

Diabetes and Retinol-Binding Protein (RBP4): The Study of Free and Exosomal RBP4 in Blood Plasma and Urine of Patients

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Diabetes is the most prevalent chronic disease worldwide. In type 2 diabetes, target tissues decrease their sensitivity to insulin. Before the onset of type 2 diabetes, insulin resistance begins to grow, and beta cells produce more insulin for a longer time until beta cells become burned out and finally develop into type 2 diabetes. In prediabetes and diabetes patients, the retinol-binding protein (RBP4) concentration is high in the blood plasma. In this study, we sought to observe free RBP4 and exosomal RBP4 in the blood plasma as well as the urine of healthy, prediabetes, and diabetes patients. Three participants with type 2 diabetes mellitus, two participants with prediabetes, and five healthy participants were involved in this research. Blood and urine were collected from each participant, and exosomes from blood plasma and urine were isolated. RBP4 was analyzed using ELISA. The results showed that RBP4 was not only found as free RBP4 but also as exosomal RBP4 in the blood plasma as well as the urine. No previous research reported urinary exosomal RBP4. Interestingly, the concentration of exosomal RBP4 in the blood plasma of diabetes, prediabetes, and healthy participants was inversely proportional to the free RBP4 concentration in diabetes, prediabetes, and healthy participants. The exosomal RBP4 concentration of healthy participants was higher than that of prediabetes and diabetes participants. In conclusion, exosomal RBP4 can be found in urine and blood plasma. The concentration of exosomal RBP4 in diabetic participants is lower than prediabetes and healthy participants.

Keywords: Exosome; Prediabetes; Plasma; RBP4; Type 2 Diabetes; Urine.

Diabetes mellitus is one of the most frequently occurring chronic diseases in almost all countries and continues to increase in numbers, mainly due to changes in lifestyles in many countries recently¹. There are two types of diabetes mellitus. In type-1 diabetes (T1D), patients suffer from absolute insulin deficiency because of the autoimmune destruction of pancreatic beta cells. In contrast, in type-2 diabetes (T2D), there is a heterogeneous metabolic disorder due to the decreased sensitivity of target tissues to insulin².

Prediabetes is a high-risk state of T2D. When insulin resistance worsens in prediabetes, the pancreatic beta cells will try to become more insulin-insensitive. This condition can occur for a long time without any symptoms until finally, the beta cells will be exhausted and will no longer be able to synthesize enough insulin; moreover, blood sugar will start to rise to above-average levels.

Prediabetic people have higher-than-normal blood sugar. When prediabetes is not treated early, prediabetes will develop to T2D. If there

are many damaged beta cells, diabetes becomes an irreversible condition³. In order to detect either insulin resistance or beta-cell dysfunction, glycemic parameters (fasting and two-hour postprandial blood glucose and HbA1c) will be measured. However, glycemic parameters alone cannot show the pathophysiology of the disease.

In the last decade, it was found that RBP4 in blood plasma (Retinol Binding Protein 4) correlated with Diabetes Mellitus and has been described as a link between impaired glucose uptake in adipocytes and systemic insulin sensitivity. [4,5,6]. Plasma retinol-binding protein (also termed as RBP or RBP4) belongs to the lipocalin family of proteins, which transport vitamin A (all-trans-retinol) from the liver to the peripheral tissues. In the blood, RBP4 circulates as a complex with thyroxine-binding protein, the transthyretin (TTR)^{7,8,9}.

Yang *et al*⁴ reported that RBP4 was increased in insulin-resistant mice with adipose tissue-specific GLUT4 knockout and in humans with obesity and type 2 Diabetes Mellitus. In this GLUT4 null mice, RBP4 was high. Overexpression of RBP4 in normal mice causes insulin resistance and glucose intolerance. On the other hand, genetic deletion of RBP4 increased insulin sensitivity¹⁰. The authors also stated that RBP4 seems to decrease glucose uptake in the muscle tissue by inhibiting phosphorylation of the insulin receptor substrate-1 (IRS-1) and phosphoinositide-3-kinase (PI-3-kinase) activity, which are essential components of the insulin signaling pathway. While in the liver, RBP4 upregulated the expression of the gluconeogenic enzyme phosphoenol-pyruvate carboxykinase (PEPCK). This condition can subsequently increase hepatic glucose output.

