Original Article

Association of *IL-6 (-174)G>C* Genetic Polymorphism to Glycemic Status and Plasma CRP in Pre-Diabetes and Type 2 Diabetes Mellitus in a Filipino Population

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Diabetes which has been described as an inflammatory disease has provided avenues for investigations on inflammatory markers that might be associated to its development. C-Reactive Protein and Interleukin-6 polymorphisms are among the objects of current investigations. *IL-6 (-174)G>C*, one of IL-6 functional polymorphisms in the promoter region, has been reported as influencing IL-6 gene transcription and has been associated with elevated CRP levels. Its relationships to diabetes and several diseases have been studied and the frequencies of its genotypes in different ethnic and population groups have been observed previously to vary from 45 to 100%. This study aimed to explore the frequencies of the IL-6 (-174) genotypes in the Filipino population and to find evidence on the association of *IL-6 (174)G>C* polymorphism to glycemic status and plasma CRP levels in pre-diabetes and Type 2 diabetes mellitus. Two hundred thirty-three (233) subjects (96 type 2 diabetics, 84 pre-diabetics and 53 normoglycemics) were used in the study group. Fasting Blood Glucose Tests and Plasma CRP tests were done using Abbott Hexokinase/G6-PDH reagents and Quantitative Immunoturbidimetric CRP Vario Kit (CRP-16-Ultrasensitive), respectively, in the Abbott Architect ci4100. Genotyping of the *IL-6 (-174)G>C* polymorphism was carried out using Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP). Data of this study showed frequencies of 95%, 5%, 0% for the *IL-6 (-174)* genotypes *GG*, *GC* and *CC*, respectively. The presence of the GC genotype was detected in the diabetic group and none in the pre-diabetic and normoglycemic groups. No adequate evidence of association was found between *IL-6 (-174)G>C* and Plasma CRP levels ranging from 0.51 mg/dL to 0.9 mg/dL. Significant association was observed between *IL-6 (-174)G>C* and Plasma CRP levels of 1.0 mg/dL and above (p-value = 0.006). In this study, significant association was shown between *IL-6 (-174)G>C* and glycemic status (p-value = 0.005) in type 2 Diabetes Mellitus in a Filipino population.

Key words:IL-6 – Interleukin-6, CRP – C-Reactive Protein, Glycemia – presence and level of blood glucose, SNP – Single Nucleotide Polymorphism

Received: March 23, 2014 Revised: June 12, 2014 Accepted: July 18, 2014
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Introduction

The Philippines ranks tenth (10^{th}) globally among countries with the highest diabetes incidence and more than three million Filipinos are afflicted with the disease [1,2]. Diabetes has remained a subject of interest in many studies and has been described as an inflammatory disease [3,4]. Inflammation, as measured by C-Reactive Protein (CRP) levels, has been shown to be associated with the development of diabetes[5]. Among the activators of intracellular pathways promoting the development of insulin resistance and Type 2 diabetes mellitus are cytokines[3]. The cytokine IL-6 predominantly induces increased CRP production[6].

IL-6 (-174)G>C (rs1800795), one of IL-6 functional polymorphisms in the promoter region, influences IL-6 gene transcription and has been associated with elevated CRP levels [7,8,9,10,11,12]. It is located 174 nucleotides upstream of the major transcription initiation site of the IL-6 gene and the presence of either guanine or cytosine at this position gives rise to two different IL-6 alleles leading to three possible genotypes: *GG, GC* and *CC*[13]. The genotype frequencies of polymorphisms are known to vary according to race or ethnicity[14]. A study done on five ethnic groups from the European part of Russia and populations from twenty-four countries of Africa and Eurasia reported that the frequency of the *-174G* allele varied from 45-100%[15].

