

# Suggestive association between *PLA2G1A* single nucleotide polymorphism rs2285714 and response to anti-vascular endothelial growth factor therapy in patients with exudative age-related macular degeneration

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**Purpose:** The use of anti-vascular endothelial growth factor (anti-VEGF) therapy, with drugs such as ranibizumab and bevacizumab, to treat neovascular age-related macular degeneration (nAMD) produces an effective but widely variable response. Identifying markers that predict differentiated response could serve as a valuable assay in developing more personalized medicine. This study aimed to identify single nucleotide polymorphisms (SNPs) that influence the outcome of treatment with anti-VEGF therapy for AMD.

**Methods:** One hundred six patients with nAMD were treated with either ranibizumab or bevacizumab as needed over a period of 12 months. Visual acuity and the presence of macular fluid were measured with optical coherence tomography at baseline, six months, and 12 months. Patients were then classified as good or poor responders based on change in visual acuity and macular fluid on follow-up visits. DNA extracted from blood was genotyped with a TaqMan-based allelic discrimination SNP assay for 21 SNPs in six candidate genes (*PLA2G1A*, *IL23R*, *STAT3*, *VEGFA*, *KDR*, and *HIF1A*). The SNPs were primarily selected based on previously reported associations with AMD and functional involvement in angiogenesis pathways. SNPs shown to be promising for association with anti-VEGF therapy were then assessed in an independent AMD case-control cohort.

**Results:** Of the 106 patients with nAMD, 77 were classified as good responders and 29 as poor responders. For rs2285714 (*PLA2G1A*), the frequency of minor allele T was 40.1% for good responders compared to 51.7% for poor responders (odds ratio: 1.60, 95% confidence interval of odds ratio: 0.87–2.94,  $p=0.13$ ). Genetic model analysis of rs2285714 (*PLA2G1A*) demonstrated an association between rs2285714 (*PLA2G1A*) and therapy response in a dominant genotypic model. Patients carrying at least one T allele of rs2285714 were 2.79 times (95% confidence interval=1.02–7.69,  $p<0.05$ ) more likely to be poor responders (79.3% of poor responders) than good responders (57.3% of good responders). However, after adjusting for multiple testing by the false discovery rate or Bonferroni correction, the initially observed association was no longer statistically significant. No association was identified between the remaining SNPs and response status. The SNP rs2285714 of *PLA2G1A* was not significantly associated with AMD in an independent AMD case-control cohort.

**Conclusions:** Data suggest a possible weak association between rs2285714 (*PLA2G1A*) and response to anti-VEGF therapy, but the association must be confirmed in additional cohorts with larger patient samples. Identifying factors that predict the differentiated response could provide a valuable assay for developing approaches in personalized medicine.

Age-related macular degeneration (AMD) is the leading cause of central, irreversible visual impairment in the elderly population of developed countries [1,2]. More than 8.5 million individuals over age 55 are affected by some degree of AMD in the United States [2]. Patients with this disease eventually develop either neovascular AMD (nAMD) or geographic atrophic AMD (GA AMD). nAMD is characterized by choroidal

neovascularization, which may ultimately lead to exudative and hemorrhagic changes in the retina, and is responsible for the majority of severe visual loss associated with this disease [3,4]. Although the pathogenesis of nAMD is not completely elucidated, vascular endothelial growth factor (VEGF) is a key contributor to disease progression. Hypoxic environments upregulate VEGF levels, which result in angiogenesis and increased vasopermeability of the microvasculature [5].

To date, the most widely used therapy for nAMD is intravitreal administration of anti-VEGF antibodies such

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as bevacizumab and ranibizumab [6-8]. Previous clinical trials, such as the Minimally Classic/Occult Trial of the Anti-VEGF Antibody Ranibizumab in the Treatment of Neovascular AMD (MARINA) study, have demonstrated an approximately 20-letter gain in visual acuity (VA) in patients given anti-VEGF therapy [6]. Although anti-VEGF therapy is effective for most patients, some do not respond effectively to treatment and experience continued presence of subretinal/intraretinal fluid and/or a deterioration of VA [8,9]. Furthermore, a single injection of ranibizumab costs approximately \$2,000, and the total costs of ranibizumab exceed \$9 billion per year in the United States alone [10]. Therefore, it is of great interest to find factors that cause differentiated response to anti-VEGF therapy and distinguish early on the individuals who are less likely to respond to the therapy.