Until now, it was known that RBP4s circulate in the blood as free RBP4s; however, there is no information about whether there is another way by which RBP4 is delivered to the target cells or non-target cells. Nowadays, it is known that exosomes serve as one of the essential intracellular communications. It can contain protein, micro RNA, and mRNA¹¹. Circulating exosomes in diabetic blood might correlate with the regulation of lipid and glucose metabolism¹². Therefore, in this research, we aim to examine free RBP4 and exosomal RBP4 in the blood plasma as well as

in the urine of healthy participants, prediabetes patients, and diabetes patients.

MATERIAL AND METHODS

Participants of the Research

This research was approved by the ethics committee of Hasan Sadikin Hospital, Bandung, Indonesia, with ethics approval agreement no. 118/UN6.C1.3.2/KEPK/PN/2017.

The participants were recruited from the staffs of Institut Teknologi Bandung in Indonesia. This study only included adult participant (>30 years old) both men and women who are willing to participate through informed consent.

These participants are then categorized into three categories (normal, prediabetes, and diabetes) based on the level of fasting glucose, postprandial (2 hour) glucose, and HbA1C (glycosylated hemoglobin). The categorization based on these parameters are summarized in Table 1.

Clinical Characteristics of Blood

Ten mL of venous blood was collected after a 14 hour overnight fast. Two hours postprandial, blood was collected again. Glycosylated hemoglobin, glucose, urea, creatinine, uric acid, and lipid profile were analyzed by Pramita clinical laboratory service (Bandung, Indonesia)

Exosome Isolation and ELISA of RBP4

For RBP4 quantification in blood plasma, another five mL of blood was collected. Two mL of it was used to isolate exosomes using Total Exosome Precipitation reagent kit for plasma (Invitrogen). The rest (3 mL) was allowed to clot to collect the blood plasma for quantification of free RBP4. Both exosomes and blood plasma were stored at -20oC for subsequent RBP4 quantification.

For RBP4 quantification in urine, exosomes were isolated from two mL of urine using Total Exosome Isolation kit for urine (Invitrogen). Free RBP4 is quantified directly using untreated urine samples.

Both in blood and urine analysis, exosomal and free RBP4 were quantified using RBP4 ELISA kit (Abcam). In this study, free RBP4 in blood plasma is only found in one participant in each diabetic (subject #1) and normal (subject #2) category.

Statistical Analysis

All statistical analysis in this study was done using GraphPad Prism 8.4.2. Comparison of free RBP4 in urine and blood samples of subject #1 and subject #2 utilized unpaired and paired T-test (* $P < 0.05$, ** $P < 0.01$). Meanwhile, comparison of exosomal RBP4 between normal, prediabetes, and diabetes categories employed two-tail ANOVA ($P < 0.05$).

RESULTS AND DISCUSSION

In this research, we found that in blood plasma and urine, there is not only free RBP4 but also exosomal RBP4 (Table 2). The concentration of free RBP4 in the blood plasma of diabetes participant is significantly higher than the healthy participant (Figure 1). This result is consistent with the previous researches, such as in Yang *et al.* and Cho *et al.* [5, 6]. Free RBP4 in blood plasma is synthesized and secreted from adipocyte and majorly from the liver¹³. This high concentration of RBP4 in the blood plasma of diabetic participants is probably correlated with its function for maintaining metabolic control. Ma *et al.*¹⁴ stated that the important role of hepatic RBP4 is to maintain metabolic control.

