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Equol's efficacy is greater than astaxanthin for antioxidants, extracellular matrix integrity & breakdown, growth factors and inflammatory biomarkers via human skin gene expression analysis

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ABSTRACT

Skin homeostasis and dermal aging can be influenced by phytochemicals. Astaxanthin is a powerful antioxidant and anti-inflammatory agent, while equol's beneficial properties have been recently reported. The purpose of this *in vitro* study was to compare astaxanthin versus equol at a 1% concentration by a single topical application using epidermal full-thickness skin cultures. After 24 h (exposure) human gene expression was quantified by qPCR-mRNA across 9 functional categories for 63 genes. For 39 biomarkers equol significantly altered the parameters compared to astaxanthin. Astaxanthin significantly influenced 6 genes compared to equol. The results revealed significantly greater effects of equol compared to astaxanthin for the antioxidants, growth factors, extracellular integrity and extracellular breakdown, and the inflammatory biomarkers. These findings indicate that equol's efficacy is greater than astaxanthin for various skin biomarkers and suggest that equol may be incorporated into topical and oral applications to improve skin health and reduce photo-aging.

Abbreviations

Genes	
AHR	aryl hydrocarbon receptor
AQP3	aquaporin3
ARNT	aryl hydrocarbon recept. translocator
BIRC5	survivin
BMP2	bone morphogenetic protein2
BMP4	bone morphogenetic protein4
CASP3	caspase3
CAT	catalase
CD44	hyaluronic acid receptor
COL1A1	collagen, type1, alpha1
COL17A1	collagen, type17, alpha1
CSF2/GM-CSF	colony stimulating factor2
CTGF/CCN2	connect tissue growth factor
DEFB1	defensin, beta 1
EDN1	endoththelin1
EGFR	epidermal growth factor receptor
ELN	elastin
F2RL1/PAP2	protease-activated receptor2
FLG	filaggrin
FOXO3	forkhead box O3
GPX1	glutathione peroxidase1
HAS2	hyaluronic acid synthase2
HBEGF	heparin-bind EGF-growth factor
HSPG2/PLC	heparan sulfate proteoglycan2
HMOX1	heme oxygenase1
ICAM1	intercellular adhesion molecule 1
IFNA	interferon alpha
IL1A	interleukin1 alpha
IL1B	interleukin 1 beta
IL-6	interleukin 6
IL-8	interleukin 8
IL-10	interleukin 10
ITGB1	integrin beta 1
ITGB4	integrin beta 4
KITLG/SCF	kit ligand/stem cell factor
KLK5	kallirein-related peptidase5
KLK7	kallirein-related peptidase7
KRT5	keratin 5
KRT14	keratin 14
MMP1	matrix metalloproteinase1
MMP2	matrix metalloproteinase2
MMP9	matrix metalloproteinase9
MT1A	metallothionein1 A
MT2A	metallothionein2A
PCNA	proliferating cell nuclear antigen
PTGS1/COX1	prostaglandin endoperoxide synthase 1/cyclooxygenase1
PTGS2/COX2	prostaglandin endoperoxide synthase 2/cyclooxygenase2
SERPINB3	serpin peptidase inhibitorB3
SERPINH1	serpin peptidase inhibitorH1
SIRT1	sirtuin1
SMPD1	sphingomyelin phosphodieasease1
SOD1	superoxidase dismutase1
SOD2	superoxidase dismutase2
SPINK5	serine peptidase inhibitor

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TGFB1	transforming growth factor beta1
TIMP1	tissue inhibitor metalloproteinase1
TLR2	toll-like receptor2
TLR3	toll-like receptor3
TNF	tumor necrosis factor alpha
TP63	protein63
TXN	thioredoxin
TXNRD1	thioredoxin reductase1
VCAN	versican
Other	
5α-DHT	5alpha-dihydrotestosterone
ANOVA	analysis of variance
CAM	complementary and alternative medicine
CNS	central nervous system
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
EFT	epidermal full-thickness
GI	gastrointestinal
HSD	honestly significantly different
LDH	lactate dehydrogenase
MTT	3-(4,5-dimethylthiazol-2yl)-2,5-diphenlytetrazolium bromide
NKkappaB	transcription factor NFkappaB
Nrf2	nuclear-factor-erythroid 2-related factor 2
PRP	platelet-rich plasma
ROS	reactive oxygen species
SAM	selective androgen modulator
SERM	selective estrogen receptor modulator
USD	United States dollars
USFDA	United States Food and Drug Administration
UV	ultra violet

1. Introduction

Among the therapeutic agents approved by the USFDA during the 30 year period between 1981 and 2010, 40% were linked to natural product/compound(s) (Newman & Cragg, 2012). Natural ingredients have been used for centuries for skin care and within the last decade the increased use of complementary and alternative medicine (CAM) was reported to be significantly higher in adults with skin disorders (at 49%) compared to the general population (at 36%) (Smith, Shin, Brauer, Mao, & Gelfand, 2009; Volmer, West, & Lephart, 2018). Approximately 10,000 phytochemicals have been identified to date (Zhang et al., 2015). Notable, within the last 10–15 years, botanical use in skin products have been developed and marketed to function as skin protectants [against: ultra violet (UV) light, inflammation / reactive oxygen species (ROS) and air-pollution], skin lightening, antioxidant, growth factor, extracellular matrix protein support, anti-aging, and antioxidant ingredients via topical or oral administration (Bosch et al., 2015; Davinelli, Nielsen, & Scapagnini, 2018; Joshi & Pawar, 2015; Kanlayavattanakul & Lourith, 2017; Lephart, 2018a; Ribeiro, Estanqueiro, Oliveira, & Lobo, 2015; Volmer et al., 2018). In this regard, the global cosmetic products market was valued at 532 billion USD in 2017 and is expected to reach a market value of 806 billion USD by 2023 with a compound annual growth rate of 7% during this period (Reuters, Orbis Research (2018), 2018).

One of the most popular skin anti-aging active ingredients in cosmetics is astaxanthin, which was isolated from lobster by Kuhn and Sorensen in 1938 (Kuhn & Sorensen, 1938). Astaxanthin gives salmon and lobster their orange-reddish color, flamingo feathers their pinkish hue, and it is used in the aquatic-farm industry to increase the color of the flesh in farm-raised salmonids (Davinelli et al., 2018; Volmer et al., 2018). Astaxanthin is a keto-carotenoid similar in chemical structure to β-carotene, however, it is not converted to vitamin A, and the sources of astaxanthin include plants, animals, and algae (Davinelli et al., 2018; Volmer et al., 2018). While there are several astaxanthin stereoisomers in nature, the major molecular species in the natural foods, dietary supplement, cosmetic and food industry appears to be the all-trans 3S, 3S′astaxanthin (Davinelli et al., 2018; Volmer et al., 2018) (Fig. 1A). Starting in the 1990s astaxanthin's powerful antioxidant properties was becoming widely accepted from numerous animal and human studies, but also, included was astaxanthin's many other human health benefits

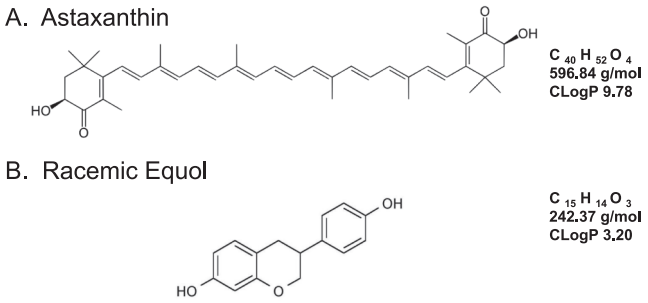

Fig. 1. Comparison of the Chemical Structures, Molecular Formulas, Molecular Weights and CLogP for Astaxanthin (A) and Racemic Equol (B). CLogP = the logP value of a compound representing its partition coefficient and lipophilicity. Astaxanthin is a keto-carotenoid similar in chemical structure to β-carotene, but is not converted to vitamin A. Equol is a polyphenolic compound classified as an isoflavonoid and phytoestrogen.

such as anti-inflammatory, anti-cancer, and especially it's skin-protective effects (Chou et al., 2016; Davinelli et al., 2018; Meephansan, Rungjang, Yingmema, Deenonpoe, & Ponnikorn, 2017; Suganuma, Nakajima, Ohtsuki, & Imokawa, 2010; Volmer et al., 2018).

Equol is a relatively new phytochemical used as an ingredient for human skin applications that has a polyphenolic chemical structure found in plant and food sources (Lephart, 2018a, 2016, 2017; Magnet et al., 2017; Oyama et al., 2012) (Fig. 1B). It is also classified as an isoflavonoid and a phytoestrogen, having selective estrogen receptor modulator (SERM) characteristics that yield an enhanced/sustained delivery into the dermal skin layers (Gopaul, Knaggs, & Lephart, 2012; Lephart, 2016, 2017, 2018a), which inhibits dermal aging and enhances facial attractiveness (Lephart, 2018b; Magnet et al., 2017; Oyama et al., 2012). Equol has a chiral carbon, resulting in two isomers or mirror image molecules (R-equol and S-equol). Both equol isomers exhibit antioxidant, anti-inflammatory, skin protectant (against ROS/oxidative stress) and specifically anti-androgen hormonal actions by binding free 5α-dihydrotestosterone (5α-DHT) as a selective androgen modulator (SAM) (Gopaul et al., 2012; Lephart, 2013, 2016, 2017; Magnet et al., 2017; Oyama et al., 2012). In this regard, a comparison among R-equol, racemic equol and S-equol for various biochemical characteristics and molecular/biomarker parameters is shown in Table 1. While R-equol, versus racemic equol versus S-equol has been examined previously in human skin gene array studies and compared to other polyphenolic phytochemicals, remarkably, astaxanthin has not been tested in a comprehensive manner using this powerful gene array technique (Chou et al., 2016; Lephart, 2017).