Previous studies have found associations between increased risk of developing AMD and smoking, hypertension, and increased body mass index [11-13]. There is extensive support for a strong genetic component in AMD pathogenesis [14]. Based on the assumption that disease-associated genetic factors might also contribute to the therapy outcome, a study investigated the association of anti-VEGF therapy and AMD-related single nucleotide polymorphisms (SNPs) and found two SNPs, rs10898563 of *FZD4* and rs1061170 of *CFH*, were associated with response to anti-VEGF treatment [15]. In this study, we hypothesized that AMD-associated SNPs and gene variation in non-AMD-associated SNPs might contribute to therapy response. Based on the importance of inflammation in AMD pathogenesis and progression and pathways involved in angiogenesis, we selected SNPs in inflammatory genes such as *PLA2G1A* and *IL23R*, as well as angiogenesis-related genes such as *VEGF*, *KDR*, and *HIF-1A* to examine their possible association with anti-VEGF response [16-18].

## METHODS

*Study subjects:* The study was conducted according to the Declaration of Helsinki and approved by the institutional review boards. All participants signed the respective informed consent forms. The study included 106 patients of Caucasian ethnicity with nAMD from the New York Eye and Ear Infirmary (n=39), Wake Forest University Eye Center (n=36), and the National Eye Institute (n=31). Patients were selected consecutively from each institution. Eligibility criteria included an age of 50 years or more and the presence of active choroidal neovascularization due to AMD. To determine the presence of active choroidal neovascularization, we required evidence of intraretinal/subretinal leakage, as identified through optical coherence tomography (OCT). Exclusion

criteria included polypoidal choroidal vasculopathy, retinal angiomatous proliferation, and a history of disciform macular scars based on fluorescein angiography and indocyanine green angiography. Patients were treated at baseline with intraocular injections of either bevacizumab (1.25 mg) or ranibizumab (1.25 mg), two comparable anti-VEGF drugs used as the first line of therapy for patients with AMD [8]. Following the initial baseline dose, subsequent injections (over a total of 12 months) were given only if persistence of active choroidal neovascularization was observed based on OCT. To increase the generalizability of our study, prior treatment other than bevacizumab or ranibizumab was not an exclusion criterion.

*Clinical data collection and responder classification:* Best-corrected visual acuity (BCVA) was recorded at baseline and six and 12 months following anti-VEGF therapy. All BCVA examinations were conducted using Early Treatment of Diabetic Retinopathy Study (ETDRS) eye charts. OCT was performed on all of the patients at each of the previously mentioned time points. The amount of fluid removal observed in each eye was determined by examining the OCT images qualitatively for changes in fluid volume. All patients were classified as either a “good responder” or a “poor responder” based on change in visual acuity and the presence of subretinal/intraretinal fluid. A “good responder” was defined as someone who demonstrated a loss of fewer than 15 ETDRS letters, absorption of previous subretinal or intraretinal fluid at six- and 12-month follow-up visits, and no development of new areas of macular fluid at six- and 12-month follow-up visits. A “poor responder” was defined as an individual who met any combination of the following criteria: 1) a loss of more than 15 ETDRS letters, 2) persistent subretinal or intraretinal fluid at six- and 12-month follow-up visits in the same area of the fundus, 3) new macular fluid at six- and 12-month follow-up visits in different areas of the retina, including macular edema with no foveal involvement via OCT findings. Other clinical information, such as the number of anti-VEGF injections, development of new lesions, diabetes status, past and current smoking status, and history of cardiovascular disease, was recorded for all patients.

*DNA extraction and single nucleotide polymorphism genotyping:* Peripheral venous blood was collected from each study participant in EDTA tubes for genomic DNA extraction. We used commercially available TaqMan-based allelic discrimination assays (Applied Biosystems, Foster, CA). Assays were performed according to the manufacturer’s recommendations, using an Applied Biosystems 7500 detection system.