Investigations on the association of *IL-6 (-174)G>C* to diabetes and other diseases have been conducted in various populations but not among Filipinos. Thus far, IL-6 polymorphisms have been associated with coronary heart disease in British patients, sporadic Alzheimer's disease, colorectal carcinoma, and peripheral arterial disease in diabetes in Italian subjects [16,17,18,13], rheumatoid arthritis in Mexican patients[14] and insulin sensitivity in a Finnish population[19].

In Asian populations, several studies on *IL-6 (-174)G>C* were conducted which include its associations with bone mineral density, coronary heart disease, endothelial dysfunction in type 2 diabetes in Chinese patients [20,21,22], with metabolic syndrome risk in North Indian women [23], with knee osteoarthritis in a Thai population [24], and with vascular access dysfunction in Korean hemodialysis patients[25]. The *GC* genotype frequency in Japanese population[15] as well as the ethnic variation of the *IL-6 (-174)G>C* polymorphism in the Malaysian population [26] were also investigated.

The frequency of *IL-6 (-174)G>C* in Filipinos needs to be explored. This study aimed to find evidence on the association of *IL-6 (-174)G>C* polymorphism to glycemic status and plasma CRP levels in pre-diabetes and type 2 Diabetes mellitus in a Filipino population.

Materials and Methods

Subjects

The subjects were Clinical Laboratory out-patients of the University of Perpetual Help System Dalta Medical Center who are natural-born Filipinos, males and females, ages 18 to 70 yrs.old, The study was approved by the University of Perpetual Help System Dalta – University of Perpetual Help System Jonelta Institutional Ethics Review Board . Informed consents from the subjects were obtained.

Fasting Plasma Glucose (FPG) tests were done on applicants. American Diabetes Association (ADA) reference ranges for diagnosis of diabetes were used as basis of classification of subjects to Normoglycemic, Pre-diabetic and Diabetic groups. The diabetic subjects used for this study were previously-diagnosed diabetics on self-report. Diabetics who had been prescribed with insulin under age 35 were excluded in the study [21]. Previous diagnoses of atherosclerosis, cancer, heart disease, hypertension, lupus erythematosus, multiple sclerosis, psoriasis, and rheumatoid arthritis were part of the exclusion criteria.

From 320 individuals that were screened, a study group composed of 96 type 2 diabetics, 84 pre-diabetics and 53 normoglycemics were used as subjects . The sample size was determined using the formula for the estimation of population proportion. It was computed as $n = [Z^2 (2PQ)] / d^2$, where P = estimate of true proportion, based on past experience or knowledge[22,23]: Q = 1 – P: Z = the normal deviate corresponding to reliability level desired for estimate = 1.64 at 90% reliability; d = maximum permissible error for the difference between estimate and actual proportion = 10%.

Specimen Collection

Venipunctures were done on patients who have undergone at least 8-hour fasting. Evacuated tubes containing EDTA were used and proper mixing of blood and anti-coagulant was done. A portion of the blood specimen was taken and centrifuged immediately. A portion

of the plasma was taken for Fasting Plasma Glucose determination and the remaining plasma was stored at -20^{0}C and thawed before the CRP test was performed. The remaining portion of anti-coagulated whole blood was stored at -20^{0}C until genotyping was done.

Fasting Plasma Glucose Tests

Fasting Glucose tests were performed using plasma samples of the participants in the Abbott Architect ci4100 using the Hexokinase/G-6-PDH Abbott Reagents.

Plasma CRP Tests

Plasma C-Reactive Protein tests were done in the samples using the Quantitative Immunoturbidimetric CRP Vario Kit (CRP 16-Ultrasensitive) in an Abbott Architect ci4100 machine. The measuring range was 0.01 to 16 mg/dL (0.1 to 160 mg/L).