Thus, the purpose of this study was to determine the efficacy of astaxanthin versus equol as potential cosmetic active ingredients by quantifying the human gene parameters across 9 major skin function classifications in a comprehensive manner, but especially examining the antioxidant, growth factor, extracellular matrix protein (support & breakdown) and anti-inflammatory characteristics of these two phytochemicals. This was accomplished by topically applying the astaxanthin or racemic equol treatments at 1% concentrations dissolved in dimethyl sulfoxide (DMSO) to epidermal full-thickness (EFT) skin equivalents for 24 h, after which the various skin biomarkers are quantified by RNA isolation and real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis. In general, equol's efficacy was better than astaxanthin among the human skin biomarkers that were tested, and the results are presented below.

2. Materials and methods

2.1. Test materials, Control(s) - untreated tissues, DMSO vehicle testing; human skin tissue cultures, viability, validation and assessment

Astaxanthin [(3S,3′S)-3,3′-Dihydroxy-β,β-Carotene-4,4′-dione, trans-astaxanthin, product number: SML0982; ≥ 97 purity] and HPLC grade

Table 1
Comparison Among R-Equol, Racemic Equol and S-Equol for Various Characteristics and Parameters.

	R-Equol	Racemic Equol	S-Equol	Reference(s)
Characteristics/Parameters				
1. Binds 5α-DHT	+	+	+	Lephart (2016, 2018a)
2. Inhibits 5α-Reductase Enzyme (type I in skin)	+	+	−	Lephart, (2016, 2017)
3. Binds Estrogen Receptor Beta	K_i = 15.4 nM	IC_{50} = 0.2 μM	K_i = 6.4 nM	Lephart (2017)
4. Binds Estrogen Receptor Alpha	K_i = 27.4 nM	IC_{50} = 1.5 μM	K_i = 15.4 nM	Lephart (2017)
5. Binds/Activates Estrogen Related Receptor gamma (γ)	ND	+	ND	Lephart (2016)
6. Present in plants/food products	fermented plants	−	plants/tofu/eggs/milk	Lephart (2016)
7. GI Intestinal Metabolite (humans)	ND	−	yes, from daidzein	Lephart (2016)
8. Metabolism in Humans	pharmacokinetics (oral) are similar for all 3 forms of equol			Lephart (2016)
9. Stimulates Collagen	+	+	+	Lephart (2013, 2017)
10. Stimulates Elastin	+	+	−	Lephart, (2013, 2017)
11. Inhibits Elastase	ND	+	ND	Gopaul et al. (2012)
12. Stimulates TIMP1	−*	+	+	Lephart, (2013, 2017)
13. Inhibits MMPs	+ + +	+ +	+	Lephart (2013 2018a)
14. Stimulates Growth Factors	+ + +	+ +	+	Lephart, (2013, 2017)
15. Strong Antioxidant	+ +	+ +	+ +	Lephart, (2013, 2017)
16. Binds Nrf2/activates other antioxidants	ND	+	ND	Lephart (2016, 2018a)
17. Inhibits NFkappaB	ND	+	ND	Lephart (2016, 2018a)
18. Inhibits Inflammatory Molecules	+ + +	+ + +	+	Lephart (2013, 2016)

+ = yes.
− = no or very low*.
ND = not determined.
5α-DHT = 5alpha-dihydrotestosterone; GI = gastrointestinal.
As shown, S-equol binds estrogen receptor (ER) beta approximately 1/5 as well as 17beta-estradiol while having low affinity for ER alpha. Conversely, R-equol has weak affinity for either ER and, in general, has weak estrogenic properties at best. TIMP 1 = tissue inhibitor of matrix metalloproteinase 1; MMP = matrix metalloproteinases; Nrf2 = Nuclear-factor-erythroid 2-related factor 2 that is a master regulator of the transcriptional response to oxidative stress; it plays a key role in the cellular defense against oxidative and xenobiotic stressors by its capacity to induce the expression of numerous genes, which encode detoxifying enzymes and antioxidant proteins; NFkappaB = is a pro-inflammatory transcription factor NFkappaB that is involved in oxidative stress mechanisms by the expression of numerous genes such as cytokines and plays a major role in the pathology of inflammatory diseases.

DMSO were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Racemic equol (R,S-equol), product number BU 1520; ≥ 98 purity) was purchased from Central Glass (Halle/Westfallen, Germany). All other chemicals: tissue culture medium, and regents, etc., were also purchased from Sigma-Aldrich Chemical Company.

The epidermal full-thickness human skin cultures (EFT-400) from MatTek (Ashland, MA, USA) were maintained at 37 °C with 5% CO_2. Since the EFT cultures represent human skin barrier equivalents, the concentration of astaxanthin or equol applied topically was 1.0% in DMSO or DMSO alone (vehicle control) or no application in untreated controls. The total volume of the DMSO control or treatments dissolved in DMSO that were applied to the EFT human skin cultures was 20 μl (single application), and the exposure time was 24 h as described previously (Gopaul et al., 2012; Lephart, 2013). Notably, DMSO is considered as a safe skin penetrating agent and has desirable uses in clinical settings for cardiac and CNS applications (Jacob & de al Torre, 2009).

A cell viability study was performed using an MTT assay as described elsewhere (Gopaul et al., 2012; Lephart, 2013), where the controls (untreated, n = 2 as the positive control; DMSO, n = 3 as the vehicle control; Triton X-100, n = 1 as the negative control) and test materials (n = 3 for the astaxanthin or equol groups). These results following 24 h exposure are displayed in Fig. 2A. The untreated control's cell viability was 100%, the DMSO controls viability was 101%, while the astaxanthin and equol treatments displayed 87% viability although, the viability within the racemic equol data was approximately 1.5-times as large compared to astaxanthin's variance (Fig. 2A). Thus, a lactate dehydrogenase (LDH) assay was performed (Takara Bio USA, Inc., Mountain View, CA) on the medium of the various controls and treatment samples to determine if any adverse or unfavorable conditions existed during the incubations. The untreated tissues displayed no unfavorable conditions, while the DMSO (vehicle control) and the astaxanthin groups showed a 12 and 10% variance, respectively. The racemic equol group exhibited a variance more than twice that of the DMSO or astaxanthin results (data not shown), which may explain the increased variability within the equol treatment group for viability in the MTT assay results. This increased variance in the equol treatment had not been seen previously in other studies (Gopaul et al., 2012; Lephart, 2013), and the reasons for this obtained finding are unknown.

To validate the integrity of all of the EFT skin cultures, after the application of the treatment(s), sections of the skin sample were prepared and stained with hematoxylin/eosin that revealed intact cellular components [epidermal layers (stratum corneum and keratinocytes), dermal (fibroblasts), and epidermal/dermal borders], as shown in Fig. 2B and this method has been reported elsewhere (Lephart, 2013).

2.2. Gene array/mRNA quantification of human skin biomarkers

This was accomplished through experiments using gene (array/mRNA levels) expression, where several skin-related genes could be examined at the same time using human skin (EFT) cultures, as preformed previously (Gopaul et al., 2012; Lephart, 2013). Ninety human biomarkers were selected across 9 different skin function categories. Of the total, 63 biomarkers are reported here (or 70%) due, in part, to the increased variability of the obtained data and the unexpected significant influence of the DMSO control vehicle on many of the quantified parameters (when compared to untreated control values). This gene expression experiment was performed to compare astaxanthin versus racemic equol at 1% (dissolved in 100% DMSO) compared to the DMSO control values for each parameter (at 24 h topical exposure), plus untreated controls, n = 6 across all treatment groups. Validation of these methods used have been reported elsewhere (Gopaul et al., 2012; Lephart, 2013). However, in brief, after the topical application of the 20 μl test samples onto the EFT cultures and at the end of the 24 h incubations, total RNA was isolated using Maxwell 16 Simply RNA Tissue kit (Promega, Madison, Wi, USA). RNA concentration and purity were determined using a Nanodrop 200 spectrophotometer, cDNA was