TABLE 1. SUMMARY OF SNPs EXAMINED AND REASONS FOR SELECTING THESE CANDIDATES.

| Gene            | # of SNPs examined | Function   | Reasons for selecting gene  |
|-----------------|--------------------|--|---|
| <i>PLA2G12A</i> | 1                  | Participates in helper T cell Immune response [16] | Association with AMD [33]   |
| <i>IL-23R</i>   | 11                 | Regulates IL-1A expression [35]                    | Regulator of IL-17 expression [35]  |
| <i>STAT3</i>    | 1                  | Transcriptional factor in IL-23/IL-17 pathway [35] | Regulator of IL-17 Expression [35]  |
| <i>VEGFA</i>    | 4                  | Promotes Neovascularization [36]                   | Higher secretion in AMD patients [37]                                       |
| <i>KDR</i>      | 1                  | Receptor for VEGFA [38]                            | Differences in receptor expression or function may affect VEGF binding [39] |
| <i>HIF-1A</i>   | 3                  | Oxygen dependent transcriptional activator [40]    | Regulates VEGF expression [40]  |

SNPs were selected based on previously reported AMD association and functional involvement in the angiogenesis pathways. All of the SNPs examined as well as their associated genes are listed in Table 1.

*Statistical analysis:* The SNP allelic association and genotypic association as a dominant model (carriers with at least one minor allele versus those with two major alleles) was analyzed using unconditional logistic regression in which good responder versus poor responder status was designated as the outcome. The p value, odds ratio (OR) of association, and Hardy–Weinberg Equilibrium (HWE) p value were assessed using a chi-square test. Multiple testing was corrected by the false discovery rate and by setting Permutation to analyze 1,000 simulations [19]. The analyses were performed using SVS software (version 7.4.1; [HelixTree Genetics Analysis Software](#), Golden Helix Inc., Bozeman, MN). Differences were considered significant when  $p < 0.05$ .

*Assessment of PLA2G12A in an independent age-related macular degeneration cohort:* *PLA2G12A* SNP genotyping was conducted on DNA samples collected from the National Eye Institute, a cohort that has been reported previously [20,21]. This patient cohort consists of 203 patients with clinically diagnosed cases of advanced AMD, including nAMD and GA AMD, as well as 158 unrelated, normal control subjects. The patients with AMD and the control subjects were evaluated by NEI ophthalmologists using the Age-Related Eye Disease Study criteria [20,21]. All patients classified as normal controls exhibited the absence of drusen or had fewer than five small drusen ( $< 63$   $\mu$ m), and had no evidence of any other retinal diseases. The disease status of all study participants was confirmed by independent grading of fundus photographs.

## RESULTS

*Patient characteristics:* In total, 106 patients were treated with anti-VEGF therapy in this study. Of these patients, 77 were classified as good responders and 29 patients as poor responders. There were no statistically significant differences in age, age of AMD diagnosis, baseline visual acuity, and number of anti-VEGF injections between good and poor responders. Gender, smoking status, history of cardiovascular disease, and diabetes status were also assessed; no significant difference was observed in clinical characteristics between good and poor responders. The clinical characteristics of the patient cohort and the distribution between good and poor responders are summarized in Table 2.

*Genetic analysis:* We first screened 84 samples, including 58 good responders and 26 poor responders due to the setting of the assay. Of the 21 SNPs examined, only rs2285714 (*PLA2G12A*) and rs10146037 (*HIF1A*) showed promise of associations with therapy response. Further genotyping of all study subjects available, including 77 good responders and 29 poor responders, for rs2285714 (*PLA2G12A*) and rs10146037 (*HIF1A*) did not show an increase in the likelihood of the association. The allelic frequencies of all SNPs tested were within the boundaries of HWE ( $p > 0.05$ ) except *PLA2G12A* rs2285714 in the poor responders group ( $p < 0.05$ ). The allelic analysis of all 21 SNPs is summarized in Table 3 and Table 4.

Genetic model analysis of rs2285714 (*PLA2G12A*) and rs10146037 (*HIF1A*) demonstrated an association between rs2285714 (*PLA2G12A*) and therapy response in a dominant mode ( $p < 0.05$ ) but not rs10146037 (*HIF1A*;  $p > 0.05$ ; Table 5). Patients carrying at least one T allele of rs2285714 were 2.79 times more likely to be poor responders (95% confidence interval [CI]= 1.02–7.69,  $p < 0.05$ ) than good responders. However, after adjusting for multiple testing either by the false discovery rate or Bonferroni correction, the initially observed association was no longer statistically significant.