IL-6 (-174)G>C Genotyping

Two hundred microliter (200 µL) whole blood was used for isolation of genomic DNA using Roche High Pure PCR Template Preparation Kit. Genotyping for SNP *IL-6 (-174)G>C* was carried out by Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR- RFLP) on blood samples from normoglycemics, pre-diabetics and diabetic subjects. A 198 bp fragment of the IL-6 gene was amplified using forward primer 5'-TGACTTCAGCTTTACTCTTTGT-3' and reverse primer 5'-CTGATTGGAAACCTTATTAGG-3' [13]. DNA was denatured for 9 mins. at 94^{0}C, then subjected to 35 amplification cycles. Each of the PCR cycle consisted of denaturation for 60 sec. at 94^{0}C, annealing for 95 secs at 55^{0}C and extension for 60 secs. at 72^{0}C, followed by final extension at 72^{0}C for 9 mins [13]. The PCR products were digested for 2 hours with 2 U/µL Sfa NI at 37^{0}C and electrophoresis was done using 2.5% agarose with 5 µL ethidium bromide and was carried out for 30 minutes. Visualization was done using ultraviolet light. Presence of a single 198 bp band corresponds to the *CC* genotype; bands at 140 bp and 58 bp correspond to *GG* genotype; and the presence of bands at 198 bp, 140 bp and 58 bp corresponds to the heterozygous *GC* genotype [13].

Statistical Analyses

For the profile of subjects, measures of central tendency (mean) and variation (standard deviation and range) were used for quantitative variables while frequency distribution were used for categorical variables.

Mean, SD and range of blood glucose levels were computed for the study population. Frequency distribution of normoglycemics, pre-diabetics and diabetics were determined. Mean, Standard Deviation and range of blood glucose levels were computed per glycemic status. Mean, Standard Deviation and range of plasma CRP level per glycemic status category were determined.

Association of *IL-6 (-174)G>C* with glycemia and plasma CRP in normoglycemics, pre-diabetic and diabetics were determined from data on allele frequency and glycemic level and plasma CRP per glycemic status. Genotype frequencies were computed using Hardy-Weinberg Equilibrium. Odds Ratios of SNP with CRP levels were computed. Fisher's Exact Test was used to determine the relationship between *IL-6 (-174)* genotype and glycemic levels (with and without SNP). The same method was used to determine the relationship between *IL-6 (-174)* genotype and CRP levels (with and without SNP) per glycemic status.

All inferential analyses were done at 5% level of significance. Stata software version 6.0 was used in the analysis.

Results

Demographic Profile

The demographic profile of the participants is shown in Table 1. Two hundred and thirty three individuals with results of genetic analysis for *IL-6 (-174)G>C* were included in the analysis. Overall mean age (± standard deviation) of the participants was 49.5 ± 10.3 years. Mean age for the diabetic group was numerically higher than both the normal and pre-diabetic groups (53.9 vs. 45.8 and 46.9 years, respectively). Majority of the participants were in the 41 – 60 year age group overall (64%) as well as per FPG status sub-group (60% for normal, 62% for pre-diabetics, and 67% for diabetics). Majority of the participants were female overall (55%) as well as among normal (66%) and pre-diabetics (55%), whereas gender distribution was even among diabetics (50% female and 50% male).

Table 1. Demographic profile of participants

Characteristic	Normal (n = 53)	Pre-diabetic (n = 84)	Diabetic (n = 96)	Total (n = 233)
Age (Years)				
<41	17 (32)	21 (25)	7 (7)	45 (19)
41 – 60	32 (60)	52 (62)	64 (67)	148 (64)
>60	4 (8)	11 (13)	25 (26)	40 (17)
Mean ± SD[1]	45.8 ± 11.2	46.9 ± 10.2	53.9 ± 8.2	49.5 ± 10.3
Range	23 - 68	23 - 68	35 - 70	23 - 70
Gender				
Male	18 (34)	38 (45)	48 (50)	104 (45)
Female	35 (66)	46 (55)	48 (50)	129 (55)