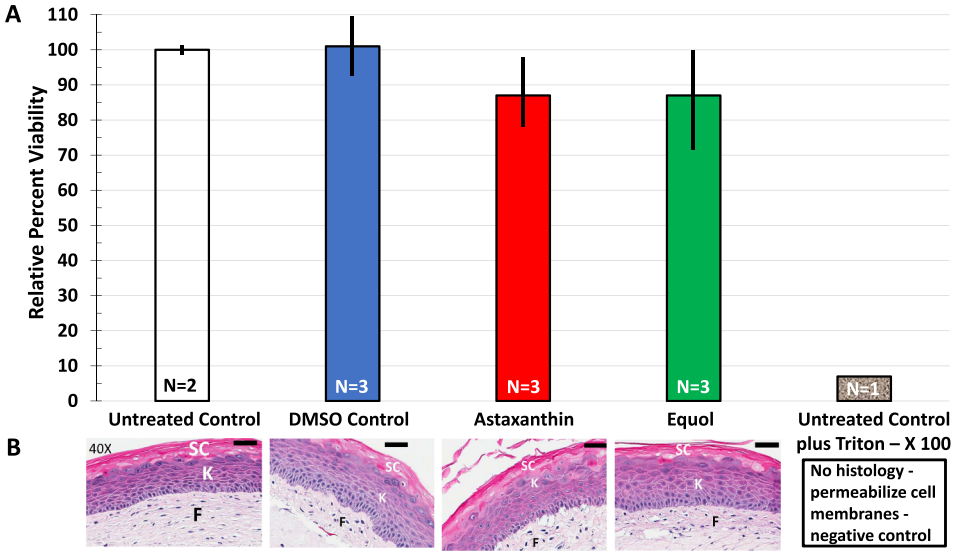

Fig. 2. A- Topical applications of the treatments (single dose of 20 μl) for 24 h to test cell viability (expressed in relative percent) via MTT assay. The data are displayed as the mean ± SEM for all treatments except the untreated control plus Triton-X 100 data. The number of replicates tested by treatment are shown at the base of each bar. B- Validation of the epidermal full-thickness (EFT) skin culture integrity. Representative histological sections across the treatment groups from the gene array experiments are displayed in 40× magnification. The skin sections were stained with hematoxylin/eosin and all treatment slides displayed intact and healthy epidermal layers (SC = stratum corneum and K = keratinocytes), dermal (F = fibroblasts) components, and epidermal/dermal borders.

synthesized using High Capacity DNA Synthesis Kits (Applied Biosystems, Foster City, CA, USA) for open array processing and qPCR reactions were run using validated Taqman gene expression assays, which were analyzed in a Life Technologies QuantStudio 12 K Flex instrument. GUSB was the most stable (control) endogenous gene (among 5 control gene tested), and statistics (unpaired t-tests) were performed using dCT values normalized to the GUSB values for each biomarker.

2.3. Data and statistical analysis

Real-Time RT-PCR data were analyzed using RealTime StatMiner software v4.2 (Thermo Fisher Scientific, Waltham, MA, USA) for statistical analysis using the relative quantitation (RQ) method. The cycle threshold (CT) value of the target was normalized to the CT value of a selected endogenous control. RQ value was calculated and converted to linear fold changes. Unpaired t-tests were performed, and a p-value of less than or equal to 0.05 was reported as statistically significant results ($p \leq 0.05$), as reported previously (Gopaul et al., 2012; Lephart, 2013). However, to reveal increased precision of the statistical analysis a second statistical cut off value of $p \leq 0.005$ was also determined and reported for some biomarkers. For comparison between the treatment groups (DMSO vs. astaxanthin or equol and especially the astaxanthin versus the equol results) the data were analyzed using analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post-hoc tests (Kim, Fischer, Dyring-Andersen, Rosner, & Okoye, 2017). Treatment groups were compared to the DMSO results, and a p-value of less than or equal to 0.05 was reported as statistically significant results ($p \leq 0.05$). Additionally, as before, a second statistical cut off value of $p \leq 0.005$ was determined to show again the increase in the precision of the statistical analysis, where appropriate. All results were expressed as mean ± standard error of the mean in all figures and tables.

3. Results

Overall, 90 skin target biomarkers were analyzed across 9 skin function categories; 63 genes (or 70% of the total) are reported here. Twenty-seven gene biomarkers were omitted due to: (a) having high variability in the obtained data, (b) being significantly altered by the DMSO control vehicle compared to untreated controls in an unexpected manner or, (c) a non-significant alteration in the quantified parameter among the obtained values from the DMSO control vehicle, versus the astaxanthin and/or equol treatments.

3.1. Anti-aging skin genes (4 biomarkers reported out of 5 tested)

When survinin (BIRC5) was examined, the DMSO control vehicle significantly inhibited this biomarker by 2-fold compared to untreated control levels, whereas, the astaxanthin treatment did not significantly alter this parameter compared to DMSO values (Fig. 3A). On the other hand, the equol treatment significantly increased survinin levels by 4.86-fold above DMSO levels (Fig. 3A).

When the forkhead box O3 (FOXO3) biomarker was tested, DMSO significantly increased these levels by 3.4-fold compared to untreated control values (Fig. 3B). But, both the astaxanthin and equol treatments were not significantly different compared to the DMSO results (Fig. 3B).

The heparan sulfate proteoglycan 2 (HSPG2/PLC) biomarker results displayed non-significantly changes for the DMSO or astaxanthin levels, while the equol treatment significantly inhibited this parameter by 1.82-fold compared to the DMSO vehicle control values (Fig. 3C).

Finally, the sirtuin 1 (SIRT1) biomarker results displayed a significant stimulation by the DMSO treatment alone by 1.82-fold over the untreated control values, while the astaxanthin treated skin cultures were not significantly different compared to the DMSO levels (Fig. 3D). However, the equol treatment significantly stimulated the SIRT1 gene by 1.81-fold over that of the DMSO levels (Fig. 3D).

3.2. Antioxidant genes (11 biomarkers reported out of 13 tested)

Surprisingly, the DMSO vehicle treatment significantly: (a) stimulated 6 of the antioxidant biomarkers, (b) inhibited 3 other genes; or did not significantly alter 2 of the biomarkers, when compared to untreated control values (Table 2A). For the aryl hydrocarbon receptor (AHR), the equol treatment significantly stimulated, while the astaxanthin treatment significantly inhibited this parameter compared to DMSO levels (Table 2A). When the aryl hydrocarbon receptor nuclear translocator (ARNT) and catalase (CAT) biomarkers were examined, both the astaxanthin and equol treatments did not significantly altered these levels compared to DMSO values (Table 2A). Among seven other antioxidant biomarkers, the equol treatment significantly stimulated glutathione peroxidase 1 (GPX1), heme oxygenase 1 (HMOX1), metallothionein 1 A and 2 A (MT1A and MT2A), superoxidase dismutase 1 (SOD1), thioredoxin (TXN) and thioredoxin reductase 1 (TXNRD1) over that of astaxanthin levels even though astaxanthin significantly stimulated MT1A and MT2A levels above that of DMSO values (Table 2A). Finally, both the astaxanthin and equol treatments significantly stimulated superoxide dismutase 2 (SOD2) above DMSO levels, but were not significantly different from each other (Table 2A).

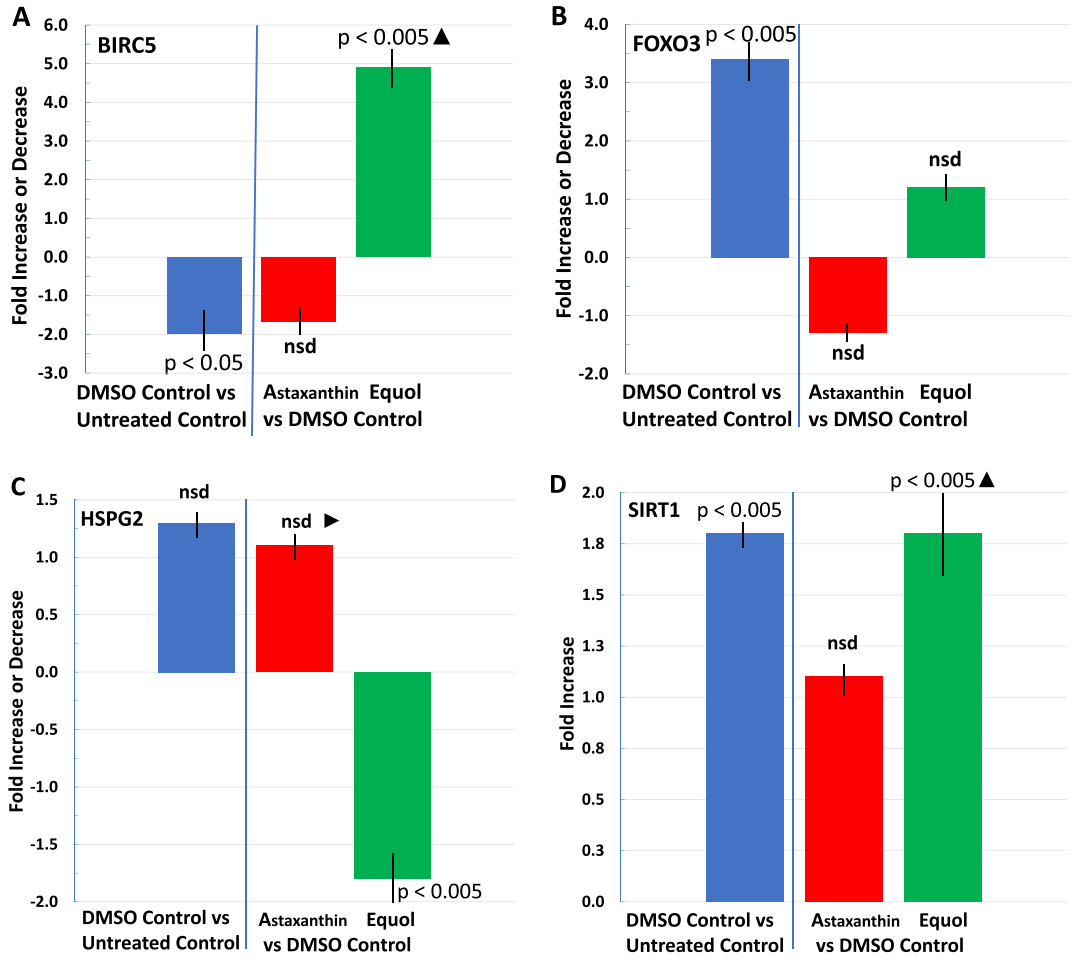

Fig. 3. Anti-aging Biomarkers. Topical application of the DMSO vehicle control versus untreated controls, plus the astaxanthin and equol treatments compared to the DMSO vehicle controls and astaxanthin versus equol levels where appropriate for survivin (BIRC5) (A), forkhead box 03 (FOXO3) (B), heparan sulfate proteoglycan 2 (HSPG2/PLC) (C) and sirtuin 1 (SIRT1) (D), shown as the mean ± SEM. nsd = not significantly different; ▲ = significantly greater compared to astaxanthin levels; ► = significantly greater than equol levels.