TABLE 2. PATIENT CHARACTERISTICS.

| Characteristic   | Good responders (n=77)       | Poor responders (n=29)       |
|--|------------------------------|------------------------------|
| Mean age   | 80.8±6.75                    | 75.7±8.49                    |
| Mean age diagnosed with AMD                              | 77.4±8.40                    | 71.8±8.86                    |
| Mean baseline BCVA in ETDRS letters (Snellen conversion) | 47.0±31.9 (20/250±6.4 lines) | 53.0±30.2 (20/160±6.0 lines) |
| Change in visual acuity over 12 months (letters)         | 2.1±15.1                     | -2.0±12.7                    |
| Number of injections over 12 months                      | 4.8±3.1                      | 5.9±2.81                     |
| Previous anti-VEGF therapy (%)                           | 19 (25%)                     | 3 (10%)                      |
| % Male   | 42%                          | 52%                          |
| Ever Smoker  | 58%                          | 48%                          |
| Cardiovascular disease                                   | 38%                          | 41%                          |
| Diabetes   | 14%                          | 12%                          |

TABLE 3. DISTRIBUTION OF ALLELIC FREQUENCIES OF IL23 SNPs AMONG RESPONDERS AND POOR RESPONDERS.

| SNP (Minor/major) | Good responders Minor/major (%) | Poor responders Minor/major (%) | P     | OR for minor allele (95% CI) |
|-------------------|---------------------------------|---------------------------------|-------|------------------------------|
| rs11465804 (G/T)  | 7/109 (6.0)                     | 3/49 (5.7)                      | 0.946 | 0.955 (0.24–3.85)            |
| rs11209026 (A/G)  | 6/108 (5.2)                     | 2/50 (3.8)                      | 0.693 | 0.72 (0.14–3.69)             |
| rs790633 (T/C)    | 29/87 (25.0)                    | 18/34 (34.6)                    | 0.199 | 1.58 (0.78–3.33)             |
| rs7539625 (A/G)   | 39/77 (33.6)                    | 14/38 (26.9)                    | 0.388 | 0.73 (0.35–1.50)             |
| rs6688383 (G/A)   | 42/74 (36.2)                    | 19/33 (36.5)                    | 0.967 | 1.01 (0.51–2.00)             |
| rs7530511 (T/C)   | 12/104 (10.3)                   | 9/43 (17.3)                     | 0.207 | 1.81 (0.71–6.42)             |
| rs1343151 (T/C)   | 42/74 (36.2)                    | 15/37 (28.8)                    | 0.352 | 0.71 (0.35–1.45)             |
| rs10889677 (C/A)  | 43/73 (37.1)                    | 16/36 (30.8)                    | 0.429 | 0.75 (0.38–1.52)             |
| rs1321157 (A/G)   | 74/80 (48.1)                    | 32/26 (55.2)                    | 0.355 | 1.33 (0.73–2.44)             |
| rs10127763 (A/G)  | 17/137 (11.0)                   | 9/49 (15.5)                     | 0.376 | 1.48 (0.62–3.54)             |
| rs11465803 (T/C)  | 11/105 (9.5)                    | 6/46 (11.5)                     | 0.683 | 1.25 (0.43–3.57)             |

All HWE p values are >0.05. All SNP assay call rates are >98%.

TABLE 4. DISTRIBUTION OF ALLELIC FREQUENCIES OF OTHER SNPs AMONG RESPONDERS AND POOR RESPONDERS.