[1] *SD – standard deviation*

Fasting Plasma Glucose and C-Reactive Protein profile

The Fasting Plasma Glucose (FPG) of the participants is presented in Figure 1. As expected, mean FPG was numerically highest in the diabetic group (179 ± 54.1 mg/dL) followed by the pre-diabetic group (107.6 ± 6.0 mg/dL) and lowest among the normal group (87.0 ± 7.1 mg/dL). Figure 2 shows the C-Reactive Protein (CRP) profile of the participants. In Figure 2-A, the mean CRP value was numerically higher among diabetics (0.51 ± 1.2) compared to the pre-diabetics (0.24 ± 0.50) and normal group (0.23 ± 0.35). Figure 2-B shows that eighteen (18) out of 96 diabetics, seven (7) out of 84 pre-diabetics and four (4) out of 53 non-diabetics exhibited elevated CRP values (0.51 – ≥1.0 mg/dL). There is numerically a greater proportion of elevated CRP among diabetics (18%) compared to pre-diabetics and normal group (both 8%).

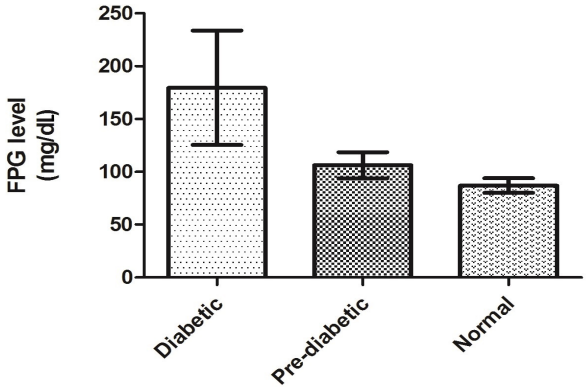
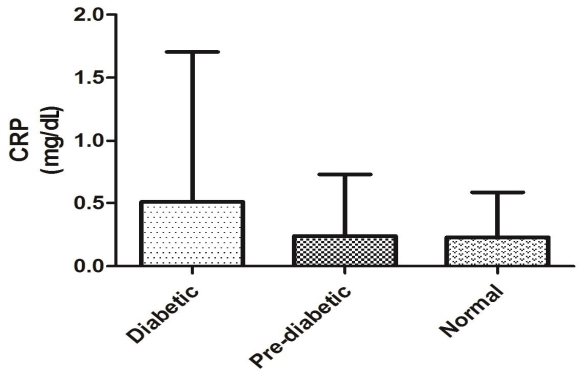


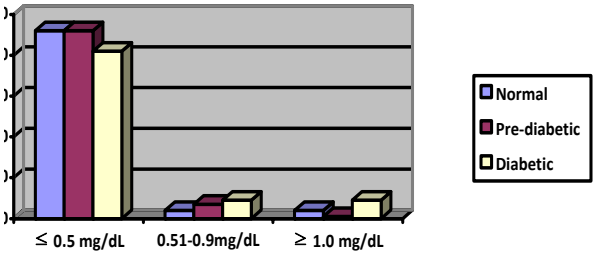
Fig.1 Fasting plasma glucose profile of participants - (Mean FPG values in mg/dL per Glycemic Status)

Genotype and Allele frequency

Of the 233 participants, 221 individuals demonstrated the *GG* genotype, 12 individuals demonstrated the *GC* genotype and no individual exhibited the *CC* genotype (Table 2-A). These findings showed 95%, 5% and 0% for the *GG, GC* and *CC* genotypes, respectively. Illustrations samples of the agarose gel electrophoresis are shown in Figs. 3-A and 3-B.,



2-A CRP values per glycemic status in mg/dL



2-B Percentage of Participants with CRP Levels per glycemic status

Fig.2 – CRP profile of participants by Fasting Plasma Glucose Status.