The concentration of free RBP4 in urine is much lower than the concentration of free RBP4 in blood plasma. However, the concentration of RBP4 in urine is nearly similar between diabetic and healthy participants (Figure 2). In contrast,

Park *et al.*¹⁵ showed that free urinary RBP4 from prediabetic and diabetic patients is higher than the healthy persons. Park *et al.*¹⁵ also showed that urinary RBP4 concentrations were correlated with several cardiometabolic parameters, such as insulin

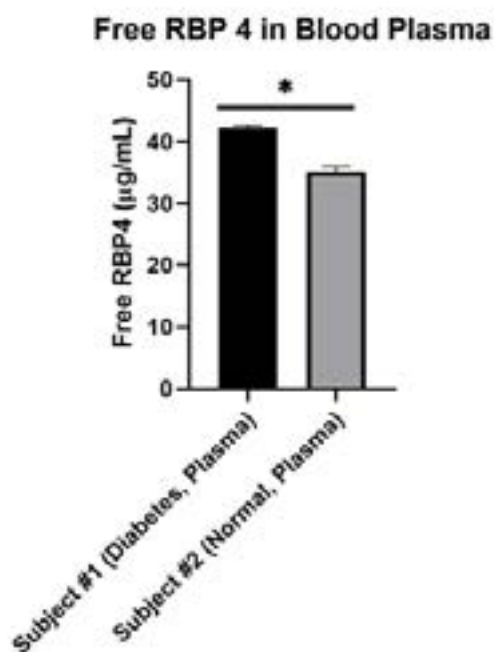


Fig. 1. Comparison of free RBP4 in blood plasma of diabetic participant (subject #1) and normal participant (subject #2). Statistic analysis used unpaired T-test (* $P < 0.05$)

Table 1. Categories of participants in the study

Category of participants	Fasting glucose (mg/dL)	Postprandial (2 hour) glucose (mg/dL)	HbA1C (percentage)	Number of participants (n)
Normal	<100	<140	<6%	5
Prediabetes	100-126	140-199	6-6.4%	2
Diabetes	>126	=200	>6.4%	3

Table 2. Free RBP4 and exosomal RBP4 concentration in plasma and urine of diabetes and normal participant (subject #1 and #2)

	Normal (Subject #2)		Diabetes (Subject #1)	
	Plasma	Urine	Plasma	Urine
Free RBP4 (mg/mL)	35.125	0.124	42.189	0.126
Exosomal RBP4 (ng/mL)	99.132	7.704	97.876	7.000

resistance, inflammation, and arterial stiffness. The inconsistency in the results between ours and Park *et al*¹⁵ might be due to the very small number of samples in this study; therefore, further research should be performed.

Table 2 showed exosomal RBP4 in blood plasma and urine. The presence of RBP4 in exosome from blood plasma has been listed in ExoCarta^{16, 17, 18}. However, there was no previous information that RBP4 can also be found in the urinary exosome. The concentration of exosomal RBP4 in urine is lower than that of blood plasma. Exosomal RBP4 will probably support free RBP4 by transporting functional RBP4 to other cells that do not express RBP4 and helping vitamin A uptake by the cells. This phenomenon was identified by Street *et al*¹⁹, who found that exosomes transported functional aquaporin to cells that did not express the protein, thus causing a significant increase in water flow.

Exosomal RBP4 in the blood plasma of health and diabetic participants might be

produced by adipocytes. Deng *et al*²⁰ and Guay and Regazzi²¹ showed that in the mice model for obesity and diabetes (ob/ob mice), exosomes containing RBP4 were secreted by adipocytes and could activate the TLR4/NF κ B pathway and stimulate macrophages (M1) to secrete of IL-6 and TNF α , which are important for inflammation. In this research, it is also found that exosomal RBP4 is produced in healthy participants. In this research, exosomal RBP4 in diabetes plasma blood is probably correlated with the stimulation of the M1 macrophage to secrete IL-6 and TNF α . This condition could reduce the insulin sensitivity of the liver and muscles. In this research, we also found exosomal RBP4 in urine; however, we still do not know the function of exosomal RBP4 in urine.

