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Prevalence of zinc-α 2-glycoprotein binding peptide among Omani blood donors

Hasson, S. S.*, Al-Balushi, M. S, Al Shaili, A. S, Al-Busaidi J. Z., Said, E. A.,Idris, M. A. and Al-Jabri, A. A.

Immunology Unit, Department of Microbiology and Immunology, College of Medicine and Health Sciences, Sultan Qaboos University, P.O. Box 35, Muscat, Oman.

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Zinc alpha-2-glycoprotien (ZAG) binding peptide is a multi-functional protein, which is structurally similar to a major histocompatibility complex class I. It has been discovered as a novel adipokine enhancing lipolysis and influencing other physiological processes such as sperm mobility and melanin production. Furthermore, ZAG level has been correlated to a variety of diseases such as atherosclerosis and diabetes type II with a potential use as a tumor biomarker in future. In this study, we aim to investigate the prevalence of ZAG among healthy blood donors attending to the Sultan Qaboos University Hospital blood bank and correlate it with their age and sex. The ZAG levels analysis of the sera from 106 (49 females and 57 males) apparently healthy donors from different regions was carried out using a competitive type of enzyme-linked immunosorbent assay (ELISA) (Abnova GmbH-Germany). Analysis was mainly based on two parameters; age and sex. Out of the 106 subjects, 78% of blood donors have high ZAG levels (>35 ng/ml), 13% have a normal level (20 to 35 ng/ml) while 9% have a level lower than 20 ng/ml. A significant association was found between ZAG level and sex (*P* = 0.012) with males showing low levels. Although high ZAG level was correlated between age and ZAG levels in the female group, higher levels were also found in donors below and above 22 years old (*P* = 0.0099). The prevalence of ZAG levels in blood donors was found to be high, especially in those between 20 to 30 years old. This emphasizes the measurement of ZAG level prior to blood transfusion to patient(s) who are clinical under weight. Gender and age significantly influences the plasma level of ZAG.

Key words: Zinc alpha-2-glycoprotien (ZAG), Oman, Sultan Qaboos University Hospital (SQUH), blood donation.

INTRODUCTION

Zinc-α-2-glycoprotein (ZAG) binding peptide is a 43 kDa soluble protein which plays multiple roles in human (Stejskal et al., 2008). The ZAG binding peptide normally produced by the epithelial cells in several tissues including the liver, adipose tissue, sweat glands, breast and the gastrointestinal tract, so that it can be found in various body fluids such as the plasma, semen, sweat, milk and the cerebrospinal fluid (Hassan et al., 2008a). The plasma concentration of ZAG binding peptide is affected by several factors including the body weight and the health status but a range between 20 to 35 ng/ml is considered normal.

Its structure has been found to be similar to the major histocompatibility complex (MHC) Class I (Stejskal et al., 2008). Both molecules share the basic three alpha chains (α1, α2, α3) structure in the same arrangement with the binding groove formed between α1 and α2. However, α3 chain in ZAG binding peptide molecule does not bind to

*Corresponding author. E-mail: shyahasson@squ.edu.om. Tel: +968 24143549.

an additional β2 microglobulin (β2M) like in the case of MHC class I. It does instead bind to a zinc molecule in the same way of metalloprotease enzyme proteins. The second major difference is the presence of a hydrophobic molecule - polyethylene glycol in the binding groove of ZAG binding peptide protein (Hassan et al., 2008b). While MHC class I can only bind to peptides, this specific feature gives ZAG binding peptide the ability to bind to different types of ligands including fatty acids.

ZAG binding peptide was first discovered by Burgi and Schmid in 1961 (Zhu et al., 2012). Yet, its function remained unclear. Recent studies showed that ZAG binding peptide has multi-functions due to its high distribution and can be used as a marker for several diseases.

