originating as a splice variant of galanin-like peptide (GALP) mRNA [7]. Alarin was initially found in ganglionic cells, and later on it has been found around the blood vessels with vasoactive actions for regulating ocular blood flow [8-10]. Subsequently, Alarin expression was found in the arcuate nucleus (ARC), a central nucleus related energy homeostasis in hypothalamus of rats [11]. Alarin peptide sequence is 92% similarity between mice and rats, and 96% between humans and monkeys [7]. In the animal study, Alarin has been reported to promote the secretion of luteinizing hormone (LH) [11] and has shown antidepressant-like and antimicrobial roles [12, 13]. In addition, peripheral and intraventricular (ICV) treatment of Alarin increased body weight and food intake, and promoted glucose uptake in muscle tissues mediated by insulin in rats [14-16]. Recently, Guo et al. also demonstrated that central treatment of Alarin increased adiponectin release, 2-(N-7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino-2-deoxyglucose uptake, glucose transporter 4 (Glut4) mRNA expression and translocation on cell membrane. During the hyperinsulinemic-euglycemic clamp (EHC), glucose infusion rates (GIR) in ICV Alarin-treated rats were increased, whereas blood glucose level and retinol-binding protein 4 (RBP4) were reduced in diabetic rats [17]. Therefore, Alarin has been considered as a cytokine related insulin resistance and energy homeostasis. However, so far, no study has demonstrated the relationship between circulating Alarin and MetS in humans.

In the current study, we investigated the possible role of circulating Alarin in humans and conducted the cross-sectional and interventional studies to evaluate whether Alarin is correlated with insulin resistance and MetS.

Materials and Methods

Cross-sectional study

This study was performed from Oct. 2015 to Oct. 2017. A total of 429 subjects (including 237 MetS and 192 healthy controls) were consecutively recruited to the study. MetS was diagnosed according to Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) criteria [18]. Patients with newly diagnosed MetS had not been treated with any medicines including lipid-lowering and hypoglycemic agents, as well as diet control or exercise. Excluded criteria included type 1 diabetic (T1D), liver cirrhosis, hepatic and renal failure, congestive heart failure, or other diseases. One hundred and ninety-two age-matched normal individuals without any major diseases were recruited from an unselected population of routine medical check-up, the community or schools as the controls. These

subjects had no family history of T2D and hypertension. Healthy individuals had no taking any medications. A written voluntary consent was obtained from all subjects before their participation. The study complied with the Declaration of Helsinki and was approved by the Human Research Ethics Committee of Chongqing Medical University.

Euglycemic-hyperinsulinemic clamp (EHC)

EHCs were performed in thirty healthy individuals [15 men and 15 women; age 24 ± 4 yr; body mass index (BMI) 22.8 ± 1.4 kg/m²] as previously reported [3]. Briefly, after an overnight fast of 12–14 h, two catheters were inserted into antecubital veins of the right and left arm for blood withdrawal or glucose and insulin infusions, respectively. Insulin (1 mU/kg/min, Humulin Regular; Eli Lilly) was infused for 2 h, and 25% glucose was infused at variable rates that were adjusted to keep blood glucose levels at the baseline. During the EHCs, blood glucose was examined every 10 min to guide the glucose infusion. Glucose disposal rate (GRD) was defined as the glucose infusion rate (GIR) at the stable state of the EHC and was related to body weight (M-value). Blood samples were obtained at indicated times (0, 80, 100, 110 and 120 min) [19].

Lipid infusion study

Twenty-two sex- and age-matched healthy subjects (11 men, 11 women) were assigned to receive a 20 % Intralipid/heparin (0.4 U/kg/min, Pharmacia and Upjohn, Milan, Italy) or a 0.9% saline/heparin infusion (1.5 ml/minute) for 300 minutes. 3-hours after the start of the lipid or saline/heparin infusion, a 2-hour EHC was performed as described above. Blood samples for further analysis were drawn before and at indicated timed intervals during lipid infusion and the EHCs.