3.3. Growth factors genes (8 biomarkers reported out of 9 tested)

The DMSO treatment alone significantly stimulated 5 of the growth factor biomarkers, significantly inhibited 2 genes and did not significantly alter the connective tissue growth factor (CTGF), when compared to untreated control levels (Table 2B). Among 4 of the growth factors examined, equol significantly stimulated these parameters such as bone morphogenetic protein 4 (BMP4), connective tissue growth factor (CTGF/CCN2), endothelin 1 (EDN1) and intercellular adhesion molecule 1 (ICAM1) over that of astaxanthin levels (Table 2B). However, the astaxanthin treatment stimulated heparin-binding EGF-like growth factor (HBEGF) by 1.95-fold over that of equol values or DMSO levels. Finally, for bone morphogenetic protein 2 (BMP2), while both astaxanthin and equol significantly stimulated this biomarker, both treatments also significantly inhibited epidermal growth factor receptor (EGFR), but did not significantly alter the gene levels of kit ligand/stem cell factor (KITLG/SCF), when compared to the DMSO results (Table 2B).

3.4. Epidermal barrier genes (5 biomarkers reported out of 9 tested)

Notably, the DMSO treatment alone significantly inhibited all the epidermal barrier genes, when compared to untreated control levels (Table 2C). Also, both biomarkers, filaggrin (FLG) and keratin 5 (KRT5) were significantly inhibited by the astaxanthin and equol treatments, while integrin beta 1 (ITGB1) levels were not significantly altered compared to DMSO levels. Conversely, the equol treatment significantly stimulated integrin beta 4 (ITGB4) and keratin 14 (KRT14) levels above astaxanthin values (Table 2C).

3.5. Hydration genes (4 biomarkers reported out of 5 tested)

For aquaporin 3 (AQP3), the astaxanthin treatment significantly stimulated gene expression over equol or DMSO control levels (Table 2D). Conversely, equol significantly stimulated hyaluronic acid receptor (CD44) by 1.33-fold over that of astaxanthin or DMSO control values. Surprisingly, the equol treatment significantly inhibited hyaluronic acid synthase 2 (HAS2) versus DMSO levels, but this equol level was not significantly different compared to astaxanthin values (Table 2D). Finally, both astaxanthin and equol significantly inhibited sphingomyelin phosphodieastease (1) levels in the 1.4–1.7 range compared to DMSO values, but were not significantly different from each other (Table 2D).

3.6. Cell renewal and cell regeneration genes (4 biomarkers reported out of 9 tested)

Caspase 3 (CASP3) was significantly stimulated by DMSO alone by 1.67-fold, while astaxanthin did not significantly alter this parameter (Fig. 4A). Equol treatment significantly stimulated CASP3 expression by 1.58-fold compared to DMSO levels (Fig. 4A). When proliferating cell nuclear antigen (PCNA) was examined, DMSO alone significantly

Table 2
Human Skin Gene Expression by Skin Function – Part 1.

Gene Symbol (name)	DMSO Control	Astaxanthin	Equol
	vs. Untreated Control	vs. DMSO Control	
A. ANTIOXIDANTS (fold increase or decrease) 11 out of 13 genes tested			
AHR (aryl hydrocarbon receptor)	1.57 ± 0.09b	−1.20 ± 0.07c	1.58 ± 0.14 d ▲
ARNT (aryl hydrocarbon receptor nuclear translocator)	−1.26 ± 0.07b	−1.07 ± 0.08 nsd	−1.08 + 0.10 nsd
CAT (catalase)	−2.87 ± 0.31b	−1.21 ± 0.07 nsd	−1.02 ± 0.06 nsd
GPX1 (glutathione peroxidase 1)	−1.43 ± 0.08 a	1.02 ± 0.06 nsd	1.62 ± 0.18 d ▲
HMOX1 (heme oxygenase 1)	1.52 ± 0.12b	−1.45 ± 0.10c	1.69 ± 0.16 d ▲
MT1A (metallothionein 1 A)	12.19 ± 1.3b	1.22 ± 0.12c	4.07 ± 0.34 d ▲
MT2A (metallothionein 2 A)	10.39 ± 0.83b	1.43 ± 0.06c	1.76 ± 0.15 d ▲
SOD1 (superoxidase dismutase 1)	−1.21 ± 0.09 nsd	1.12 ± 0.05 nsd	2.01 ± 0.19 d ▲
SOD2 (superoxidase dismutase 2)	2.64 ± 0.14b	2.33 ± 0.17 d	2.00 ± 0.18c
TXN (thioredoxin)	−1.15 ± 0.06 nsd	−1.02 ± 0.08 nsd	1.30 ± 0.12c ▲
TXNRD1 (thioredoxin reductase 1)	2.02 ± 0.15b	1.18 ± 0.05 nsd	2.50 ± 0.17 d ▲
B. GROWTH FACTORS (fold increase or decrease) 8 out of 9 genes tested			
BMP2 (bone morphogenetic protein 2)	8.12 ± 0.55 a	1.35 ± 0.09c	1.32 ± 0.16c
BMP4 (bone morphogenetic protein 4)	−2.56 ± 0.19b	−1.64 ± 0.07c	4.44 ± 0.49 d ▲
CTGF (connective tissue growth factor)	1.03 ± 0.05 nsd	1.14 ± 0.06 nsd	9.40 ± 1.2 d ▲
EDN1 (endothelin 1)	1.97 ± 0.24 a	−1.36 ± 0.08c	3.97 ± 0.42 d ▲
EGFR (epidermal growth factor receptor)	1.21 ± 0.06 a	−1.30 ± 0.09c	−1.52 ± 0.15 d
HBEGF (heparin-binding EGF-like growth factor)	13.04 ± 1.1 a	1.95 ± 0.12b ►	−1.99 ± 0.25c
ICAM1 (intercellular adhesion molecule 1)	4.01 ± 0.29 a	1.34 ± 0.09c	2.34 ± 0.22 d ▲
KITLG/SCF (kit ligand/stem cell factor)	−1.63 ± 0.13 a	−1.06 ± 0.05 nsd	1.02 ± 0.74 nsd
C. EPIDERMAL BARRIER (fold increase or decrease) 5 out of 9 genes tested			
FLG: (filaggrin)	−3.38 ± 0.26b	−7.63 ± 0.89 d	−6.31 ± 0.94 d
ITGB1 (integrin beta 1)	−1.57 ± 0.17b	−1.01 ± 0.08 nsd	−1.30 ± 0.20 nsd
ITGB4 (integrin beta 4)	−3.93 ± 0.37b	−3.01 ± 0.28c	1.32 ± 0.18 d ▲
KRT5 (keratin 5)	−3.56 ± 0.31b	−1.54 ± 0.15c	−1.23 ± 0.19c
KRT14 (keratin 14)	−2.80 ± 0.18b	−1.03 ± 0.09c	1.70 ± 0.29 d ▲
D. HYDRATION (fold increase or decrease) 4 out of 5 genes tested			
AQP3 (aquaporin 3)	−2.74 ± 0.24b	1.65 ± 0.19c ►	−1.21 ± 0.31 nsd
CD44 (hyaluronic acid receptor)	−1.11 ± 0.32 nsd	−1.10 ± 0.14 nsd	1.33 ± 0.19 d ▲
HAS2 (hyaluronic acid synthase2)	−1.56 ± 0.24b	−1.11 ± 0.30 nsd	−1.65 ± 0.28c
SMPD1 (sphingomyelin phosphodiesterase 1)	1.37 ± 0.31 nsd	−1.35 ± 0.28c	−1.72 ± 0.26c

Topical application of the DMSO vehicle control versus untreated controls plus the astaxanthin and equol treatments compared to the DMSO vehicle controls and comparison of astaxanthin to equol levels where appropriate.
a = $p < 0.05$; significantly different than untreated control values.
b = $p < 0.005$; significantly different than untreated control values.
c = $p < 0.05$; significantly different than DMSO control values.
d = $p < 0.005$; significantly different than DMSO control values.
▲= $p < 0.005$; significantly greater than astaxanthin values.
►= $p < 0.005$; significantly greater than equol values.
nsd = not significantly different than control values.
data displayed as the mean ± SEM.

inhibited this expression by 1.23-fold, while the astaxanthin treatment was not significantly different compared to DMSO levels (Fig. 4B). However, the equol treatment significantly stimulated PCNA expression by 1.78-fold over DMSO control values (Fig. 4B). For transforming growth factor beta 1 (TGFB1), DMSO alone and astaxanthin significantly increased expression by 1.37-fold and 1.56-fold, respectively, while the equol treatment significantly inhibited TGFB1 by 1.61-fold compared to DMSO control levels (Fig. 4C). Finally, for protein 63 (TP63) gene expression, DMSO alone significantly inhibited TP63 levels by 5.16-fold, while the astaxanthin or equol treatments were not significantly different versus DMSO levels (Fig. 4D).