The discovery of ZAG binding peptide as a novel adipokine has a major role in understanding the effect of this protein in the normal and pathological health conditions. Researchers linked the high levels of ZAG binding peptide in the plasma to high rates of lipolysis (Hassan et al., 2008b). It was found that the level of ZAG binding peptide is directly proportional to the level of cholesterol and fatty acids both in the human body and animal models (Gong et al., 2010). Therefore, ZAG binding peptide is considered to be a key player in obesity and obesity-linked diseases especially type 2 diabetes (Olofsson et al., 2010). It is suggested that it will be used as a future treatment of obesity (Choi et al., 2012). On the other hand, ZAG binding peptide level has a high correlation to the fat and muscle wasting condition termed cachexia which cancer patient suffer from (Rolli et al., 2007).

The exact mechanism by which ZAG plays its function through is still unclear. Researchers suggest that it works through β3- adrenoceptors leading to an increase in the concentration of cyclic adenosine monophosphate (cAMP), which enhances lipolysis (Russell and Tisdale, 2012). Moreover, ZAG binding peptide expression is found to be primarily controlled by another adipokine, which is the tumor necrosis factor (TNF-α) (Bao et al., 2005). In addition to that the semen level of ZAG binding peptide, it is reported to be 6 folds higher than the plasma level (Hassan et al., 2008b). The effect of ZAG binding peptide in fertilization is focused in the role it plays in sperm motility, as sperms are independent on the metabolism of lipid to initiate and maintain the motility of their flagella. Moreover, ZAG binding peptide has showed an ability to bind to the sperm membrane and significantly increasing the cAMP concentration (Fei Qu, 2007). The cAMP-dependent phosphorylation is counted as one of the most important pathways for regulating sperm motility.

This protein, ZAG binding peptide, is normally produced by keratinocytes in the epidermis layer of the skin. A very low level of ZAG binding peptide is required for the production of melanin (Hassan et al., 2008b). However, researchers found that ZAG binding peptide generally has an inhibitory effect in melanin production. The primarily mechanism is through decreasing the level of tyrosinase enzyme which plays a key role in the process of melanin production (Hale, 2002). Other studies suggest indirect mechanisms through TNF-α, which is also known to decrease this process (Hassan et al., 2008b).

One of the recent discoveries of ZAG binding peptide properties indicates its potential usage as an early biomarker for more than one type of cancer. Although studies showed that ZAG can suppress the proliferation of malignant cells by activating apoptosis (Hassan et al., 2008b), researches showed that a number of tumors especially, breast and prostate cancer produce ZAG in the early stages. About 40 to 50% of breast cancers show high levels of ZAG binding peptide while up to 73% of prostate cancers are ZAG-producing tumors (Bondar et al. 2007). However, in both types the production of ZAG binding peptide is inversely related to the differentiation of the tumor, as poorly differentiated tumors showed very low levels or absence of ZAG production (Hale et al., 2001).

The multi-functions of ZAG binding peptide are still under study worldwide. The high levels of ZAG and its links to different diseases and conditions such as metabolic syndromes (Stejskal et al., 2008), chronic hemodialysis (Philipp et al. 2011; Leal et al., 2012), insulin resistance (Ceperuelo-Mallafré et al., 2009) and cardiovascular diseases (Tedeschi et al., 2012), suggested that ZAG binding peptide level will be a good candidate to be involved in a variety of diagnostic procedures and as well as it may be considered also to be used therapeutically.

Despite the global interest in this unique and significantly useful protein, no research has been published neither in Oman nor in the Middle East region. To the best of our knowledge, this study is possibly the first in the region to investigate the prevalence of ZAG binding peptide in Omani blood donors. For the first time, this study aims to correlate the levels of ZAG binding peptide to the gender and age.