Exercise testing procedures

A subgroup of 12 healthy volunteers (6 men and 6 women, aged 20–25; BMI: 21.0 ± 1.2 kg/m²) was assigned to a 45-min bout of treadmill exercise. After an overnight fast for 10–12 h, individuals were asked to arrive at the laboratory between 8:00 and 9:00 on the morning of the treadmill exercise study. All study subjects performed a 45-min bout of treadmill exercise using ParvoMedics Metabolic Measurement System (ParvoMedics, Sandy, Utah, USA) at 60% of VO₂ max. Blood samples were obtained from a forearm intravenous catheter at 4 time points including baseline, 45 min following treadmill exercise, 60 and 120 min at rest after the treadmill exercise.

The daily secretion measurement

Twelve healthy volunteers (6 men and 6 women) aged 20–30 were arranged to the ward at 0740 and were given with a standardized breakfast. During at indicated times (0800, 1000, 1200, 1600, 2000, 2400, and 0400), blood samples were drawn from the forearm vein over a period of 24 h to observe the daily varieties of Alarin concentration. Blood samples were immediately centrifuged, separated and stored at -80°C for measuring Alarin concentration.

Measurements of adipokines

Serum Alarin levels were measured with a commercial ELISA Kit following the manufacturer's protocol (Phoenix Pharmaceuticals, Inc., Belmont, CA, USA). The kit has a sensitivity of 0.08 ng/ml and a linear range of 0.08 - 0.78 ng/ml. Intra-assay and inter-assay variations were <10% and <15%, respectively. The ELISA kit had been validated by the dealer, showing high sensitivity and excellent specificity for detection of human Alarin, but no significant cross-reactivity with other members of the galanin peptide family. Serum tumor necrosis factor- α (TNF α) concentrations were measured by ELISA Kit (4A Biotech Co. Ltd, Beijing, China). The linear range of the ELISA assay was 4.3–1000 ng/L. The intra- and inter-assay coefficients (CV) were both < 10 %.

Anthropometric and biochemical evaluation

Anthropometric and body composition were measured by a trained dietician in all subjects after an overnight. BMI and the waist-to-hip ratio (WHR) were calculated as reported previously [20]. The homeostasis model assessment of insulin resistance (HOMA-IR) and the area under the curve for glucose (AUC_{glucose}) and insulin (AUC_{insulin}) were calculated as reported previously [21]. Blood glucose, HbA1c, Free fatty acids (FFAs), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglyceride (TG) were determined as published previously [20].

Statistical Analysis

All analyses were performed using SPSS version 19.0 (SPSS, Chicago, IL, USA). Normal distribution of the data was tested using Kolmogorov-Smirnov test. The variables of non-normal distribution were skewed and logarithmically transformed to obtain a normal distribution. Continuous variables were presented as

mean and standard deviation (SD) or median and interquartile range (IQR). Comparisons between groups were performed using ANOVA, an unpaired *t*-test or a paired *t*-test. Simple and multiple linear regression analyses were used to assess the association between Alarin and the other biomarkers. The utility of circulating Alarin to predict MetS and insulin resistance was evaluated by receiver operating characteristic (ROC) curve analysis. Sample size was calculated using the following equation: $N = [Z_{\alpha/2} \sigma / \epsilon \mu]^2$ (σ , standard deviation; μ , mean; $Z_{\alpha/2} = 1.96$, $\alpha = 0.05$, $\epsilon = 4\%$). In all statistical tests, *P* values of < 0.05 were considered significant.

Results

Circadian rhythm of circulating Alarin in healthy controls

Twelve normal controls were given a standardized breakfast (0730), and blood samples were collected from 0800 on day 1 to 0400 on day 2 to observe whether serum Alarin levels are regulated by circadian factors. Data showed that serum Alarin decreased to nadir at 12:00 h, and then started to rise at 16:00 h, peaked at 24:00 h. Alarin started to decrease from 4:00 h to 8:00 h (Fig. 1A). Therefore, these data showed that the circadian rhythm of circulating Alarin was smooth and there is no big fluctuation.