3.7. Extracellular matrix (ECM) integrity genes (6 biomarkers reported out of 13 tested)

Four of the biomarkers were significantly inhibited by DMSO alone ranging from 1.38 to 6.2-fold (Fig. 5A–F). Whereas, for tissue inhibitor of metalloproteinase 1 (TIMP1), DMSO alone significantly stimulated the expression of this biomarker by 3.49-fold, and there was no significant alteration of gene expression for serpin peptidase inhibitor H1 (SERPINH1), when DMSO levels were compared to untreated controls (Fig. 5A–F). For collagen 1A1 (COL1A1) and collagen 17A1 (COL17A1), the equol treatment significantly stimulated gene expression by 1.8- and 2.1-fold, respectively, while the astaxanthin treatment showed no significant alteration in COL1A1 and significantly inhibited COL17A1 by 1.44-fold (Fig. 5A and B). Both the astaxanthin and equol treatments significantly stimulated elastin (ELN) gene expression by 1.51-fold and 2.70-fold, respectively; and TIMP1 gene levels by 1.25-fold and 2.95-fold, respectively, plus the equol levels were significantly higher compared to the astaxanthin results for these parameters (Fig. 5C and D). For the SERPINH1 biomarker, the equol treatment significantly stimulated gene expression by 2.31-fold, while astaxanthin levels were not significantly different compared to DMSO controls (Fig. 5E). Finally, for versican (VCAN) gene expression the equol treatment significantly increased expression by 1.48-fold, while astaxanthin levels were not significantly different compared to DMSO controls (Fig. 5F).

3.8. Extracellular matrix (ECM) breakdown genes (8 biomarkers reported out of 9 tested)

The DMSO control vehicle significantly decreased the expression of protease-activated receptor 2 (F2RL1/PAR2), kallikrein peptidase

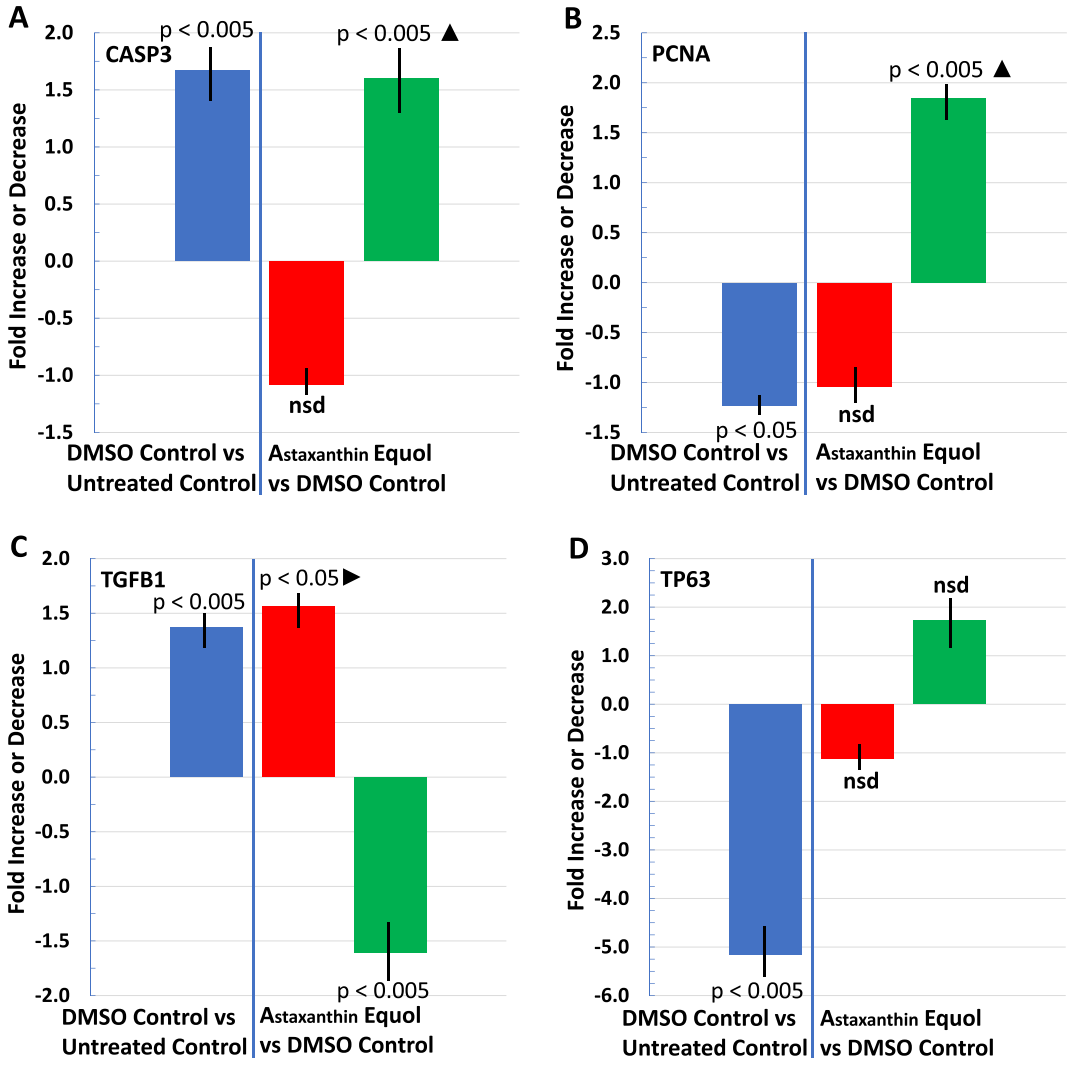

Fig. 4. Cell Renewal and Cell Regeneration Biomarkers. Topical application of the DMSO vehicle control versus untreated controls, plus the astaxanthin and equol treatments compared to the DMSO vehicle controls and astaxanthin versus equol levels where appropriate for caspase 3 (CASP3) (A), proliferating cell nuclear antigen (PCNA) (B), transforming growth factor beta 1 (TGFB1) (C) and protein 63 (TP63) (D), shown as the mean ± SEM. nsd = not significantly different; ▲ = significantly greater compared to astaxanthin levels; ► = significantly greater than equol levels.

5 and 7 (KLK 5 and KLK 7) and serine peptidase inhibitor 5 (SPINK5), and significantly increase matrix metalloproteinase 1 and 2 (MMP 1 and MMP 2), but did not alter gene expression levels of serpin peptidase inhibitor, clade B member 3) (SERPINB3) compared to untreated controls (Table 3A). Both the astaxanthin and equol treatments significantly inhibited F2RL/PAR2, KLK5 and KLK7, MMP 1, SERPINB3 and SPINK5 gene expression compared to DMSO vehicle control values (Table 3A). However, for the biomarkers, MMP 2 and MMP 9, the astaxanthin treatment did not significantly alter gene expression levels compared to DMSO controls (Table 3A). Finally, for all the extracellular matrix breakdown genes, the equol treatment resulted in significantly greater inhibition in expression levels compared to the astaxanthin treatment (Table 3A).

3.9. Inflammation and immune genes (13 biomarkers reported out of 18 tested)

For all the inflammation/immune biomarkers, the DMSO treatment alone significantly stimulated gene expression, except for toll-like receptor 3 (TLR3), which displayed a significantly inhibition and interleukin-10 (IL-10) that showed no significant alterations compared to untreated control values (Table 3B). Among eight of the biomarkers [colony stimulating factor 2 (CSF2/GM-CSF), interleukin 1 alpha (IL1A), interleukin 6 (IL-6), interleukin 8 (IL-8), prostaglandin endoperoxide synthase 1/cyclooxygenase 1 (PTSG1/COX1), toll-like receptors 2 and 3 (TLR2 and TLR3) and tumor necrosis factor alpha (TNF)] both the astaxanthin and equol treatments significantly inhibited gene expression compared to DMSO control vehicle levels (Table 3B). However, for six of these biomarkers, CSF2/GM-CSF, IL1A, IL-6, PTGs/COX1, TLR2 and TNF the equol treatment displayed significantly greater inhibition compared to the obtained astaxanthin levels (Table 3B). There were no significant alterations in gene expression for the biomarkers, interferon alpha (IFNA), interleukin 1 beta (IL1B) and interleukin 10 (IL-10) in either the astaxanthin or equol treated skin cultures, when compared to the DSMO vehicle control levels (Table 3B). Notably, the astaxanthin treatment significantly increased gene expression of defensin beta 1 (DEFB1) above both the DMSO vehicle control and equol levels (Table 3B). Finally, for the prostaglandin endoperoxide synthase 2/cyclooxygenase 2 (PTGS2/COX2) biomarker the astaxanthin treatment significantly inhibited gene expression compared to both the DMSO vehicle controls or equol levels (Table 3B).