MATERIALS AND METHODS

The study was carried out at the Immunology Unit, Department of Microbiology and Immunology, Sultan Qaboos University (SQU), College of Medicine and Health Sciences. Subjects of this research included 106 healthy blood donors who attended to the Sultan Qaboos University Hospital (SQUH) blood bank from different regions in Oman. The group comprised of 98 males and 49 females and 57 male with an age range of 18 to 57 years. The majority (68%) of subjects' age was between 20 to 30 years old (median 22 years). Analysis of ZAG binding peptide levels was carried out using a competitive enzyme-linked immunosorbent assay (ELISA) (Abnova GmbH-Heidelberg, Germany). The Kit (KA1689 kit detects ZAG 278aa) is an *in vitro* assay for detecting ZAG binding peptide based on the principle of competitive enzyme immunoassay. The micro-plate in the kit is pre-coated with anti-rabbit secondary antibody. After a blocking step and incubation of the plate with anti-ZAG antibody, both biotinylated ZAG peptide and peptide standard or targeted peptide in sera samples interacts competitively with the

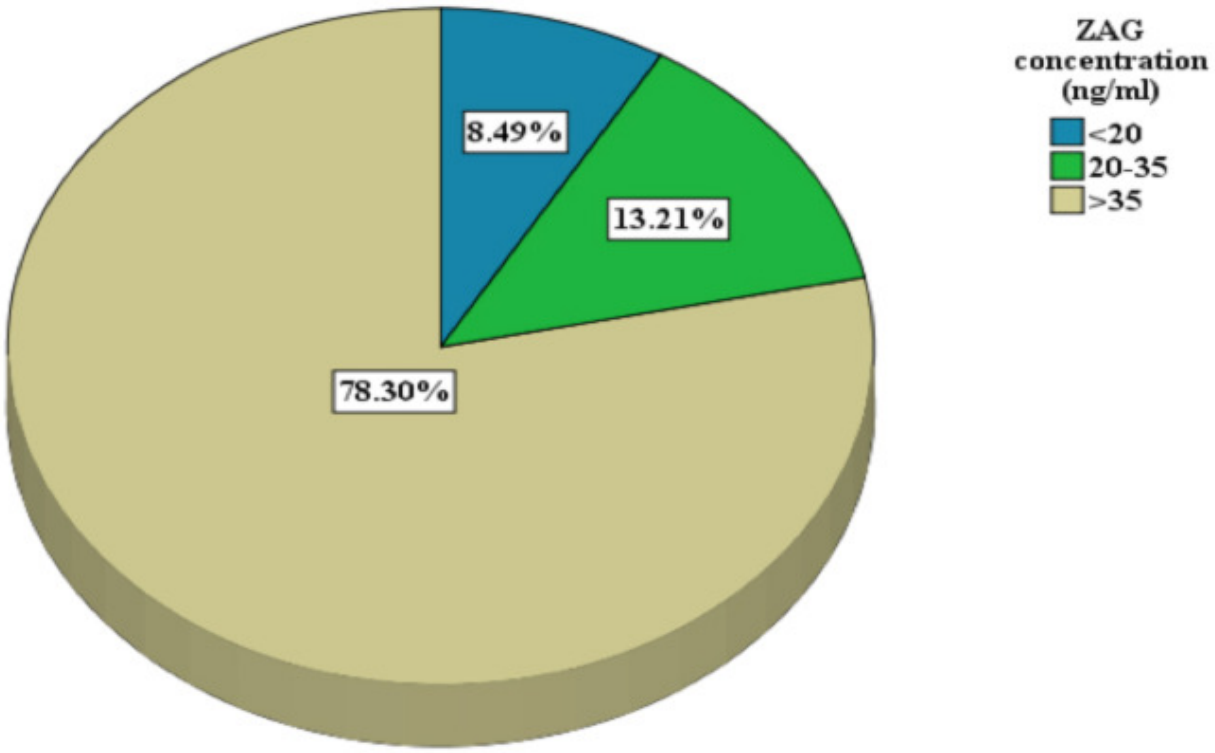

Figure 1. The distribution of ZAG level among 106 Omani blood donors attending SQUH, classified into low (<20 ng/ml), normal (20 to 35 ng/ml) and high (>35 ng/ml).

ZAG antibody.