Circulating Alarin levels and its association with other parameters

Anthropometric and biochemical parameters of the 429 subjects were shown in Table 1. MetS patients had higher BMI, WHR, blood pressure (BP), TG, FFA, fasting blood glucose (FBG), 2-h post-glucose load blood glucose (2h-BG), HbA1c, fasting insulin (FIns), 2-h insulin after glucose overload (2h-Ins), HOMA-IR, AUC_{glucose} and TNF- α , and lower HDL-C than the

Fig. 1. Concentration of circulating Alarin in study population. A. Circadian of circulating Alarin variations in healthy subjects. B. Circulating Alarin levels according to MetS and controls. C. Circulating concentrations of Alarin elevated with increasing number of components of the MetS. Values were given as means $\pm$ SD, * $P < 0.05$, ** $P < 0.01$ compared with controls or 0 component. D-E. ROC curve analyses were performed for the prediction of circulating Alarin for MetS. D. and insulin resistance E.

controls. Importantly, circulating Alarin concentrations were higher in MetS subjects than in controls ($P < 0.01$, Fig. 1B). The difference remained significant after adjustment for sex and age (Table 1). Next, the association of Alarin with other variables was analyzed by partial correlations. In all study population, circulating Alarin closely correlated with waist circumference (WC) ($P < 0.05$), BP ($P < 0.05$), parameters of blood glucose (FBG and HbA1c, both $P < 0.05$), insulin resistance indices (increased HOMA-IR and AUC_{glucose} , $P < 0.05$ or $P < 0.01$), an adverse lipid profile (increased TG, $P < 0.01$) and inflammatory markers (TNF- α , $P < 0.05$; Table 2). These associations remained statistically significant even after adjusting for age and BMI, except for TNF- α .

In multiple stepwise regression analyses, we found that serum Alarin levels were independently associated with the two important components of MetS, SBP ($\beta = 0.001$, $P < 0.05$) and FBG ($\beta = 0.028$, $P < 0.01$), and AUC_{glucose} ($\beta = 0.011$, $P < 0.001$). Multivariate logistic regression analysis found that serum Alarin concentrations were markedly related MetS and insulin resistance, even after adjustment for anthropometric parameters, age, gender and BP (Table 3).

To further investigate the association between Alarin and the occurrence of MetS, we stratified the concentrations of serum Alarin by the number of MetS components. The results showed that serum Alarin levels were significantly elevated when the number of MetS components was equal to 4 or more (Fig. 1C). Individuals with 4 or more MetS components had Alarin levels (mean $\pm$ SD) of 0.51 ± 0.26 $\mu\text{g/L}$, or higher. The ORs for MetS components associated with circulating Alarin were showed in Table 4. Of the five MetS components, hypertriglyceridemia, hyperglycemia and central obesity seem to have the positive associations with circulating Alarin levels in women, while hyperglycemia and HOMA-IR in men.

Finally, we performed the ROC curve analyses. The results showed that the best cut-off value for Alarin to predict MetS was 0.47 $\mu\text{g/L}$ (sensitivity 65%, specificity 89%, and AUC 0.702; Fig. 1D), and to predict insulin resistance was 0.41 $\mu\text{g/L}$ (sensitivity 65 %, specificity 71 %, and AUC 0.711, Fig. 1E).

Table 1. Main clinical features and circulating Alarin levels in study population MetS, metabolic syndrome; BMI, body mass index; WHR, waist-to-hip ratio; FAT%, visceral fat%; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; FFA, free fatty acid; FBG, fasting blood glucose; 2h-BG, 2 h post-glucose load blood glucose; FIns, fasting insulin; 2h-Ins, 2 h serum insulin after glucose overload; HOMA-IR, HOMA-insulin resistance index; AUC_{insulin} , the area under the curve for insulin; AUC_{glucose} , the area under the curve for glucose; TNF- α , tumor necrosis factor- α . Data are present as mean (standard deviation) or median (interquartile range).¹ Mean and standard error by general linear model with adjustment of age and gender