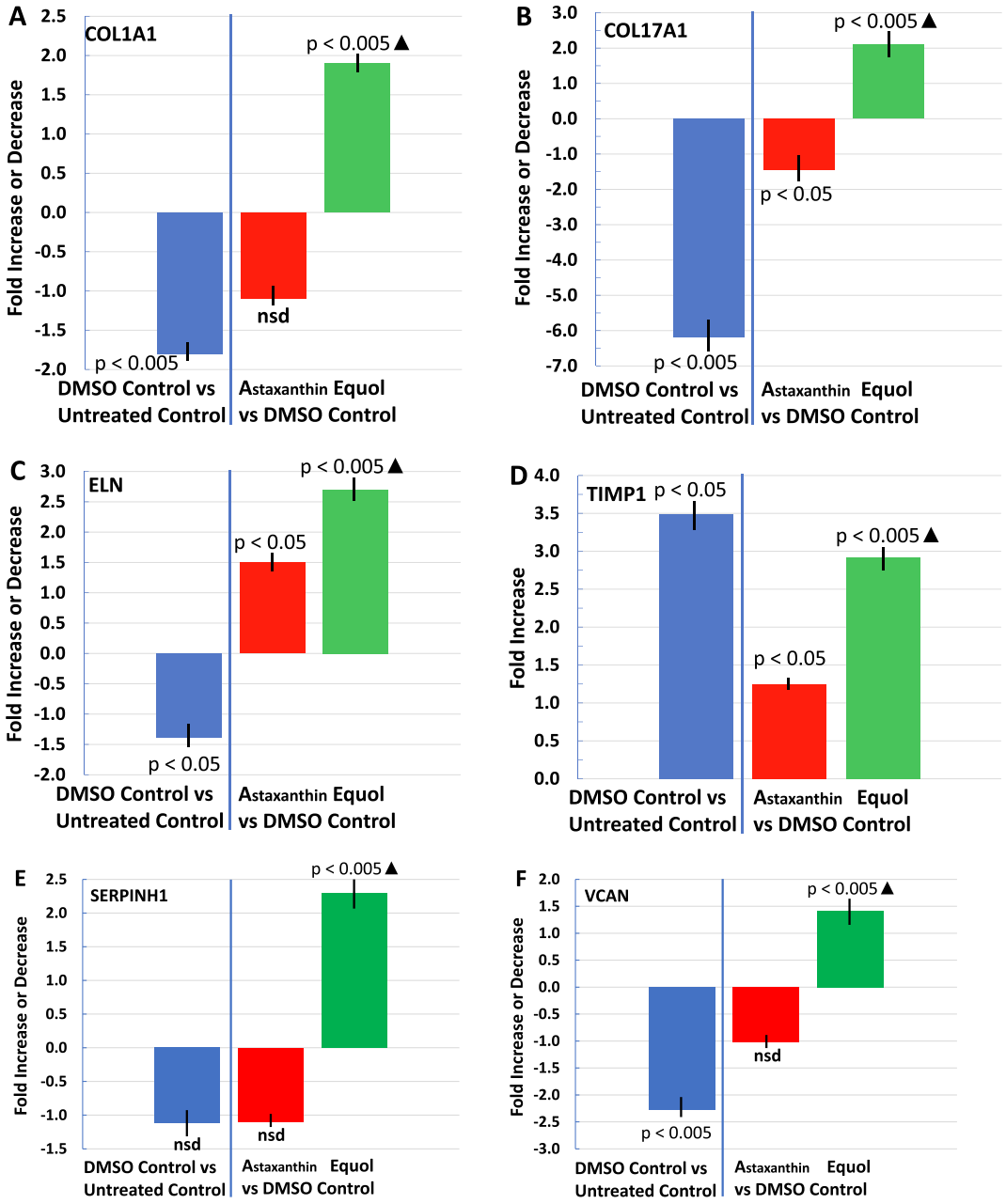

Fig. 5. Extracellular Matrix (ECM) Integrity Biomarkers. Topical application of the DMSO vehicle control versus untreated controls, plus the astaxanthin and equol treatments compared to the DMSO vehicle controls and astaxanthin versus equol levels where appropriate for collagen, type 1, alpha 1 (COL1A1) (A), collagen, type 17, alpha 1 (COL17A1) (B), elastin (ELN) (C) tissue inhibitor of metalloproteinase 1 (TIMP 1) (D), serpin peptidase inhibitor H 1 (SERPINH1) (E) and versican (VCAN) (F) shown as the mean ± SEM. nsd = not significantly different; ▲ = significantly greater compared to astaxanthin levels;

3.10. Grand summary: human skin gene expression- effects of astaxanthin versus equol by skin function categories

As shown in Table 4 (Alam, Sehgal, Kundu, Dalal, & Vaidya, 2011; Antonini et al., 2008; Breiden & Sandhoff, 2014; Brown & McLean, 2012; Giangreco, Goldie, Failla, & Watt, 2009; Hoste, 2011; Ismail & Yusuf, 2014; Jensen, 2010; Jiang, Sanders, Ruge, & Harding, 2012; Li et al., 2017; Pastore, Mascia, Mariani, & Girolomoni, 2008; Picardo & Cardinali, 2011; Saxena et al., 2015; Tanaka, Narazaki, & Kishimoto, 2014), all 63 genes are reported across nine skin function categories. For each gene symbol/name by skin function category in the mid-portion of this table, a brief description of the gene-product's function is described followed to the right by the reference(s) cited. Each gene symbol is color-coded, which indicates whether astaxanthin (in red font) or equol (in green font) displayed the greatest (stimulation or inhibition) compared to each other [subsequent to statistical analysis against DMSO vehicle control levels]. Each gene symbol in black font indicates that no significant difference between the astaxanthin versus equol values was detected. In 39 skin biomarkers the equol treatment (shown in the green font gene symbols) significantly altered (increased or decreased) the parameters in a positive manner compared to astaxanthin. Conversely, the astaxanthin treatment (shown in the red font gene symbols) significantly influenced 6 dermal genes compared to equol treated skin cultures, while 18 of the skin biomarkers (shown in black font) were not significantly different between the two treatment groups. The data shown in Table 4 is discussed below in relation to the importance/impact upon the obtained results by treatment and skin function by gene category.

Table 3
Human Skin Gene Expression by Skin Function – Part 2.

Gene Symbol (name)	DMSO Control vs. Untreated Control	Astaxanthin vs. DMSO Control	Equol
A. EXTRACELLULAR BREAKDOWN (fold increase or decrease) 8 reported out of 9 genes tested			
F2RL1/PAR2 (protease-activated receptor 2)	−1.42 ± 0.06 a	−1.55 ± 0.05c	−3.10 ± 0.23 d ▼
KLK5 (kallikrein peptidase5)	−1.56 ± 0.11b	−2.27 ± 0.18c	−3.58 ± 0.30 d ▼
KLK7 (kallikrein peptidase7)	−1.48 ± 0.12 a	−1.49 ± 0.10c	−4.24 ± 0.68 d ▼
MMP1 (matrix metallopeptidase 1)	7.80 ± 0.59b	−1.40 ± 0.09c	−2.20 ± 0.19 d ▼
MMP2 (matrix metallopeptidase 2)	1.30 ± 0.10 a	1.10 ± 0.08 nsd	−1.60 ± 0.20 d ▼
MMP9 (matrix metallopeptidase 9)	1.10 ± 0.05 nsd	−1.16 ± 0.11 nsd	−5.50 ± 0.23 d ▼
SERPINB3 (serpin peptidase inhibitor, clade B member 3)	−1.28 ± 0.18 nsd	−1.70 ± 0.13c	−14.80 ± 1.7 d ▼
SPINK5 (serine peptidase inhibitor5)	−1.88 ± 0.13b	−1.82 ± 0.17c	− 4.61 ± 0.59 d ▼
B. INFLAMMATION/IMMUNE (fold increase or decrease) 13 reported out of 18 genes tested			
CSF2/GM-CSF (colony stimulating factor 2)	8.60 ± 0.79 a	−3.30 ± 0.18c	−15.6 ± 1.8 d ▼
DEFB1 (defensin, beta1)	1.43 ± 0.16b	1.51 ± 0.07c►	−1.48 + 0.15 nsd
IFNA (interferon alpha)	2.88 ± 0.16 a	−1.25 ± 0.09 nsd	−1.63 ± 0.32 nsd
IL1A (interleukin 1 alpha)	5.51 ± 0.27 a	−1.08 ± 0.05c	−4.84 ± 0.63 d ▼
IL1B (interleukin 1 beta)	14.14 ± 3.4 a	1.00 ± 0.24 nsd	−1.14 ± 0.39 nsd
IL-6 (interleukin 6)	6.95 ± 0.60 a	−1.51 ± 0.17 d	−2.26 ± 0.28 d ▼
IL-8 (interleukin 8)	12.24 ± 1.3 a	−2.21 ± 0.12 d	−2.10 ± 0.18 d
IL-10 (interleukin 10)	1.00 ± 0.06 nsd	1.01 ± 0.04 nsd	−1.14 ± 0.09 nsd
PTGS1/COX1 (prostaglandin endoperoxide synthase 1/ cyclooxygenase 1	2.18 ± 0.19 a	−1.04 ± 0.11c	−2.60 ± 0.34 d ▼
PTGS2/COX2 (prostaglandin endoperoxide synthase 2/ cyclooxygenase 2	13.80 ± 1.9b	−1.38 ± 0.07c●	1.55 ± 0.34 nsd
TLR2 (toll-like receptor 2)	1.85 ± 0.15b	−1.67 ± 0.09 d	−6.32 ± 0.59 d ▼
TLR3 (toll-like receptor 3)	−2.58 ± 0.24b	−1.52 ± 0.07c	−1.70 ± 0.10c
TNF (tumor necrosis factor alpha)	32.56 ± 2.8 a	−1.99 ± 0.15c	−6.65 ± 0.67 d ▼

Topical application of the DMSO vehicle control versus untreated controls, plus the astaxanthin and equol treatments compared to the DMSO vehicle controls and astaxanthin versus equol levels where appropriate.
a = $p < 0.05$; significantly different than untreated control values.
b = $p < 0.005$; significantly different than untreated control values.
c = $p < 0.05$; significantly different than DMSO control values.
d = $p < 0.005$; significantly different than DMSO control values.
▼= $p < 0.005$; significantly less than astaxanthin values.
► = $p < 0.05$; significantly greater than equol values.
● = $p < 0.05$; significantly less than equol values.
nsd = not significantly different compared to control values.
all data are displayed as the mean ± SEM.

