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T Allele of *FOXO3 rs2802292* Increases CCL2 Concentration and Slightly Decreases TGF-β Concentration in Indonesian Elderly

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Background: Cellular senescence and the senescence-associated secretory phenotype (SASP) are pivotal factors influencing aging and age-related diseases. SASP secretes cytokines, chemokines, metalloproteinases, and growth factors that cause chronic inflammation. C-C ligand 2 (CCL2) and transforming growth factor-beta (TGF-β) are SASP markers secreted by senescent cells. This study investigated the relationship between the *FOXO3* variant *rs2802292* and SASP markers, focusing on CCL2 and TGF-β.

Materials and methods: A cross-sectional study involving 72 elderly individuals from Jakarta was conducted. A sandwich enzyme-linked immunosorbent assay (ELISA) was used to quantify CCL2 and TGF-β concentrations. Random blood glucose, blood pressure, and *FOXO3 rs2802292* genotyping data were obtained from a previous study. Differences in CCL2 and TGF-β concentrations between genotype groups were analyzed using one-way ANOVA and the Kruskal-Wallis test. Meanwhile, differences in CCL2 and TGF-β concentrations between allele groups were analyzed using the Mann-Whitney test.

Results: The CCL2 and TGF-β concentrations of the subjects were 66.5 (10.58-190.9) pg/mL and 6,319 (2,379-13,846) pg/mL, respectively. There were significant differences in CCL2 concentrations among the *FOXO3 rs2802292* genotypes (p=0.041). However, there were no significant differences in TGF-β concentrations among *FOXO3 rs2802292* genotypes (p=0.955). Subjects with the G allele had significantly lower CCL2 concentrations compared with those with the T allele (p=0.033). TGF-β concentrations did not significantly differ between G and T alleles (p=0.771).

Conclusion: CCL2 concentrations are associated with the *FOXO3* variant *rs2802292* in the elderly population. The T allele of *FOXO3 rs2802292* increased CCL2 concentration and slightly decreased TGF-β concentration in elderly individuals.

Keywords: *aging, SASP, CCL2, TGF-β, SNP, FOXO3, rs2802292*

Introduction

Human aging and longevity are influenced by several major factors, such as genetics, environment, and lifestyle.[1] At the cellular level, aging occurs due to the accumulation of senescent cells. Cellular senescence refers to a permanent halt in the cell cycle, accompanied by a pro-inflammatory phenotype known as the senescence-associated secretory

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phenotype (SASP), and their accumulation in aged tissues causes tissue damage.[2,3] The secretory component of SASP consists of cytokines, chemokines, metalloproteinases, and growth factors, most of which are inflammatory factors.[4,5]

Among the SASP components, C-C motif ligand 2 (CCL2), also known as monocyte chemoattractant protein-1 (MCP-1), has emerged as a key player in age-related inflammation and disease progression.[6] In general, senescent cells tend to overexpress CCL, including CCL2.[7] Many studies have found an association between CCL2 and aging. Chronic inflammation, marked by CCL2, can promote secondary aging in healthy cells.[6] Inflammatory indicators like CCL2 are linked to age-related diseases.[8] A study has shown that plasma proteome analysis of elderly mice reveals elevated aging-related components, including CCL2. Immune modulation and inflammation are mediated by CCL2. It exhibits chemotactic activity for monocytes and basophils.[9] Chemokines modulates cell infiltration and migration, which is critical for non-specific and adaptive immunity, inflammation, tissue regeneration, and central nervous system function. The major CCL2 receptor is CCR2, which is expressed in many cell types and organs.[10]

Transforming growth factor-beta (TGF-β) is a growth factor that has anti-inflammatory properties and exerts various effects on hematopoietic cells. TGF-β has been demonstrated to be involved in the regulation of inflammation, immune system maintenance, and homeostatic responses.[11] Regulatory T cells are cells that secrete TGF-β as a response to maintain peripheral tolerance and suppress the adaptive immune response.[12] Multiple studies have demonstrated a substantial rise in the concentration of TGF-β in the plasma of elderly adults compared to younger individuals. The TGF-β signaling pathway plays a role in the mechanisms underlying various diseases associated with aging.[13] Modulation of the downstream signaling target of TGF-β has a role in multiple facets of aging, including cellular proliferation, regulation of the cell cycle, formation of reactive oxygen species (ROS), DNA damage repair, regulation of telomeres, activation of oncogenes, and autophagy.[14] SASP induces the synthesis and release of numerous signaling molecules, including TGF-β. TGF-β can both initiate and sustain aging characteristics and age-related diseases either through direct cell-to-cell communication or by acting on neighboring cells.[13] A study found that the integrin β3 (ITGB3) has a role in regulating the aging process. Specifically, it enhances aging by activating the TGF-β signal in an autocrine or paracrine manner and promotes SASP in human fibroblasts. The study also demonstrated a direct relationship between ITGB3 expression rate and components of the TGF-β signaling pathway, including TGF-β receptor (TβR)I, TβRII, Smad3, and Smad4, as well as the aging process.[15]