| Gene     | SNP (Minor/major) | Good Responders Minor/major (%) | Poor responders Minor/major (%) | P     | OR for minor allele (95% CI) |
|----------|-------------------|---------------------------------|---------------------------------|-------|------------------------------|
| PLA2G12A | rs2285714 (T/C)   | 61/91 (40.1)                    | 30/28 (51.7)                    | 0.13  | 1.60 (0.87–2.94)             |
|          | rs10146037 (C/T)  | 6/144 (4.0)                     | 5/53 (8.6)                      | 0.182 | 2.26 (0.66–7.72)             |
| HIF1A    | rs11549467 (A/G)  | 4/112 (3.4)                     | 2/50 (3.8)                      | 0.898 | 1.12 (0.20–6.32)             |
|          | rs11549465 (T/C)  | 14/100 (12.3)                   | 6/46 (11.5)                     | 0.892 | 0.93 (0.34–2.58)             |
| STAT3    | rs744166 (C/T)    | 51/63 (44.7)                    | 26/26 (50.0)                    | 0.528 | 1.24 (0.64–2.38)             |
| VEGF     | rs699947 (A/C)    | 54/62 (46.6)                    | 27/25 (51.9)                    | 0.519 | 1.24 (0.64–2.39)             |
|          | rs833060 (T/G)    | 20/96 (17.2)                    | 11/41 (21.1)                    | 0.546 | 1.29 (0.57–2.93)             |
|          | rs3025010 (C/T)   | 50/66 (43.1)                    | 17/35 (32.7)                    | 0.203 | 0.64 (0.32–1.27)             |
|          | rs833069 (G/A)    | 22/94 (19.0)                    | 12/40 (23.1)                    | 0.54  | 1.28 (1.57–2.84)             |
| KDR      | rs2071559 (C/T)   | 59/55 (51.8)                    | 21/29 (42.0)                    | 0.25  | 0.67 (0.34–2.58)             |

All HWE p values are >0.05 except PLA2G12A rs2285714 in poor responders group. All SNP assay call rates are >98%

TABLE 5. GENOTYPIC MODEL ANALYSIS OF PLA2G12A rs2285714 AND HIF1A rs10146037.

| Gene               | Genotype               | Good responders<br>N (%) | Poor responders<br>N (%) | P value | OR (95% CI)       |
|--------------------|------------------------|--------------------------|--------------------------|---------|-------------------|
| PLA2G12A rs2285714 | CC                     | 32 (42.7)                | 6 (20.7)                 | 0.041*  | 2.79 (1.02- 7.69) |
|                    | CT+TT (Dominant model) | 43 (57.3)                | 23 (79.3)                |         |                   |
| HIF1A rs10146037   | TT                     | 69 (92.0)                | 24 (80.0)                | 0.17    | 2.44 (0.67–9.09)  |
|                    | TC+CC (Dominant model) | 6 (8.0)                  | 5(20.0)                  |         |                   |

\*:  $p > 0.05$  after adjusting for multiple testing either by the false discovery rate (FDR) or Bonferroni correction

*PLA2G12A* in age-related macular degeneration versus control: To test rs2285714 (*PLA2G12A*) association with AMD disease status in general, we genotyped the SNP in an independent AMD case-control cohort. Of the 203 patients with AMD, 45 (22%) were diagnosed with GA AMD while 158 (78%) were diagnosed with nAMD. The T allelic frequency was 43.6% in patients with AMD compared to 38.0% in normal controls ( $p > 0.05$ , OR=1.25; 95% CI=0.93–1.67). No statistically significant association was found via dominant model genotype analysis. Furthermore, no differences in genotypic or allelic frequencies were found between nAMD and GA AMD (Table 6).

## DISCUSSION

This study aimed to identify associations between candidate SNPs and response to anti-VEGF therapy. Previous studies have examined AMD-associated SNPs in *CFH* [15,22-25], *CFB* [15], *C3* [24], *HTRA1* [25], *AMRS2* [25], *VEGFA* [24], *KDR* [15], *LRP5* [15], and *FZD4* [15] and their effects on anti-VEGF therapy response. The most consistent polymorphism associated with anti-VEGF treatment response is rs1061170 (*CFH*) [15,23,26]. These studies found that patients with nAMD who had the risk allele (C) of rs1061170 were at

higher risk of responding poorly to anti-VEGF treatment and required additional anti-VEGF injections. To identify novel SNPs associated with treatment response, we selected several SNPs implicated in AMD pathogenesis and functionally involved in the angiogenesis pathway for genetic analysis. Of the SNPs examined, only rs2285714 (*PLA2G12A*) showed marginal association with anti-VEGF therapy response. Even though the association was rendered non-significant after multiple testing adjustments, the odds of having a TT or TC genotype among poor responders were three times greater than the odds of having the same genotypes among good responders