Table 2 IL-6 (–174) Genotype and Allele Frequencies

2-A Genotype and allele frequency among participants by Fasting Plasma Glucose Status

	Genotypes			Alleles	
	GG	***GC***	***CC***	***G***	***C***
Normal (n=53)	53 (100)	0 (0)	0 (0)	106 (100)	0 (0)
Pre-diabetic (n=84)	84 (100)	0 (0)	0 (0)	168 (100)	0 (0)
Diabetic (n=96)	84 (88)	12 (12)	0 (0)	180 (93.8)	12 (6.2)
Total (n=233)	221 (95)	12 (5)	0 (0)	454 (97.4)	12 (2.6)

2-B Genotype and allele frequency among participants by C-Reactive Protein Levels and Fasting Plasma Glucose Status

CRP Level/ FPG	Genotypes			Alleles	
	GG	***GC***	***CC***	***G***	***C***
CRP ≤ 0.5					
Normal FPG	49	0	0	98	0
Pre-Diabetic	77	0	0	154	0
Diabetic	70	8	0	148	8
Total	196 (96)	8 (4)	0 (0)	400 (98)	8 (2)
CRP 0.51-0.9					
Normal FPG	2	0	0	4	0
Pre-Diabetic	6	0	0	12	0
Diabetic	8	1	0	17	1
Total	16 (94)	1 (6)	0 (0)	33 (97.1)	1 (2.9)
CRP ≥ 1.0					
Normal FPG	2	0	0	4	0
Pre-Diabetic	1	0	0	2	0
Diabetic	6	3	0	15	3
Total	9 (75)	3 (25)	0 (0)	21 (87.5)	3 (12.5)
Grand Total	221 (95)	12 (5)	0 (0)	454 (97.4)	12 (2.6)

All (100%) of the alleles for normal and pre-diabetics consist of *G* alleles. Out of 96 diabetic subjects, 84 were homozygous for the *G* allele while 12 had single nucleotide polymorphism (SNP) or were heterozygous. For these diabetic participants, 93.8% are *G* alleles which comprise 97.4% of total alleles while *C* alleles are 6.2% of diabetics and 2.6% of total alleles.

The single nucleotide polymorphism (SNP) or the heterozygote condition *GC* was only found among the diabetics. Of the diabetics with normal CRP level (≤0.5 mg/dL), 10% have SNP corresponding to a *C* allele frequency of 5.1%. For those with elevated CRP (5.1 – 0.9 mg/dL), 11% have SNP corresponding to a *C* allele frequency of 5.6%. And among those who have highly elevated CRP (≥1.0 mg/dL), 33% have SNP corresponding to a *C* allele frequency of 16.7% (Table 2-B) .

Association of *IL-6 (-174)G>C* with CRP and FPG

Table 3 presents the determination of association between *IL-6 (-174)G>C* and CRP status and Fasting Plasma Glucose. There is no adequate evidence of association between SNP status and CRP status among those with normal vs. elevated CRP of 0.51 – 0.9 mg/dL (Odds ratio = 1.53, p-value = 0.696) and among those with elevated 0.51 – 0.9 mg/dL vs. highly elevated CRP of ≥1.0 mg/dL (Odds ratio = 5.33, p-value = 0.173). There is, however, around 8 times greater likelihood of having SNP among those with highly elevated CRP (≥1.0 mg/dL) compared to those with normal CRP (Odds

ratio = 8.17, p-value = 0.006).

For the association of SNP and Fasting Plasma Glucose, Odds ratios were not computed since the *GC* phenotype was only found in the diabetics and none in the normal or pre-diabetic participants. The p-value computation for normal vs. pre-diabetics was also not applicable because of the same reason. The statistical determination, however, for the association of SNP with FPG among diabetics vs. pre-diabetics showed that there is a greater proportion of SNP *IL-6 -174)G>C* among diabetics compared to pre-diabetics (12% vs. 0%, Fisher's exact p-value = <0.001). There is also a greater proportion of SNP *IL-6 (-174)G>C* among diabetics compared to participants with normal FPG (12% vs. 0%, Fisher's exact p-value = 0.005).