The human *FOXO3* gene encodes the forkhead box transcription factor O3. *FOXO3* is a regulatory gene involved in the insulin-like growth factor-1 (IGF-1) signaling system, which has been initially linked to human lifespan.[16] *FOXO3* activity modulates responses to DNA damage, oxidative stress, calorie restriction, and nutritional deprivation. Studies reveal that *FOXO3* plays a vital role in regulating various cellular processes, including cell cycle progression, cell homeostasis, DNA repair, ROS detoxification, and apoptosis.[17] The *rs2802292* variant, found in intron 2 of *FOXO3*, has been linked to longevity and is associated with healthy biomarkers and a lower incidence of age-related diseases.[18] The G allele at single nucleotide polymorphism (SNP) *rs2802292* creates a heat shock factor-1 (HSF-1) binding DNA site. Activation of this enhancer area upregulates *FOXO3*. Stress-induced expression of *FOXO3* and HSF-1 stimulates the transcription of antioxidant and DNA repair genes (Figure 1).[19] The longevity-associated G allele protects against coronary artery disease by reducing chronic cardiometabolic adverse effects on intracellular processes, thereby lowering cardiovascular risk.[20]

In previous research, *FOXO3 rs2802292* has been shown to correlate with the longevity of elderly subjects in Jakarta, but there was no relationship between *FOXO3 rs2802292* and cytokines (interleukin (IL)-1α, IL-6, IL-8, dan IL-10) as SASP parameters.[21] Given that SASP parameters include chemokines (e.g., CCL2) and growth factors (e.g., TGF-β), there is a possibility of a correlation between *FOXO3 rs2802292* and these factors. Therefore, this study was conducted to analyze the expression of CCL2 and TGF-β in elderly individuals residing in Jakarta, serving as markers for SASP, based on the *FOXO3* SNP *rs2802292*.

Materials and methods

Study Design, Subjects, and Data Collection

This cross-sectional research study was conducted on 72 elderly individuals from Jakarta, specifically from three distinct sub-districts: Cilandak, Kebayoran Baru, and Pesanggrahan. The 72 subjects were included from our previous study involving 92 subjects.[21] The 72 subjects were included due to the availability of sufficient plasma samples

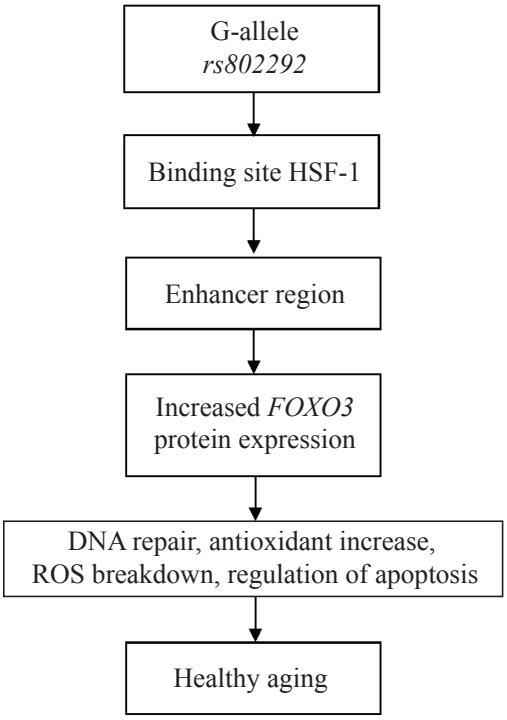

Figure 1. The relationship between the G allele of *FOXO3 rs2802292* and healthy aging.

for analysis using the enzyme-linked immunosorbent assay (ELISA) method; only these samples had adequate volume remaining.