4. Discussion

The purpose of this study was to compare the influence of astaxanthin to that of racemic equol by quantifying the expression of sixty-three skin genes across nine different biomarker categories to determine whether these phytochemicals may benefit skin health by their anti-aging and anti-photo-aging properties. Both astaxanthin and equol are known as potent antioxidant and anti-inflammatory molecules along with many other reported human health benefits (Davinelli et al., 2018; Lephart, 2016; Magnet et al., 2017; Oyama et al., 2012; Volmer et al., 2018). However, while several journal articles/reviews have reported on astaxanthin's positive properties on human dermal health (Chou et al., 2016; Davinelli et al., 2018; Volmer et al., 2018) it is surprising that astaxanthin has not been examined previously in a nutrigenomics perspective of its actions on gene expression of important human skin biomarkers in a comprehensive manner using gene microarray technology. To date, this is the first journal report known to examine astaxanthin in this fashion. In this perspective, it is known that many phytochemicals are active ingredients in topical or oral cosmetics, cosmeceuticals or even treatments for skin cancers that have been used to lessen the burden of clinical skin disorders and disease, which in the U.S. has an estimated direct cost of 75–86 billion USD (Dorni, Amalraj, Gopi, Varma, & Anjana, 2017; Grunebaum & Baumann, 2014; Ijaz et al., 2018; Lim et al., 2017; Namkoong, Kern, & Knaggs, 2018; Penta, Somashekar, & Meeran, 2018; Volmer et al., 2018).

The first skin function category covered four anti-aging genes. Survivin (BIRC5) as an anti-apoptotic molecule important for normal epidermal renewal/homeostasis (Bongiovanni, Muller, & Della Salda, 2011) was significantly stimulated by the equol treatment. Forkhead box 03 (FOXO3) involved in aging and longevity (Kim, Choi, Cho, & Lee, 2014; Martins, Lithgow, & Link, 2016) was not altered by either treatment, and heparan sulfate proteoglycan 2 (HSPG2/PLC) that encodes perlecan, which regulates assembly of ECM components including basement membranes and binds growth factors (Iozzo et al., 1997), was significantly inhibited by the equol treatment. Finally, sirtuin 1 (SIRT1) involved in aging-protecting against cellular senescence (via a pyruvate mitochondrial/lysosomal mechanism in dermal fibroblasts), oxidative stress and inhibiting MMPs (Garcia-Peterson et al., 2017; Kim et al., 2018; Lephart, Sommerfeldt, & Andrus, 2014) was examined. The equol treatment significantly increased survivin while astaxanthin did not, which corresponds to previous findings that suggest equol is involved in fibroblast renewal influencing dermal cell viability (Gopaul et al., 2012). The significantly stimulated SRIT1 levels by 1.8-fold (by equol, while astaxanthin did not) was surprising, since this has not been reported previously, but it is known that equol can enhance the actions of resveratrol in SIRT1 activation to protect dermal components (Lephart, 2017).

In the antioxidant skin function category astaxanthin did significantly alter some of the antioxidant genes (four in total), however, the equol treatment significantly stimulated eight of the biomarkers above that of the astaxanthin results. Several of the endpoints (either directly or indirectly) have been reported previously for both astaxanthin and equol that correspond to the present findings (e.g., glutathione peroxidase 1 (GPX1), metallothionein 1 A and 2 A (MT1A and

TABLE 4. Summary: Human Skin Gene Expression - Effects of Astaxanthin vs. Equol by Skin Function

	Gene Symbol: name	Skin Function	References
Antiaging	1 BIRC5: survivin	normal epidermal homeostasis, prevents sun damage	Bongiovanni 2011
	2 FOXO3:forkhead box O3	longevity/apoptosis/senescence & anti-melangoenic	Kim 2014, Martins 2016
	3 HSPG2/PLC: heparan sulfate proteoglycan2	component of basement membranes & binding factor	Iozzo 1996
	4 SIRT1: sirtuin1	anti-aging, repair, ↓MMPs via increasing TIMP	Garcia-Peterson 2017
Antioxidants	5 AHR: aryl hydrocarbon receptor	phytochemicals bind AhR & activate Nrf2/antioxidants	Furue 2014
	6 ARNT: aryl hydrocarbon recept. translocator	binds to AhR involved in ROS production/activates EGFR	Furue 2014
	7 CAT: catalase	important detoxifying enzyme of reactive oxygen species	Lephart 2016
	8 GPX1: glutathione peroxidase1	important detoxifying enzyme of reactive oxygen species	Lephart 2016
	9 HMOX1: heme oxygenase1	mediates oxidative stress, activated by Nrf2	Numata 2009, Vile 1999
	10 MT1A: metallothionein1 A	blocks metal toxicity & oxidative stress/photo-protective	Lephart 2016
	11 MT2A: metallothionein2A	blocks metal toxicity & oxidative stress/photo-protective	Lephart 2016
	12 SOD1: superoxidase dismutase1	important detoxifying enzyme of reactive oxygen species	Lephart 2016
	13 SOD2: superoxidase dismutase2	important detoxifying enzyme of reactive oxygen species	Lephart 2016
	14 TXN: thioredoxin	protects against free radicals, oxidative stress/UV damage	Arner 2009
	15 TXNRD1: thioredoxin reductase1	protects against free radicals, oxidative stress/UV damage	Lephart 2016
Growth Factors	16 BMP2: bone morphogenetic protein2	skin morphogenesis/repair/wound healing	Botchkarev 2003, Liang 2016
	17 BMP4: bone morphogenetic protein4	associated with skin brightening/pigmentation	Singh 2012
	18 CTGF/CCN2: connect tissue growth factor	collagen and fibronectin production & repair	Kiwanuka 2013, Oliver 2010
	19 EDN1: endoththelin1	regulates UV-induced melanocyte homeostasis	Hyter 2013, Zhang 2013
	20 EGFR: epidermal growth factor receptor	involved in skin homeostasis/ repair and inflammation	Pastrore 2008
	21HBEGF: heparin-bind EGF-growth factor	promotes keratinocyte migration/wound healing	Shiradata 2005, Stoll 2012
	22 ICAM1: intercellular adhesion molecule 1	cell adhesion/transmigration during inflammation/repair	Gay 2011
	23 KITLG/SCF: kit ligand/stem cell factor	regulates melanogenesis/ pigmentation / ↑mast cells	Picardo 2011
Barrier	24 FLG: filaggrin	assembly of keratins/↓ levels..↑ natural moisturizing	Brown 2012, Hoste 2011
	25 ITGB1: integrin beta 1	decreases with aging/links ECM to cytoskeleton	Giangreco 2009, Hegde 2013
	26 ITGB4: integrin beta 4	decreases with aging/links ECM to cytoskeleton	Giangreco 2009, Hegde 2013
	27 KRT5: keratin 5	with KRT14- filament assembly/differentiation	Alam 2011, Bouameur 2014
	28 KRT14: keratin 14	intermediate filament assembly/proliferation	Alam 2011, Bouameur 2014
Hydration	29 AQP3: aquaporin3	channels transport water & glycerol (epidermis)	Qin 2011
	30 CD44: hyaluronic acid receptor	cell adhesion, migration, lymphocyte activation	Papakonstaninou 2012
	31 HAS2: hyaluronic acid synthase2	enzyme that makes hyaluronic acid for lubrication	Li 2017
	32 SMPD1: sphingomyelin phosphodieasease1	converts sphingomyelin to ceramide/UV protection	Breiden 2014

(continued on next page)