Fig. 3-A GC = bands at 198 bp, 140 bp and 58 bp; GG = bands at 140 bp and 58 bp, CC = band at 198 bp GC is seen in this figure in Sample 10; GG is demonstrated by Samples 3, 4, 6, 7, 8, 9, 11, 13, 16, 17, 18, 19, 20. Samples 2, 5, 12, 14, 15 were repeated in subsequent run and turned out to be GG genotype.

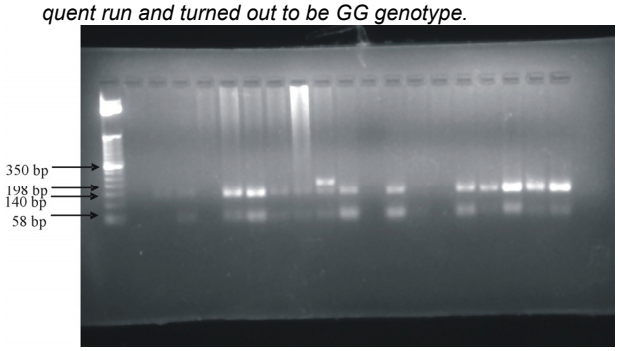


Fig. 3-B DNA ladder at Well 1 and Well 20. All samples are of GG genotype

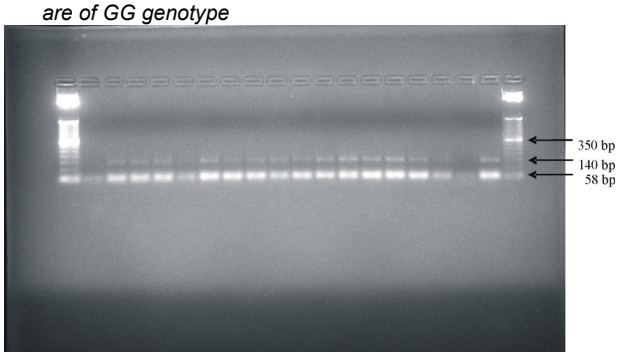


Fig.3 Agarose Gel Electrophoresis Results

Table 3 - Association of *IL-6 (174)G>C* with C-reactive protein (CRP) status and Fasting Plasma Glucose Status

CRP Status	W/out SNP	With SNP	Odds Ratio	p-value
Normal (≤ 0.5) (n=204)	196 (96)	8 (4)		
Elevated (0.51-0.9) (n= 17)	16 (94)	1 (6)	1.53	0.696
Elevated (n = 17)	16 (94)	1 (6)		
Highly elevated (≥ 1.0) (n = 12)	9 (75)	3 (25)	5.33	0.173
Normal (n=204)	196 (96)	8 (4)		
Highly elevated (n = 12)	9 (75)	3 (35)	8.17	0.006
FPG Status	Without SNP	With SNP		p-value
Pre-diabetic (n = 84)	84 (100)	0		
Diabetic (n = 96)	84 (88)	12 (12)		<0.001
Normal (n = 53)	53 (100)	0		
Diabetic (n = 96)	84 (88)	12 (12)		0.005

Discussion

Results presented herein showed that of the two hundred thirty-three (233) subjects in this Filipino population, only twelve (12) individuals were positive for *GC* genotype. Two hundred twenty-one (221) individuals were positive for *GG* genotype and no individual (0) was positive for the *CC* genotype. These findings showed 95%, 5% and 0% frequencies for the *GG, GC* and *CC* genotypes, respectively. These figures are consistent with the IL-6 (–174) frequencies of other Asian populations. It has been reported that in the study of inflammatory markers in 232 Han Chinese, only one (1) individual carried the *GC* genotype[30]. Investigation done on 259 Southern Chinese coal miners showed one (1) *GC* genotype and no *CC* genotype[31]. Researchers reported that the *C* allele was not found in 80 Korean patients who had undergone hemodialysis. Findings of a study on the ethnic variation in *IL-6 (-174)G>C* polymorphism in the Malaysian population showed 4%, 19% and 0% *C* allele frequencies in Malays, Indians and Chinese ethnic groups, respectively[26]. Previous analyses that showed *GC* genotype frequencies of 0.2% for Eastern Asians, 0.0% for Japanese, 0.6% for Koreans and 0.2% for Southern Chinese were also cited[25]. In a study of osteoarthritis on Thai subjects, IL-6 (-174) polymorphism was found to be 77%, 23%, 0% for *GG*, *GC* and *CC*, respectively[24]. It appears that on the reported studies on Asian populations, the *CC* genotype was not found and the *GC* genotype ranges from 0% to 23%.