The inclusion criteria for this study were individuals of both genders, aged 60 years and above, who expressed their willingness to participate in the research and provided written informed consent. Subjects that were excluded from this study were those who smoked, consumed alcohol within the past year, or had acute infection. Smoking and alcohol consumption were excluded because they can stimulate inflammation and adversely affect the health of the elderly, leading to the issues such as smoking-related lung disturbances from smoking and alcohol-related fatty liver.

Random blood glucose, blood pressure, and *FOXO3 rs2802292* genotyping data were obtained from the previous study.[21] Random blood glucose levels were categorized into two groups based on the National Medical Guidelines for the Management of Type 2 Diabetes Mellitus in 2020. The normal group (random blood glucose <200 mg/dL) and the high blood glucose group (random blood glucose >200 mg/dL).[22] Blood pressure was categorized into two groups, normal and high blood pressure (systole >140 mmHg or diastole >90 mmHg), based on National Guidelines for Medical Service in the Management of Adult Hypertension in 2021.[23] Sanger sequencing was used for *FOXO3 rs2802292* genotyping purpose, and the methods was adjusted according to the number of plasma samples. The Ethics Committee of Faculty of Medicine, Universitas Indonesia has granted ethical clearance with the reference number: KET-100/UN.2F1/ETIK/PPM.00.022/2023.

Enzyme-linked immunosorbent Assay (ELISA) of CCL2 and TGF-β

Sandwich ELISA method was used to measure plasma CCL2 and TGF-β concentrations. Plasma samples were collected from elderly subjects in the previous study. The samples were stored at -20˚C until laboratory analysis was performed.[21] Plasma CCL2 concentration was measured using Human MCP-1 (Monocyte Chemotactic Protein 1) ELISA kit, (Cat. No: EH0222, Fine Test, Hubei, China. Standard concentration range of the kit was 15.625-1,000 pg/mL. Plasma TGF-β concentration was measured using Human TGF-β1 (Transforming Growth Factor Beta 1) ELISA Kit, (Cat. No: EH0287, Fine Test, Hubei, China. Standard concentration range 31.25-2,000 pg/mL. When measuring the TGF beta concentration, the sample is diluted fifty times.

Statistical Analysis

Data was analyzed using SPSS 26 (IBM, Armonk, NY, USA). Data normality was assessed with Kolmogorov-Smirnov test. The descriptive analysis and measured parameters were shown as the mean±standard deviation (SD) for normally distributed data and as the median (minimum-maximum) for not normally distributed data. Differences of CCL2 and TGF-β concentrations between genotype groups were analyzed using one-way ANOVA and Kruksal-Wallis test. Meanwhile, differences of CCL2 and TGF-β concentrations between allele groups were analyzed using Mann-Whitney test. A significant result was set $p<0.05$.

Results

Characteristics of the Subject

The subjects in this study consisted of 72 elderly with an average age of 70.5±6 years (Table 1). The male cohort consisted of 17 individuals, accounting for 23.6% of the total sample. Conversely, the female cohort comprised 55 individuals, representing 76.4% of the total sample

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Table 1. Characteristic of the subject.

Characteristic	Value
Age (years old)	70.5±6
Gender	
Man	17
Woman	55
Systolic blood pressure (mmHg)	130 (100-170)
Diastolic blood pressure (mmHg)	75 (50-110)
Random blood glucose level (mg/dL)	112.5 (85-333)

regarding health indicators, 54.2% of subjects had normal blood pressure, while 45.8% of subjects showed signs of hypertension. In addition, 90.3% of subjects had normal blood glucose levels, while 9.7% experienced elevated levels.

CCL2 and TGF-β Concentration

CCL2 and TGF-β concentrations were measured using the sandwich ELISA method. The CCL2 concentration of the subjects in this study was 66.5 (10.58-190.9) pg/mL. While, the TGF-β concentration was 6,319 (2,379-13,846) pg/mL.

Distribution of FOXO3 rs2802292

The frequency distribution of the *FOXO3 rs2802292* genotype was as follows: GG in 14 subjects (19.4%), GT in 29 subjects (40.3%), and TT in 29 subjects (40.3%). The frequency distribution of the *FOXO3 rs2802292* alleles was 57 (39.6%) for the G allele and 87 (60.4%) for the T allele.[21]

CCL2 and T GF-β Concentrations Based on FOXO3 SNP rs2802292

One-way ANOVA results showed significant differences in CCL2 concentration among *FOXO3 rs2802292* genotype groups (Table 2, Figure 2). Based on the Kruskal-Wallis test, there was no significant difference in TGF-β concentrations among *FOXO3 rs2802292* genotype groups. Based on the Bonferroni's post hoc test, subjects with the GG genotype had a significantly lower CCL2 concentrations compared with those with the TT genotypes (p=0.042). However, there was no significant difference in the CCL2 concentration between the GG and GT genotype groups (p=0.645), and between the GT and TT genotypes groups (p=0.363). Although there were no significant differences in TGF-β concentration between genotype groups, subjects with the GG genotype had higher TGF-β concentrations compared with those with the GT and TT genotypes.