Group	Controls (N=192)	MetS (N=237)	P value
Age (yr)	54.2 (9.6)	55.4 (9.9)	0.239
BMI (kg/m ²)	22.8 (2.8)	25.1 (3.1)	< 0.001
WHR	0.87 (0.08)	0.92 (0.05)	< 0.001
TNF- α	7.58 (5.40-15.71)	8.64 (5.38-16.95)	< 0.05
SBP (mmHg)	118.0 (15.3)	132.3 (16.7)	< 0.001
DBP (mmHg)	74.8 (10.2)	81.5 (11.0)	< 0.001
TG (mmol/L)	1.23 (0.85-1.40)	1.89 (1.49-2.75)	< 0.001
TC (mmol/L)	5.09 (0.94)	5.08 (1.04)	0.901
HDL-C (mmol/L)	1.42 (0.28)	1.17 (0.31)	< 0.001
LDL-C (mmol/L)	3.02 (0.79)	2.95 (0.90)	0.439
FFA ($\mu\text{mol/L}$)	0.49 (0.39-0.69)	0.59 (0.43-0.89)	< 0.001
FBG (mmol/L)	6.23 (2.76)	7.73 (3.27)	< 0.001
2h-BG (mmol/L)	7.41 (6.04-10.67)	11.86 (8.59-19.10)	< 0.001
FIns (mU/L)	9.06 (5.09)	13.1 (7.50)	< 0.001
2h-Ins (mU/L)	46.82 (30.92-71.98)	69.47 (37.20-117.10)	< 0.001
AUC_{insulin}	100.4 (67.21-146.42)	119.9 (68.07-165.14)	0.229
AUC_{glucose}	17.0 (13.60-22.03)	24.9 (18.92-32.66)	< 0.001
HbA1c (%)	6.19 (1.66)	7.07 (1.97)	< 0.001
HOMA-IR	1.97 (1.33-2.93)	3.62 (2.60-5.70)	< 0.001
Alarin ($\mu\text{g/L}$)	0.41 (0.14)	0.46 (0.22)	< 0.001
Alarin ($\mu\text{g/L}$) ¹	0.42 (0.01)	0.47 (0.01)	< 0.001

The effects of acute exercise and glucose challenge on circulating Alarin levels in healthy subjects

To explore which factors regulate serum Alarin concentrations *in vivo*, we examined the effects of a single exercise and glucose challenge on Alarin in healthy controls. For acute exercise study, a 45-min bout of treadmill exercise was performed in 12 subjects at 60% of VO_2 max, and then rested for 120 min. After a 45-min exercise, there was no obvious change in the circulating levels of Alarin as compared to pre-exercise (0.36 ± 0.09 vs. 0.35 ± 0.10 $\mu\text{g/L}$, Fig. 2A). During the 2-h OGTT, in response to oral glucose challenge-induced hyperglycemia and hyperinsulinemia, Alarin concentrations gradually increased from 0.36 ± 0.10 $\mu\text{g/L}$ at 0 min, and to 0.43 ± 0.09 $\mu\text{g/L}$ at 30 min, and then to 0.42 ± 0.10 $\mu\text{g/L}$ at 60 min, and finally to 0.43 ± 0.11 $\mu\text{g/L}$ at 120 min (vs. 0 min $P < 0.05$, Fig. 2B).

The effects of hyperinsulinemia and lipid-induced insulin resistance on Alarin levels in normal subjects

To examine whether serum Alarin is impacted by euglycemic-hyperinsulinemic state, we performed EHCs in 30 normal controls. During the steady-state of clamp, blood glucose was maintained at ~ 5 mmol/L and insulin was elevated from 44.5 ± 10.2 to 315.3 ± 48.2 pmol/L. In response to hyperinsulinemia, the GIR needed to maintain euglycemia (expressed as M-values) elevated from 0 to 10.4 ± 2.8 mg/kg/min in these subjects. However, in the euglycemic-hyperinsulinemic state, serum Alarin levels showed a transient decrease, and then returning to pre-EHC levels (from 0.30 ± 0.05 $\mu\text{g/L}$ at 0 min, to 0.28 ± 0.05 $\mu\text{g/L}$ at 80 min, and to 0.26 ± 0.06 $\mu\text{g/L}$ at 100 min, vs. 0 min $P < 0.05$, and then increasing to 0.29 ± 0.07 $\mu\text{g/L}$ at 110 min, and finally to 0.29 ± 0.05 $\mu\text{g/L}$ at 120 min) (Fig. 2C). Fig. 2D showed the average level of circulating Alarin during the EHC, suggesting that Alarin levels were decreased by insulin stimulated.