The concentration of exosomal RBP4 in healthy, prediabetes, and diabetes participants is shown in Table 3. In this study, only exosomal RBP4 in blood plasma has been observed. Interestingly, we observed an opposing trend in which exosomal blood RBP4 in healthy participants

Comparison of free RBP4 in Plasma and Urine of Diabetes and Healthy Participant

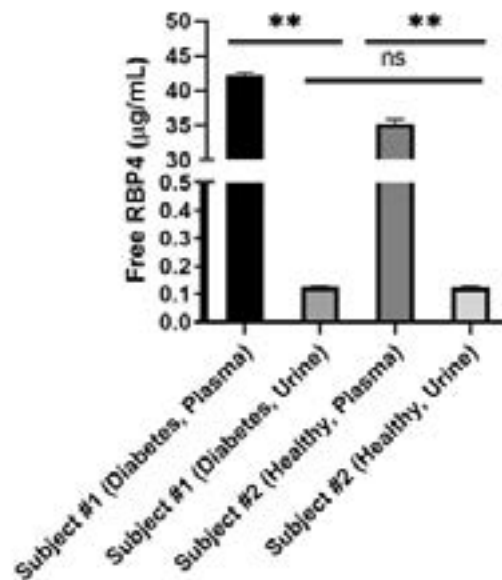
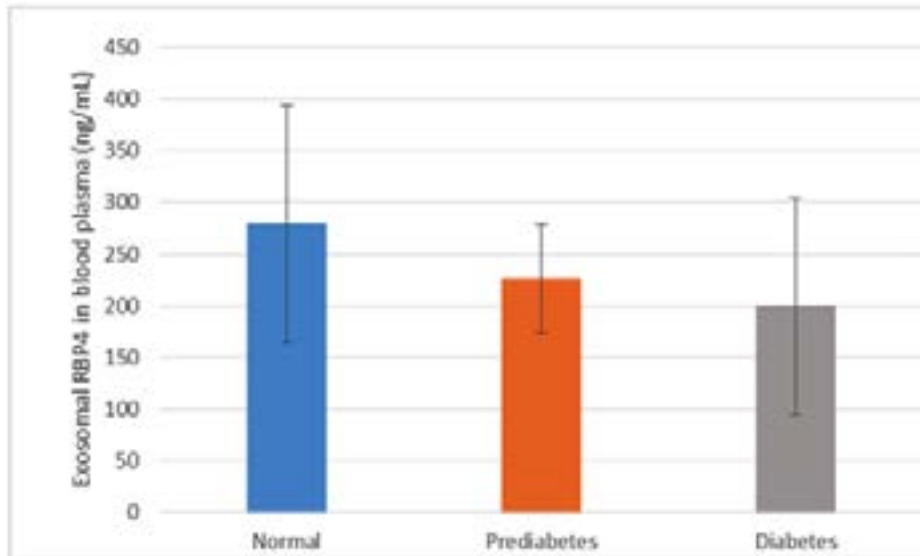


Fig. 2. Comparison of free RBP4 in plasma and urine of diabetes and healthy participants. Free RBP4 is significantly higher in plasma compared to urine, both in diabetes and healthy participant (paired T-test, $**P < 0.01$). However, there is no significant difference in RBP4 level among diabetes and healthy participant (unpaired T-test, $P < 0.05$)

is lower compared to those in prediabetes as well as diabetes patient (Figure 3), while free RBP4 in the blood of healthy participant is significantly higher compared to diabetes participant (Figure 1). It is still unclear why the concentration of exosomal blood RBP4 is higher in healthy participants. The low concentration of exosomal RBP4 in prediabetes and diabetes patients might

be associated with a high concentration of free RBP4 in blood plasma. Xiao *et al*²² stated that exosomes in T2D can participate in regulating various physiological and pathological processes *in vivo* by being transported between cells.