Based on Mann-Whitney test, there was a difference in CCL2 concentration between allele groups (Table 3, Figure 3); subjects with the G allele had significantly lower CCL2 concentrations compared with those with the T allele. However, TGF-β concentrations did not significantly differ between allele groups.

Discussion

FOXO3, a transcription factor on chromosome 6 arm q21 (Ch6q21), is implicated in cell proliferation, apoptosis, oxidative stress response, cancer, cell cycle regulation, metabolism, and longevity.[24] The *rs2802292* (G>T) variant is located in the intronic part of the *FOXO3*.[25] Previous research found the *FOXO3 rs2802292* genotype frequency distribution to be 17.4% GG, 42.4% GT, and 40.2% TT. This study found no significant differences in the concentration

Table 2. Concentration of CCL2 and TGF-β based on genotype *FOXO3 rs2802292.*

FOXO3 rs2802292 Genotype	n (%)	CCL2 (pg/mL)	TGF-β (pg/mL)
GG	14 (19.4)	52.5 ± 25.5	7,022 (2,379-13,846)
GT	29 (40.3)	66.9 ± 26.5	6,251 (4,188-12,979)
TT	29 (40.3)	81.4 ± 45.5	6,694 (3,818-10,534)

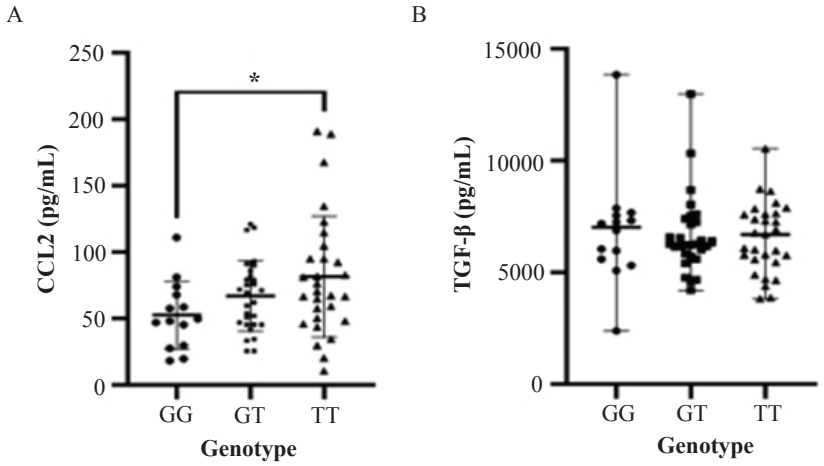

Figure 2. A: Distribution of CCL2 concentration based on *FOXO3 rs2802292*, difference in CCL2 concentrations between genotype groups were analyzed using the one-way ANOVA ($p<0.05$) and Bonferroni post hoc test. B: Distribution of TGF-β concentration based on *FOXO3 rs2802292* genotypes, differences in TGF-β concentrations between genotype groups were analyzed using Kruskal-Wallis test ($p>0.05$).

of SASP markers (IL-1α, IL-6, IL-8, and IL-10) among the *FOXO3 rs2802292* SNP genotype groups.[21] The present study focused on the concentration of CCL2 and TGF-β as SASP markers based on the *FOXO3* SNP *rs2802292*. CCL2 is one of the SASP markers associated with the occurrence of age-related diseases, while TGF-β is a growth factor that plays a role in aging.

SASP is a complex secretome produced by cells that have degenerated into a senescent state undergoing characteristic changes including transcriptional, epigenetic, morphological, and metabolic alterations.[26] The present study revealed a significant difference in CCL2 concentration across *FOXO3 rs2802292* genotype groups ($p<0.05$). The *FOXO3* SNP *rs2802292* may indirectly reduce pro-inflammatory cytokines. The observed differences in CCL2 concentrations among different genotype groups highlight the importance of genetic factors in shaping the inflammatory milieu within aging populations. This suggests that while *FOXO3* may play a role in modulating certain aspects of the SASP, its influence on other components of the phenotype, such as TGF-β, maybe more nuanced or require further investigation.