Uncompeted (bound) biotinylated ZAG peptide then interacts with streptavidin-horseradish peroxidase (SA-HRP), which catalyzes a color development reaction. The intensity of colorimetric signal is directly proportional to the amount of biotinylated peptide-SAHRP complex and inversely proportional to the amount of ZAG peptide in the standard or samples. This is due to the competitive binding to ZAG antibody between biotinylated ZAG peptide and peptides in standard or samples. The concentration of ZAG peptide in the samples was calculated accordingly using a standard curve of known concentration of ZAG binding peptide. Chi-square test was used to test for statistically significant associations between the level of ZAG binding peptide and the subjects' age and sex. The presence of the significant correlations was assessed using Spearman test. The results data analyzed here was determined using IBM statistical package for social sciences (SPSS) Statistics software.

RESULTS

The general distribution of ZAG binding peptide levels based on normal, low or high, among the 106 Omani blood donors included in this study is demonstrated in Figure 1. Surprisingly, it clearly states that the majority of donors (78%) were found to have high ZAG binding peptide levels, that is > 35 ng/ml (mean 177.53 ng/ml), while only a percentage of 13% showed ZAG binding peptide levels within the normal range of 20 to 35 ng/ml mean 29.35 SD ± 4.75), which leaves around 9% who had ZAG binding peptide level of less than 20 ng/ml (mean 9.11 SD± 4.56).

In order to associate the ZAG binding peptide level to the gender of the donors additional subdivisions are illustrated in Figure 2. A significant correlation between the age and the levels of ZAG binding peptide was found only in female ($\rho = 0.5$; $p = 0.0003$) as illustrated in Figure 3. Although both gender showed a close percentage of high ZAG binding peptide level, a significant association was found between the normal and low ZAG binding peptide level with the gender. Out of the 106 donors, 20.4% of female donors were within the normal range of ZAG binding peptide level, while this percentage fell to 7% in male participants. On the other hand, the female percentage of low ZAG binding peptide level was only 2% in comparison to male where it raised to 14%. Chi-square test showed a significant association between ZAG level and sex ($p = 0.012$) with 99.9% level of confidence. Although ZAG binding peptide level was correlated with age in the female group as shown in Figure 3, higher levels was also found in donors below and above 22 years old ($p = 0.0099$) (Figure 4). As stated before, the majority of donors lie in the category of high ZAG binding peptide level (>35 ng/ml). Furthermore, the histogram (Figure 5) clearly shows that in this category more than half of the donors (~56%) are between 20 to 30 years old. Yet, correlation was statistically not significant ($p = 0.08$).