Ying *et al*²³ stated that adipocytes from healthy animals produce exosomes containing certain miRNAs that regulate the sensitivity of the



Exosomal RBP4 in Blood Plasma

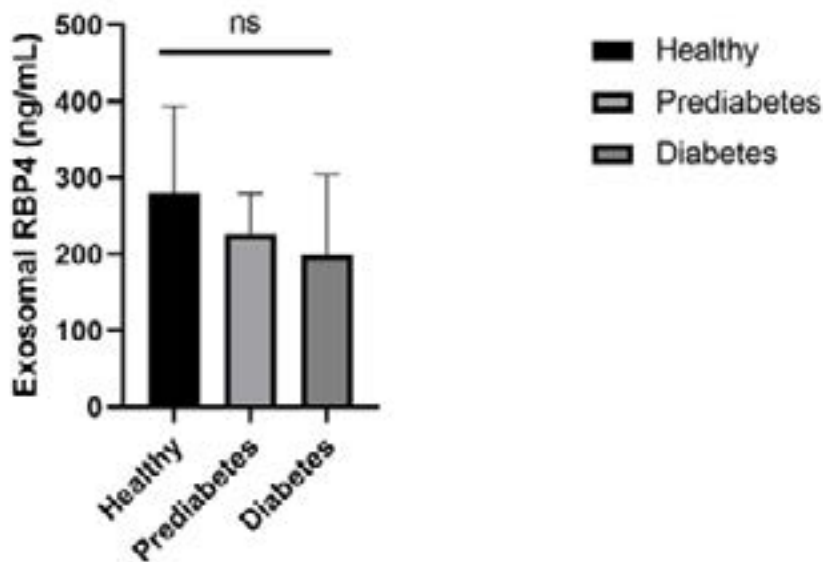


Fig. 3. Comparison of exosomal RBP4 in blood plasma of healthy (normal), prediabetes, and diabetes participants (one-way ANOVA. $P < 0.05$)

Table 3. Clinical characteristics of participants

	Healthy (n=5)	Prediabetes (n=2)	Diabetes (n=3)
Age	46±15.7	55.5±2.12	57±10.15
Gender M/F	5/0	1/1	3/0
Urea (mg/dL)	29.28±10.62	18.6±3.68	24.33±6.03
Creatinine (mg/dL)	1.02±0.22	0.8±0	0.93±0.11
Uric acid (mg/dL)	6.78±0.96	6.55±0.64	4.2±0.89
Lipid profile:			
• Cholesterol (mg/dL)	190.2±44.60	192±16.97	183±39.28
• Triglyceride (mg/dL)	125.4±72.73	120±11.31	116.33±36.20
• HDL cholesterol (mg/dL)	41.6±7.70	43.5±6.36	46.67±9.02
• LDL cholesterol (mg/dL)	123.6±37.65	125±25.46	113±39.95
Glucose fasting (mg/dL)	89.6±15.95	105.5±4.95	160±42.79
Glucose 2hours (mg/dL)	125.8±29.48	186±12.73	328.33±117.49
HbA1C (%)	5.42±0.29	6.2±0	8.17±1.69
Exosomal RBP4 in blood plasma (ng/mL)	279.2±114.74	226.5±53.03	199.67±104.61

liver and muscles to insulin. However, in obese mice, exosomes containing miRNA 155 from adipocytes decrease the sensitivity of muscles and the liver to insulin²³. Moreover, Lippai and Szabo²⁴ and Mahesh and Biswas²⁵ showed that miRNA 155 is the major inflammatory regulator. As we know, T2D is accompanied by chronic low-grade inflammation in several tissues, such as adipose tissue, liver tissue, and muscle tissue²⁶.²⁷ Therefore, together with RBP4 in exosome of prediabetes and diabetes, probably miRNA 155 in exosome supports inflammation in peripheral organs. Further observation to investigate the mechanism underlying exosomal and free RBP4 is required.

CONCLUSION

This study attempted to examine the level of free and exosomal RBP4 in the blood plasma and urine of prediabetic, diabetic, and healthy participants. We found that RBP4 is secreted as free RBP4 and exosomal RBP4 in blood plasma as well as in urine of healthy and diabetes patients. We also found that the concentration of exosomal RBP4 in healthy participants is higher than it is in prediabetic and diabetic patients, although not significant. Interestingly, we observed an inverse trend in the level of free and exosomal RBP4 in the healthy and diabetes patient. However, the reason behind this finding is still unclear. Further

observation might have to be done in order to elucidate the mechanism causing this result.

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