To investigate the effect of lipid-induced insulin resistance on circulating Alarin *in vivo*, we examined the alterations of serum Alarin level before and after lipid infusion combined with EHCs in 22 normal individuals. As expected, GIR was significantly decreased in lipid

Table 2. Partial correlations analysis of variables associated with circulating Alarin levels in study population MetS, metabolic syndrome; BMI, body mass index; WHR, waist-to-hip ratio; FAT %, visceral fat %; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; FFA, free fatty acid; FBG, fasting blood glucose; 2h-BG, 2 h post-glucose load blood glucose; FIns, fasting insulin; 2h-Ins, 2 h serum insulin after glucose overload; HOMA-IR, HOMA-insulin resistance index; $\text{AUC}_{\text{insulin}}$, the area under the curve for insulin; $\text{AUC}_{\text{glucose}}$, the area under the curve for glucose; TNF- α , tumor necrosis factor- α . Data are present as mean (standard deviation) or median (interquartile range)

Item	Alarin		Alarin (age-adjusted)		Alarin (age-and BMI-adjusted)	
	R	P value	R	P value	R	P value
Age	0.003	0.980				
BMI	0.124	0.216	0.125	0.213		
WC	0.221	< 0.05	0.222	< 0.05	0.200	< 0.05
WHR	0.081	0.417	0.082	0.417	0.054	0.593
SBP	0.222	< 0.05	0.236	< 0.05	0.208	< 0.05
DBP	0.199	< 0.05	0.199	< 0.05	0.177	0.078
FBG	0.249	< 0.05	0.249	< 0.05	0.249	< 0.05
FIns	0.049	0.627	0.049	0.624	-0.004	0.971
HOMA-IR	0.143	< 0.05	0.145	< 0.05	0.097	< 0.05
HbA1c	0.241	< 0.05	0.243	< 0.05	0.238	< 0.05
TG	0.271	< 0.01	0.272	< 0.01	0.250	< 0.05
TC	0.083	0.406	0.083	0.408	0.070	0.491
HDL-C	-0.150	0.121	-0.156	0.120	-0.129	0.199
LDL-C	0.040	0.689	0.040	0.691	0.026	0.800
FFA	0.032	0.746	0.033	0.746	0.025	0.804
$\text{AUC}_{\text{insulin}}$	0.086	0.390	0.087	0.385	0.060	0.555
$\text{AUC}_{\text{glucose}}$	0.298	< 0.01	0.298	< 0.01	0.284	< 0.01
TNF- α	0.133	< 0.05	0.133	< 0.05	0.124	0.068

Table 3. Association of circulating Alarin with MetS and insulin resistance in fully adjusted models CI, confidence interval; OR, odds ratio; MetS, metabolic syndrome; IR, HOMA-IR. Results of multivariate logistic regression analysis are presented as the odds ratio of being in MetS and IR status increase in serum Alarin

Model adjust	Mets			Insulin resistance		
	OR	95% CI	P value	OR	95% CI	P value
Age	4.27	1.40-12.99	< 0.01	4.01	1.29-12.44	< 0.01
Age, Sex	4.47	1.45-13.78	< 0.01	3.83	1.23-11.89	< 0.01
Age, Sex, BP	4.19	1.23-14.26	< 0.05	3.89	1.21-12.46	< 0.01
Age, Sex, BP, BMI	4.22	1.17-15.17	< 0.05	3.74	1.12-12.47	< 0.01
Age, Sex, BP, BMI, WHR	4.44	1.21-16.34	< 0.05	3.79	1.13-12.68	< 0.01
Age, Sex, BP, BMI, WHR, IR	2.50	0.68-9.23	0.169			

Table 4. Odds ratios and 95 % confidence interval (CI) of circulating Alarin for individual components of MetS in male and female subjects Data are present as median and interquartile range

Individual components of MetS	Men (N=165)		Women (N=264)		Total (N=429)	
	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
All components	2.01 (0.39-10.37)	0.40	9.07 (1.81-45.48)	< 0.001	4.35 (1.43-13.24)	< 0.01
Hypertension	1.66 (0.32-8.61)	0.54	1.26 (0.34-4.57)	0.72	1.51 (0.55-4.15)	0.41
High HDL-C	0.46 (0.08-2.60)	0.38	2.50 (0.62-10.08)	0.19	1.09 (0.40-2.96)	0.86
Hypertriglyceridemia	1.59 (0.31-8.17)	0.57	5.61 (1.38-22.75)	< 0.01	3.63 (1.27-10.38)	< 0.01
Hyperglycemia	351.52 (13.53-9126.95)	< 0.001	10.44 (1.87-58.16)	< 0.001	33.90 (7.16-160.51)	< 0.01
Central obesity	1.27 (0.25-6.39)	0.76	10.15 (1.48-69.20)	< 0.01	2.35 (0.78-7.06)	0.12
HOMA-IR	8.61 (1.19-62.21)	< 0.05	2.414 (0.615-9.474)	0.414	3.99 (1.29-12.340)	< 0.05