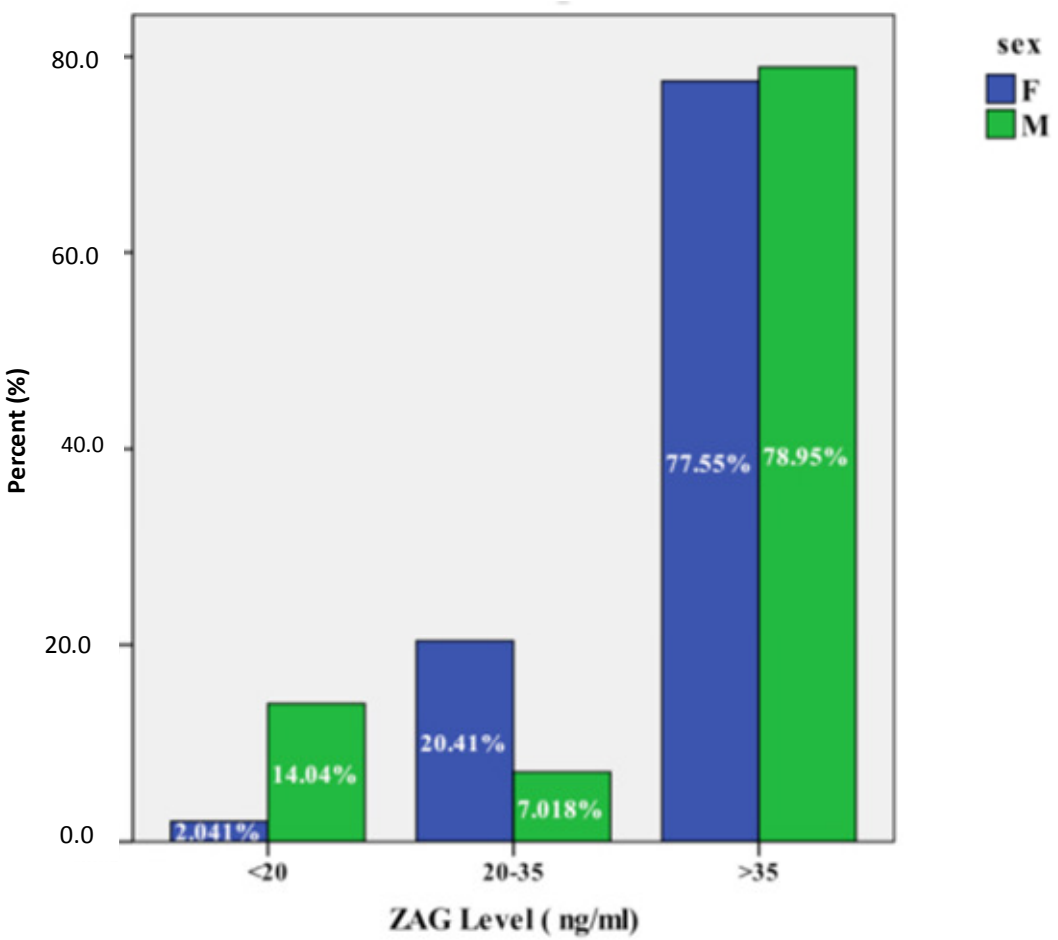

Figure 2. The distribution of ZAG level between 106 Omani males and females into low (<20 ng/ml), normal (20-35 ng/ml) and high (>35 ng/ml).

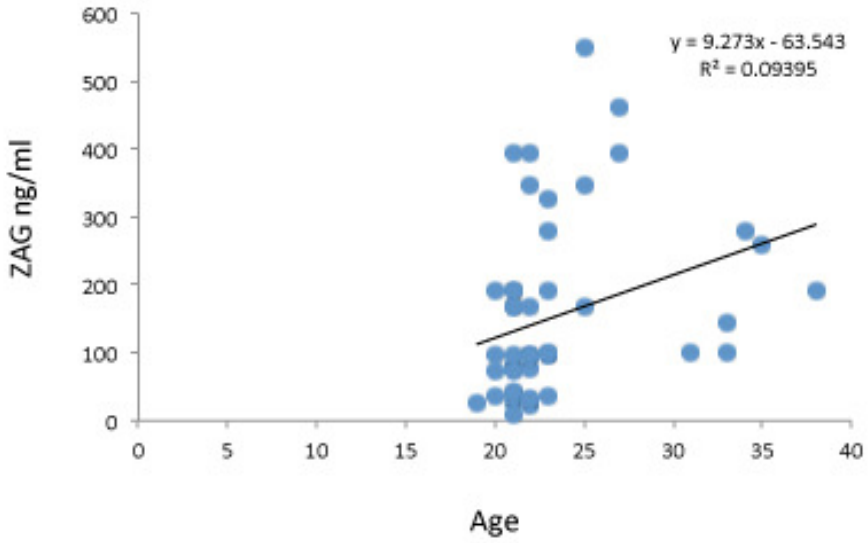

Figure 3. Showed a correlation of the levels of ZAG level among the female group with different ages. A significant association was found between the normal and low ZAG binding peptide level within the female group (R^2 = 0.09395).