A study examined the impact of *FOXO3 rs2802292* on SASP parameters. The study found that older female individuals who carry the G allele showed a slight decrease in levels of the pro-inflammatory cytokine IL-6 as they aged, compared with those carrying the T allele.[27] This is consistent with the findings of our investigation, which demonstrated that individuals carrying the G allele had lower levels of the pro-inflammatory CCL2 than individuals carrying the T allele.

The immune system often eliminates cells that undergo damage as a result of aging. Senescent cells produce specific molecules called SASP factors, which include cytokines and chemokines capable of affecting the local immunological environment. In this particular scenario, SASP has been observed to stimulate inflammation.[28] Senescent cells are subjected to immune surveillance. Senescent cells exhibit autophagy, a process in which they eliminate themselves by releasing CCL2, a chemokine that attracts and stimulates natural killer (NK) cells.[29] NK cells play a role in the immune surveillance of senescent cells during tissue repair. Senescent cells can attract them by releasing CCL2, which is dependent on p53.[30] High circulating levels of CCL2

Table 3. The concentration of CCL2 and TGF-β based on *FOXO3 rs2802292* allele.

FOXO3 rs2802292 Allele	n (%)	CCL2 (pg/mL)	TGF-β (pg/mL)
G	57 (39.5)	48.8 (18.22-110.84)	7,022 (2,379-13,846)
T	87 (60.5)	70.0 (10.58-190.96)	6,251 (4,188-12,979)

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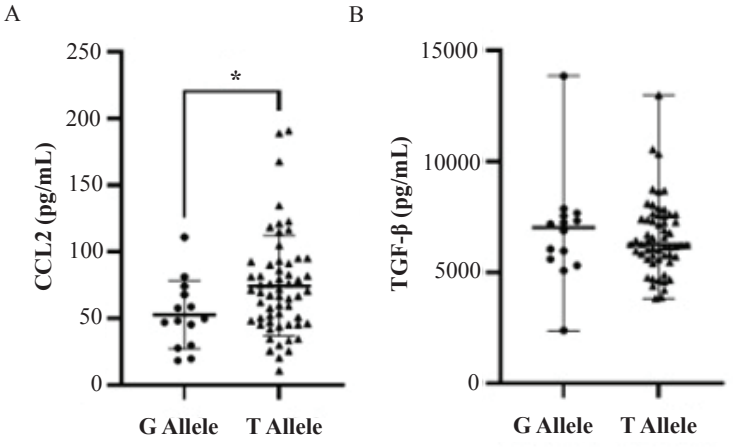

Figure 3. A: Distribution of CCL2 concentration based on *FOXO3 rs2802292* alleles, differences in CCL2 concentrations between allele groups was analyzed using Mann-Whitney test ($p<0.05$). B: Distribution of TGF-β concentration based on *FOXO3 rs2802292* alleles, differences in TGF-β concentrations between allele groups was analyzed using Mann-Whitney test ($p>0.05$).

frequently occur in diseases associated with tissue damage and have also been shown to correlate with age. This association suggests an increased burden of senescent cells that could be a potential marker for measuring the impact of interventions aimed at prolonging healthy aging.[6] In the present study, subjects with the GG genotype exhibited lower concentrations and reduced inflammatory conditions in the body, suggesting a potential for healthier aging among these subjects. Understanding how genetic variants like *rs2802292* influence the expression of inflammatory cytokines such as CCL2 could provide valuable insights into the underlying mechanisms underlying age-related pathologies and guide the development of targeted therapeutic interventions. However, it is noteworthy that no direct impact on TGF-β concentration was observed on the *FOXO3 rs2802292* genotype in this study.