Fig. 2. Circulating Alarin levels in interventional studies. A. Circulating Alarin in healthy individuals in response to acute exercise. B. Circulating Alarin concentrations in healthy subjects during an OGTT with 75 g glucose. C. Time course of circulating Alarin changes in healthy subjects during EHC. D. Circulating Alarin levels in the basal and insulin- stimulated state. E. Time course of circulating Alarin changes in healthy subjects during lipid infusion combined with EHC. F. The changes of circulating Alarin levels at baseline and during lipid infusion in healthy subjects. Data are means $\pm$ SD. * $P < 0.05$ vs. baseline.

infused individuals than in controls (4.53 ± 1.86 vs. 9.58 ± 2.40 mg/kg/min, $P < 0.01$). Importantly, serum Alarin levels were significantly increased from 0 to 180 min during lipid infusion [from 0.31 ± 0.07 to 0.35 ± 0.07 μg/L; $P < 0.05$; Fig. 2E]. The average Alarin levels during intralipid infusion were shown in Fig. 2F ($P < 0.05$). During the last 3 hour of lipid infusion and 2 hour of EHC, hyperinsulinemia led to a transient decrease of circulating Alarin levels (from 0.35 ± 0.07 at 260 min to 0.33 ± 0.07 μg/L at 280 min, Fig. 2E).

Discussion

In animal studies, the beneficial effects of Alarin on ameliorating insulin resistance, decreasing insulin levels and blood glucose have been revealed in the *vivo* and *vitro* [16, 17].

However, the relationship between circulating Alarin and metabolic diseases has not been reported in humans. To better demonstrate the association of the circulating Alarin levels with MetS and to avoid the interference of clinical confounding factors, newly diagnosed MetS individuals were recruited in the current study. Therefore, the effects of disease duration, diet and medicine were excluded. Here, we demonstrated that circulating Alarin levels were markedly increased in MetS individuals relative to healthy controls and circulating Alarin is independently associated with MetS and insulin resistance. These results reveal a relationship between circulating Alarin and MetS and suggest that a high level of Alarin might be beneficial to MetS in humans. However, the mechanisms underlying increased Alarin levels in MetS subjects remain elusive.

In a previous study, we reported that circulating Sfrp5, an adipokine, was significantly lower in MetS individuals and it may predict for MetS [22]. In the current study, we observed that circulating Alarin levels were significantly higher in MetS individuals. Therefore, when an individual has higher circulating Alarin levels and low Sfrp5 levels, it is more likely to be diagnosed as MetS.

Alarin has recently been found to be relative to the regulation of energy balance [23]. We speculate that the elevation of circulating Alarin in MetS individuals might be a compensatory up-regulation *in vivo* for counteracting the metabolic stress produced by obesity, hyperglycemia, or hyperlipemia. Furthermore, it is also possible that adiposity or metabolic disorder may cause the resistance of Alarin actions, like insulin or leptin resistances, leading to the increase of Alarin secretion and release.

Our cross-sectional study has showed an independent association of the circulating Alarin with other metabolic risk factors, including abdominal obesity, insulin resistance, dyslipidemia, hyperglycemia and hypertension. These findings indicate that Alarin might have a regulatory role in glucose and lipid metabolism *in vivo*, consistent with the results of two animal studies [16, 17]. Therefore, the elevation of circulating Alarin levels in MetS subjects might be beneficial to the improvement of metabolism *in vivo*.

To investigate the possible association of the five components of MetS and circulating Alarin, we performed logistic regression analysis. Hypertriglyceridemia, hyperglycemia and central obesity seemed to have the positive associations with Alarin in women. In keeping with this finding, animal study reported that Alarin was associated with body weight [15], glucose uptake and blood glucose levels [16]. Therefore, the results suggest an awareness of the need to screen population with MetS for the presence of high Alarin levels.