Phospholipase A2 (PLA2) is a family of enzymes that catalyzes the hydrolysis of glycerophospholipids to arachidonic acid and lysophospholipids [16]. PLA2s are also important signaling molecules and responsible for various pathologies, such as inflammation and tissue injury [27-29]. Furthermore, PLA2 has been shown to exert proangiogenic effects by inducing VEGF production in vascular endothelial cells [30]. PLA2s are secreted primarily into the extracellular matrix and can act on cell membranes in an autocrine or paracrine fashion [16]. Increased PLA2 activity may result in the increased conversion of membrane fatty acids to arachidonic

TABLE 6. THE ASSOCIATION OF PLA2G12A SNP rs2285714 WITH AMD IN A CASE-CONTROL COHORT.

| Group                                | nAMD N (%)   | GA AMD N (%) | Controls N (%) |
|--------------------------------------|--|--------------|----------------|
| N                                    | 158  | 45           | 158            |
| Average Age                          | 79.8   | 79           | 66.2           |
| % Male                               | 47   | 42           | 42             |
| C                                    | 179 (56.6)   | 50 (55.6)    | 196 (62.0)     |
| T                                    | 137 (43.4)   | 40 (44.4)    | 120 (38.0)     |
| Allelic analysis p value/OR (95% CI) | 0.1318/1.25 (0.93–1.67; AMD at-large versus control) |              |                |
| CC Genotype                          | 52 (32.9)  | 14 (31.1)    | 60 (38.0)      |
| CT Genotype                          | 75 (47.5)  | 22 (48.9)    | 76 (48.1)      |
| TT Genotype                          | 31 (19.6)  | 9 (0.20)     | 22 (13.9)      |
| Dominant Model p value/OR (95% CI)   | 0.3018/1.25 (0.82–1.90; AMD at-large versus control) |              |                |

acids. Subsequently, augmented production of arachidonic acids may then lead to higher levels of prostaglandins (PGE<sub>2</sub>), stimulating increased microvascular permeability through a cyclooxygenase-1-dependent pathway [31,32]. However, no evidence currently exists linking rs2285714 to altered PLA2 activity. We were unable to measure PGE<sub>2</sub> levels due to the lack of localized ocular specimens. There is no report on the correlation between plasma PGE<sub>2</sub> level and PLA2 SNPs.

PLA2G1A has been found to be marginally associated with AMD [27-29,33]. In addition to identifying a suggestive association between rs2285714 and anti-VEGF therapy response, we also attempted to replicate the possible association of the SNP with AMD disease status [33]. We genotyped 203 patients with clinically diagnosed cases of advanced AMD and 158 unrelated normal controls and found a higher frequency of the T risk allele in patients with AMD (0.436) compared to normal controls (0.380), with an OR of 1.25 (0.93–1.67). However, our results were again only suggestive ( $p=0.1318$ ). Our findings were consistent with a previous study showing increased frequency of the T allele in AMD cases (0.464) compared to normal controls (0.395), OR of 1.31 [33].

*Although the overall trend of our data points toward a suggestive association between rs2285714 (PLA2G1A) and anti-VEGF treatment response, there were limitations to our study. First, including patients with a previous history of anti-VEGF therapy may have confounded our SNP association results. We decided to include patients with previous anti-VEGF therapy to mimic a real-world clinical situation where therapy response could be predicted with a genetic test, regardless of previous therapies. In our patient cohort, 25% of good responders and 10% of poor responders had received previous anti-VEGF therapy. Given that a history of multiple anti-VEGF injections may suggest poorer treatment response, we expected a higher percentage of patients in the poor responders group to have received prior treatment. Interestingly, this was not the case in our patient cohort, suggesting previous anti-VEGF treatment did not affect the responsiveness to anti-VEGF in our cohort. Another major limitation of this study was small sample size, which decreases the statistical power available to identify statistically significant associations. The suggestive association is of high OR value. Due to the restriction of our clinical protocols, we are unable to extend the study. The association between PLA2G1A SNP rs2285714 and anti-VEGF response must be further confirmed in additional and larger patient cohorts.*

With the discovery and confirmation of novel SNPs associated with anti-VEGF therapy response, we may eventually be able to identify patients with nAMD who may benefit

from alternative therapies. Alternative therapeutic approaches include the use of aflibercept, combination anti-VEGF treatment with intraocular corticosteroids, and photodynamic therapy [34]. Furthermore, the continued development of novel nAMD drugs may eventually provide patients with additional alternatives and more personalized care.

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