The levels and activity of the *FOXO3* protein may influence the mechanisms associated the longevity of *FOXO3* SNPs. These mechanisms include inflammation, autophagy, glycolysis, cell cycle arrest, oxidative stress, and DNA damage repair. *FOXO3* preserves homeostasis and fights aging by improving age-related disorders. Increased levels of *FOXO3* protein promote processes that contribute to the maintenance of healthy aging.[31] By managing stress responses in response to oxidative stress, hypoxia, and DNA damage, *FOXO3* can regulate cellular homeostasis, immune response, and longevity. Depending on the stress stimulus and the subcellular location, *FOXO3* can activate a variety of genes, including those that inhibit the cell cycle, promote apoptosis, enhance autophagy, and regulate gluconeogenic enzymes.[32] This may contribute to healthier aging and longer lifespan. The G allele of SNP *rs2802292* creates a binding site for HSF-1, which in turn promotes *FOXO3* expression and stress response by enhancing the regulation of specific target genes, such as superoxide dismutase 2 (*SOD2*), catalase (*CAT*), growth arrest and DNA-damage-inducible 45 alpha (*GADD45A*), heat shock protein family A member 1A (*HSPA1A*).[33] The presence of the G allele at *rs2802292* is associated with elevated *FOXO3* expression, suggesting that the 90 base pairs around this SNP possess enhancer capabilities. Furthermore, the G allele generates a novel HSF-1 binding site that triggers the production of *FOXO3* in response to stress. Functional studies investigate the mechanisms by which the *HSF-1-FOXO3-SOD2*/*CAT*/*GADD45A* cascade enhances the removal of ROS, maintains a balanced redox state, and repairs DNA during cellular stress responses and survival. These findings suggest that there may be a relationship between *HSF-1* and *FOXO3* in human cells, influencing stress response pathways that regulate longevity and disease risk.[19] In addition, besides nuclear factor κB (NF-κB), other transcription factor regulate the expression of SASP. The expression and synthesis of SASP are controlled by multiple pathways, including the p53 pathway, the CCAAT/enhancer binding protein (C/EBP) pathway, and the GATA binding protein 4 (GATA4) pathway.[34] On the other hand, *FOXO3*, as a transcription factor, plays a role in regulating various cellular processes that contribute to healthy aging, including DNA repair, ROS breakdown, and apoptosis regulation (Figure 4). Therefore, further exploration of this pathway and its relationship to the *FOXO3* SNP *rs2802292* could provide valuable insights.

The number of subjects in the present study was 72 subjects; perhaps a larger sample size is needed to analyze the effect of the *FOXO3 rs2802292* on TGF-β expression. Additionally, this study did not include data on ethnicity, diet patterns, lifestyle, or environment of each subject, despite

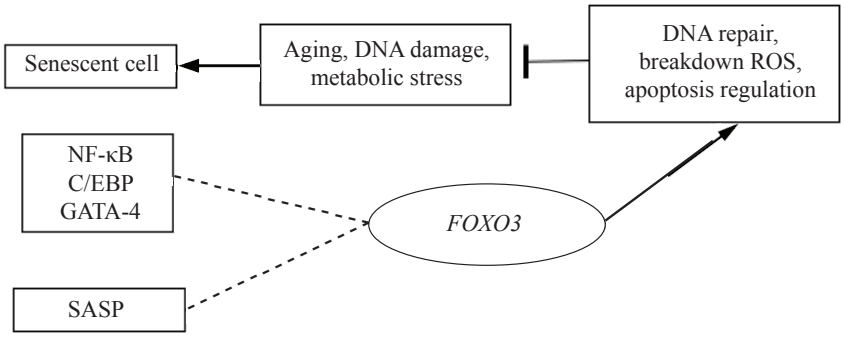

Figure 4. Relationship of *FOXO3* signaling pathway with SASP.

these factors potentially influencing SASP expression. *FOXO3* may exert its effects through various pathways, which can vary depending on age, gender, and other factors influencing the aging process.

The study identified a significant association between *FOXO3 rs2802292* genotype and CCL2 concentration. The lack of direct impact on TGF-β concentration suggests a nuanced regulatory relationship between *FOXO3* and TGF-β signaling pathways in the context of aging. Further research is warranted to elucidate the mechanisms underlying this relationship and its implications for age-related diseases.

Conclusion

CCL2 concentration as a component of SASP in the elderly is associated with *FOXO3* variant *rs2802292* in the elderly population. Individuals carrying the T allele of *FOXO3 rs2802292* have increased CCL2 concentration. Furthermore, the T allele of FOXO3 *rs2802292* slightly decrease TGF-β concentration. Understanding the interplay between genetic factors like FOXO3 and molecular pathways involved in SASP regulation could offer promising avenues for interventions aimed at promoting healthy aging and preventing age-related diseases.

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Authors Contributions

NSH and FCI were involved in conceptualizing and planning the research, WN performed the data acquisition/ collection, FCI, NSH, and WN calculated the experimental data and performed the analysis, WN and FCI drafted the manuscript and designed the figures, FCI, NSH, and WN aided in interpreting the results. FCI, NSH, and WN took parts in providing critical revision of the manuscript.

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