In our ROC curve analysis, the results show that circulating Alarin may predict both MetS and insulin resistance in our study population. In one aspect, the range of AUC (0.6-0.7) was considered to be mild-to-moderate significance, which may be due to the influence of the sample size and a non-normal distribution of Alarin levels in the studied population. In another aspect, the circulating Alarin may not be a good marker for predicting MetS and insulin resistance. Therefore, further extensive study is necessary for making a solid conclusion.

An important finding of this study is a significant increase of serum Alarin levels after an oral glucose challenge in normal subjects. This pattern is similar to insulin and glucose during OGTT, suggesting a reciprocal regulation among these factors in response to metabolic changes. The increase of circulating Alarin during OGTT may be due to increased glucose and/or insulin. To rule out possible effects of elevated insulin levels produced by oral glucose challenge on circulating Alarin, we performed EHC (a euglycemic-hyperinsulinemic state) on 30 normal subjects. During the steady-state of EHC, circulating Alarin levels showed a transient change. It decreased at 100 min of EHC, followed by a gradual return to baseline. These results lead us to speculate that short-term hyperinsulinemia may have an inhibitory effect on Alarin secretion and/or release. Therefore, the elevated Alarin concentrations during oral glucose challenge are likely due to the increased glucose levels rather than insulin, as increased blood glucose may be sufficient to eliminate the effect of insulin on circulating Alarin.

Insulin resistance is an important feature of some metabolic disorders like obesity, dyslipidemias, T2D, hypertension and MetS, all of which are risk factors for CAD [24]. It is well documented that lipid infusion induces insulin resistance in humans *via* inhibition of glucose transport activity [3, 25]. To further investigate whether circulating Alarin is related to insulin resistance in humans, intralipid infusion was performed in normal individuals. We observed that intralipid infusion led to an increase in serum Alarin concentrations and then a decrease during EHC. Therefore, circulating Alarin showed a biphasic change during lipid infusion with EHC. These findings revealed that acute elevation of FFA by lipid infusion contributed to the elevated Alarin levels, and subsequent hyperinsulinaemia during EHC led to a reduction in Alarin levels. These data demonstrate that FFA regulate Alarin release and support a role for Alarin in FFA-induced insulin resistance. Insulin resistance induced by FFAs is principally due to substrate competition and intracellular biochemical pathways including glucose phosphorylation and regulation of pyruvate dehydrogenase complex. Thus, it is possible to consider that Alarin may exert a role in insulin resistance during T2D independent of hyperinsulinaemia.

It is generally accepted that the metabolic regulation in obesity, T2D and MetS patients is influenced by their diet and physical activity, and physical activity protects against several types of disease including CAD, T2D and MetS [26, 27]. Recently, muscle tissues have been found to be an endocrine organ that releases cytokines, named myokines. Myokines have been shown to modulate several physiological pathways [28]. To explore whether Alarin secretion was increased by muscle tissue through physical activity, we investigated the effect of acute exercise on circulating Alarin. However, we observed that a 45-min bout of exercise in healthy individuals was not effective in modulating Alarin levels. This finding suggests that the release of Alarin may not be regulated by muscle tissues and this peptide may not be an exercise-responsive myokine. However, the effects of long-term exercise on circulating Alarin levels are unknown, and further studies are necessary.

There are some limitations in our research. First, the cross-sectional design of this study does not allow us to deduce a causal relationship between Alarin and MetS. Prospective studies are needed to clarify their precise interrelationship. Second, Alarin concentrations were not a pre-specified end point for recruited MetS subjects. Finally, our results could be influenced by some outliers due to the sample size. Despite these limitations, this study demonstrated clinically significant associations and differences between groups.

Conclusion

In summary, the present study provided evidence that circulating Alarin levels was significantly elevated in newly diagnosed MetS individuals and the circulating Alarin was impacted by oral glucose challenge, acute hyperinsulinemia and lipid infusion. Therefore, the data indicated that Alarin might be a cytokine related to nutrition and metabolism. Further large-scale prospective studies including different ethnic groups have been taken into our consideration.

Abbreviations

EHCs (euglycemic-hyperinsulinemic clamp); TNF- α (tumor necrosis factor- α); (WC, waist circumference); FBG (fasting blood glucose); HOMA-IR (homeostasis model assessment of insulin resistance); AUC_{glucose} (the area under the curve for glucose).

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Disclosure Statement

The authors declare that there are no conflicts of interest.

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