The result of our IL-6 (-174) genotyping as well as the reports from other Asian populations differ from the genotype frequencies of non-Asian population groups. For a Finnish study population, genotyping yielded a report of 26%, 44% and 26% for *GG*, *GC* and *CC* genotypes, respectively[19]. Investigation on European patients showed 37% for *GG*, 53% for *GC* and 10% for *CC* genotypes[16]. Frequency data of the *-174G* allele was 77% for the southern regions of Italy and 58-59% for Germany[15]. Results of our study confirmed earlier observations of the geographic allele and genotype variations of the -174 polymorphism in the IL-6 gene[15,17,25].

Findings of several studies in Caucasian subjects describe the *IL-6 (-174)* polymorphism as an influencing factor to CRP levels and the *C* allele associated with elevated concentrations of circulating CRP [7,12,32]This study showed evaluations with different ranges in CRP levels. It has taken into consideration the classical reference of 0.5 or 1.0 mg/dL as well as recent levels that refer to higher values in type 2 diabetes patients[6,35]. Our results showed no adequate evidence of association between *IL-6 (-174)G>C* and plasma CRP levels that range from 0.51 mg/dL to 0.9 mg/dL. Significant association of *IL- 6 (-174)G>C* with plasma CRP level of 1.0 mg/dL and above was demonstrated with Fisher's exact p value = 0.006. Statistical evaluation of the data in this study indicates association of the *GC* genotype to highly elevated CRP levels.

The results of our study showed presence of the *GC* genotype in diabetic patients only. The pre-diabetic and normoglycemic subjects did not exhibit the *GC* genotype. It was stated that DNA polymorphism is a useful marker to analyze disorders with genetic background even though the genetic cause of the disease has not been elucidated[33]. A number of DNA polymorphisms have been used to examine their possible linkage with a hereditary predisposition to common polygenic disorders such as diabetes mellitus[34] and it has been confirmed that individual polymorphisms were predisposing to type 2 diabetes[35]. It has been indicated in a recent observation that the level of pathogen prevalence is one of the environmental factors that determine different adaptive values of the *IL-6 (–174)G>C* allele[15]. The Philippines has a high incidence of diabetes mellitus and the data obtained in this investigation support previous observations. The present study shows significant association between the *IL-6 (-174)G>C* polymorphism and glycemia in type 2 diabetes mellitus. This relationship has been observed in a Filipino population and reflects a relationship that might not be present in any other population.

Conclusion:

IL-6 (-174)G>C polymorphism shows no association with plasma CRP levels in the range of 0.51 mg/dL to 0.9 mg/dL and significant association with plasma CRP level of 1.0 mg/dL and above. There is significant association between the *IL-6 (-174)G>C* polymorphism and glycemic status in type 2 diabetes mellitus. The presence of the *GC* genotype in diabetics and absence in pre-diabetics and normoglycemics in the Filipino population might be interesting to pursue.

Acknowledgements:

University of Perpetual Help Systems Dalta Medical Center Abbott Diagnostics Philippines for supporting the Glucose and CRP testing. Philippine Association of

Medical Technologists,Inc.

Jennifer Maries G.Yap of the Research Center for Natural and Applied Sciences, University of Santo Tomas, for sharing her expertise and valuable time in the genotyping procedures.

Friends Cecile Quimno and Leila Armonia and colleagues Perla Tan, Luella Vertucio and Ronaldo Puno for moral and technical support.

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