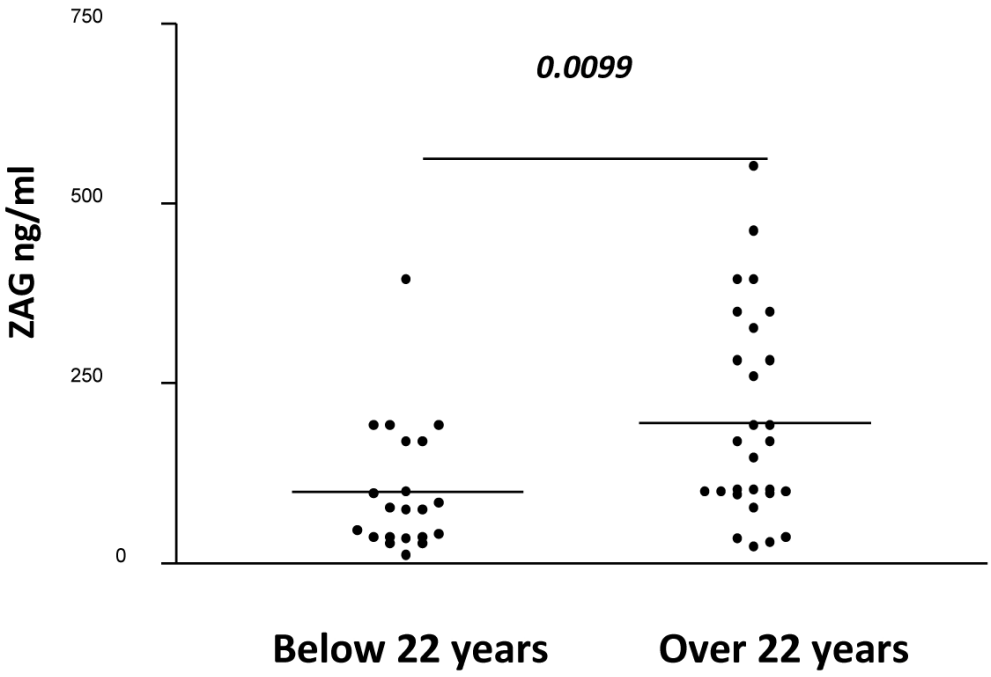

Figure 4. The prevalence of high, normal and low ZAG levels in relation to age among 106 Omani blood donors. The histogram has shown a significant association between the level of ZAG binding peptide and the donors' sex as both sex groups showed a very close percentage of high ZAG level with a significant variation within the low and normal levels of ZAG binding peptide.

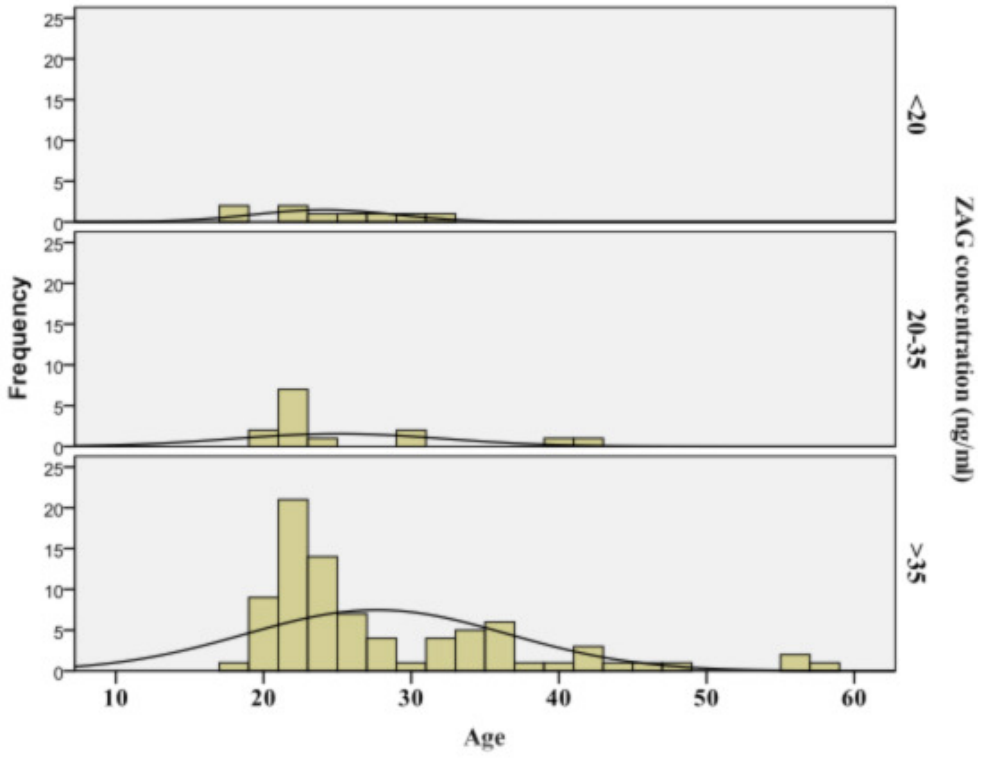

Figure 5. Showed higher levels of ZAG were found in blood donors within the age group below and above 22 years old (p = 0.0099).

DISCUSSION

In this study, the majority of blood donors attending to the SQUH blood bank were found to have high ZAG levels. This has a significant implication not only in the aim to study the pattern of ZAG binding peptide level among healthy Omani individuals, but also in the process of donating blood to those in need which will indeed be affected by such high levels, as some of the recipient are clinically under weight. The out come of this study raised two major points worth to be discussed. First is to find an explanation of the high ZAG binding peptide level expressed by 78% of the donors. As ZAG binding peptide is highly correlated to the metabolism of fat, it is estimated that its high level in the blood of the donors is directly related to their body weight, that is, increase fat metabolism. Other factors which may have influenced the ZAG binding peptide level include chronic diseases, cardiovascular or kidney diseases. It may also suggest an early development of cancer or it can be related to the genetic variation within the population. Hence, it is worthwhile to have a medical history of the blood donors which can explain such threshold of this protein.

The second point to be raised is the impact of this high level in patients receiving the blood. It is important to keep in mind that patients who have physiological abnormalities and/or clinical complications such as patients who are already losing weight due to a chronic disease especially hematological diseases, as they require frequent transfusion of blood and patients with atherosclerosis and cachectic cancer patients receiving such transfusion of blood with high level of ZAG binding peptide, can deteriorate their heath status. Therefore, due to such significant findings, we suggest that ZAG binding peptide should be assigned as a routine investigation in blood donation to assure and implement the safety issues regarding whether or not to accept or reject blood donation based on ZAG binding peptide level.

Interestingly, the ZAG binding peptide level was correlated with age in the female group as shown in Figure 3. Moreover, higher levels were also found in donors below and above 22 years old. Results have also shown a significant association between the level of ZAG binding peptide and the donors' sex. Interestingly, both sex groups showed a very close percentage of high ZAG level with a significant variation within the low and normal levels of ZAG binding peptide. The high percentage level with the male group in comparison to that shown by a low level in females can be explained by the study done by

Hong et al. (2009) on mice where the male mice have higher tendency to gain weight than female. Furthermore, the study suggested that this "obesity-protection" is caused by the ovarian hormones as ovariectomized female mice showed an equal tendency to gain weight to males (Hong et al., 2009). The anti-atherosclerotic affect of estrogen, an ovarian hormone, may also explain the relatively high percentage of female with normal ZAG binding peptide level (Marsh et al., 1999).

Moreover, an association between the age of the donors and their ZAG binding peptide level showed that participants between 20 to 30 years old form of more than half the cases of high ZAG binding peptide level. Such result is accepted if we consider the fact that the metabolic rate including lipid metabolism in people, between age group of 20 to 40 years old, is higher than those older/younger (Shock, 1955). Finally, it would be an advantage if the sera samples were analyzed using an automated analysis in parallel with the manual ELISA kit protocol. This may improve the interlaboratory variability of both the assay and/or the results obtained.

Conclusion

More than 70% of blood donors included in this study were found to have high sera ZAG binding peptide level suggesting that its measurement should be tested routinely. Furthermore, our study emphasizes the measurement of ZAG binding peptide level prior to blood transfusion to patient(s) who are clinically under weight. Gender and age significantly influences the plasma level of ZAG binding peptide.

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