Cell Regen.	33 CASP3: caspase3	antiapoptotic/protects stressed tissues against cell death	Khalil 2012
	34 PCNA: proliferating cell nuclear antigen	dermal protection, DNA repair & decreases with aging	Lephart 2016
	35 TGFB1: transforming growth factor beta1	regulates ECM collagen/remodeling/wound healing	Zaher 2009, Lephart 2018a
	36 TP63: protein63	maintains cell proliferation/ cell adhesion & differentiation	Antonini 2008
ECM Integrity	37 COL1A1: collagen, type1, alpha1	primary dermal structural protein, decreases with aging	Arseni 2018
	38 COL17A1: collagen, type17, alpha1	structural protein at epidermal/dermal membrane zone	Loffek 2014
	39 ELN: elastin	primary elastic fibers maintain skin shape, ↓ with age	Lephart 2016
	40 TIMP1:tissue inhibitor metalloproteinase1	inhibits MMPs, protects collagen, ↓ with aging	Hornebeck 2003, Lephart 2016
	41 SERPINH1: serpin peptidase inhibitorH1	or heat shock protein 47 involved in collagen synthesis	Windmer 2012
	42 VCAN: versican	a proteoglycan forms lattice/assembly of proteins for stability	Carrino 2000
ECM Breakdown	43 F2RL1/PAP2: protease-activated receptor2	proteolytic/stimulates IL-8/proinflammatory	Hou 1998, Rothmeier 2012
	44 KLK5: kallirein-related peptidase5	proteolytic/digestion: collagens, fibronectin & laminin	Yamaski 2016
	45 KLK7: kallirein-related peptidase7	IL-4 & IL-13 ↑ KLK7 which ↓ flaggrin (atopic dermatitis)	Morizane 2012
	46 MMP1: matrix metalloproteinase1	breaksdown collagens: I, II, III, VII, and X	Pittayapruek 2016, Seltzer 2003
	47 MMP2: matrix metalloproteinase2	gelatinase, degrades collagen IV, V, VII, elastin & laminin	Seltzer 2003
	48 MMP9: matrix metalloproteinase9	degrades collagens: IV, V, VII, X, XIV, elastin & fibronectin	Seltzer 2003
	49 SERPINB3: serpin peptidase inhibitorB3	↑ levels are proinflammatory/associated with psoriasis	Sivaprasad 2015
	50 SPINK5: serine peptidase inhibitor	encodes LEKTI which can inhibit KLK5 & KLK7	Briot 2009, Eissa 2008
Inflammation/Immune	51 CSF2/GM-CSF: colony stimulating factor2	pro-inflammatory/immune & ECM modulator	Hamilton 2008, Mascia 2010
	52 DEFB1: defensin, beta 1	antimicrobial, modulate immune, inflammation	van Kilsdonk 2017
	53 IFNA: interferon alpha	anti-proliferative, anti-angiogenesis, immune modulator	Ismail 2014
	54 IL1A: interleukin1 alpha	proinflammatory, synergism with TNF, immune effects	Lephart 2016
	55 IL1B: interleukin 1 beta	proinflammatory, activates NFk-B & AP-1 & skin diseases	Jensen 2010
	56 IL-6: interleukin 6	proinflammatory, ↑ with aging & UV exposure	Tanaka 2014
	57 IL-8: interleukin 8	proinflammatory, ↑ with aging & UV exposure	Jiang 2012
	58 IL-10: interleukin 10	anti-inflammatory cytokine, regulates immune response	Saxena 2015
	59 PTGS1/COX1: cyclooxygenase1	proinflammatory, formation of prostaglandins/edema	Gopaul 2012
	60 PTGS2/COX2: cyclooxygenase2	proinflammatory, formation of prostaglandins/edema	Lee 2003
	61 TLR2: toll-like receptor2	detect microbial elements/immune & inflammation	Hari 2010, Miller 2008
	62 TLR3: toll-like receptor3	mediates UVB-induced MMP-3 & MMP-13 expression	Yao 2017
	63 TNF: tumor necrosis factor alpha	inflammatory, ↑ with UVB exposure & psoriasis	Bashir 2009

Each gene symbol/name is color-coded indicating whether **Astaxanthin** or **Equol** displayed the greatest (stimulation or inhibition) compared to each other via pairwise comparisons [subsequent to testing against DMSO control values ($p < 0.005$)]. Each gene symbol/name in **black** font indicates that no significant difference between the Astaxanthin vs. the Equol values was detected. The results for all the genes (mean ± SEM) are displayed in Figs. 2–4 and Tables 2 and 3. A total of 90 target genes were tested, n = 6 per treatment group, 63 genes are reported here (70% of the total number of genes tested). Five 5 control genes were analyzed where GUSB was the most stable endogenous control gene. Antiaging genes # 1–4; Antioxidant genes # 5–15; Growth Factor genes # 16–23; Epidermal Barrier genes # 24–28; Hydration genes # 29–32; Cell Renewal/Regeneration genes # 33–36; Extracellular Matrix Integrity genes # 37–42; Extracellular Breakdown genes # 43–50; and Inflammation/Immune genes # 51–63. To the right of each gene symbol/name its skin function is displayed along with the associated cited reference(s) by first author's surname (family or last name) and the date published.

MT2A), superoxide dismutase 1 and 2 (SOD1 and SOD2), thioredoxin (TXN) and thioredoxin reductase 1 (TXNRD1), which all have important antioxidant properties such as ROS-/oxidative stress- and UV-protection, activating Nrf2, the master gene for antioxidants and, blocking metal toxicity (Arner, 2009; Chou et al., 2016; Davinelli et al., 2018; Gopaul et al., 2012; Lephart, 2016, 2017; Meephansan et al., 2017; Suganuma et al., 2010; Volmer et al., 2018). Interestingly, equol stimulated the oxidative stress target gene heme oxygenase 1 (HMOX1), and in this regard, the aryl hydrocarbon receptor (AHR) was increased, which in turn is known to in induce the cytochrome P450 CYP1A1 gene, however, certain phytochemicals have been shown to activate both the thioredoxin genes (TXN and TXNRD1) in this pathway along with Nrf2 to stimulate antioxidant production in the mechanism associated with ROS protection, protein repair and redox hemostasis (Arner, 2009; Furue, Takahara, Nakahara, & Uchi, 2014; Lephart, 2016, 2017; Namkoong et al., 2018; Numata et al., 2009; Vile, Basu-Modak, Waltner, & Tyrrell, 1994).

Platelet-rich plasma (PRP) is a current popular treatment for a broad spectrum of medical conditions including facial aesthetics and alopecia due to the abundance of growth factors in these preparations (Alver & Grimalt, 2018; Motosko, Khouri, Poudrier, Sinno, & Hazen, 2018). For the growth factor skin function category, 8 genes out of 9 tested were reported in the present study, where both the astaxanthin and equol treatments significantly increased the expression of bone morphogenetic protein 2 (BMP2) involved in skin repair and wound healing (Botchkarev, 2003; Liang et al., 2016), and intercellular adhesion molecule 1 (ICAM1), which promotes cell adhesion and transmigration during inflammation and tissue repair/healing (Gay et al., 2011). However, four growth factor genes in the equol-treated group displayed significantly greater stimulation of these biomarkers compared to the astaxanthin treatment, which included: (a) bone morphogenetic protein 4 (BMP4) that is associated with skin brightening (Singh, Abbas, & Tobin, 2012), (b) connective tissue growth factor (CTGF/CCN2) involved in collagen and fibronectin production and repair (Kiwanuka et al., 2013; Oliver, Stemlicht, Gerritsen, & Goldschmeding, 2010), (c) endothelin 1 (EDN1) that regulates UV-induced melanocyte homeostasis (Hyter et al., 2013; Zhang et al., 2013) and, (d) ICAM (Gay et al., 2011, see above). Exceptionally, heparin-binding EGF-like growth factor (HBEGF) that promotes keratinocyte migration and wound healing was the only biomarker that the astaxanthin treatment significantly stimulated above the equol-treated skin cultures (Shirakata et al., 2005; Stoll, Rittie, Johnson, & Elder, 2012). Thus, both phytochemicals were able to stimulate the growth factor biomarkers, but the equol treatment apparently was more effective compared to astaxanthin in this regard.

While not the focus of the present study, the epidermal barrier, hydration and cell renewal and cell regeneration categories were tested, and the obtained results between the astaxanthin and equol treatments

were somewhat mixed. Two epidermal barrier biomarkers: (a) integrin beta 4 (ITGB4) involved in linking ECM components to the cellular cytoskeleton, which is also known to decrease with aging, and (b) keratin 14 (KRT14) that makes up intermediate filament assembly for maintaining cell resilience and cytoarchitecture (Alam et al., 2011; Bouameur et al., 2014; Hegde & Raghavan, 2013) were significantly stimulated by only the equol treatment.

For the hydration biomarkers, aquaporin 3 (AQP3), is an aquaglyceroporin that transports water and glycerol and is expressed in the epidermis among other epithelial tissues, was significantly stimulated by astaxanthin above that of equol values (Qin et al., 2011). On the other hand, the equol treatment significantly stimulated hyaluronic acid receptor (CD44), which is a transmembrane glycoprotein that regulates cell adhesion and migration (Papakonstantinou, Roth, & Karakiulakis, 2012).

The cell renewal and cell regeneration biomarker results were also mixed, where the equol treatment only significantly increased caspase 3 (CASP3) and proliferating cell nuclear antigen (PCNA) levels, which are known to be anti-apoptotic and protect stressed tissues against cell death and mediate dermal protection via DNA repair (Khalil et al., 2012; Lephart, 2016). The PCNA results in the present study confirm previous findings (Gopaul et al., 2012; Lephart, 2013). The astaxanthin treatment alone significantly stimulated transforming growth factor beta 1 (TGFB1) expression, which is known to regulate ECM collagen, remodel cellular components and advance wound healing that are mediated via smad2/3 and smad7 (Lephart, 2018a; Park et al., 2017; Zaher et al., 2009), while keratinocytes are known to suppress TGFB1 from dermal fibroblasts (Le Poole & Boyce, 1999).

Remarkably, all the ECM integrity genes were significantly stimulated, while all the ECM breakdown biomarkers were significantly inhibited by the equol treatment. It was not that astaxanthin did not stimulate elastin levels or inhibit many of the ECM breakdown biomarkers, but overall equol's impact was significantly greater compared to the astaxanthin treatment. For example, the collagens (COL1A1 and COL17A1), elastin (ELN), tissue inhibitor of metalloproteinase 1 (TIMP1) were stimulated (Arseni, Lombardi, & Orioli, 2018; Hornebeck, 2003; Lephart, 2016; Loffek et al., 2014), and the matrix metalloproteinases (MMP 1, MMP 2 and MMP 9) that are known to degrade collagens and elastin were inhibited by the equol treatment (Pittayapruek, Meephansan, Prapapan, Komine, & Ohtsuki, 2016; Seltzer & Eisen, 2003). These present findings confirm equol's influence on these parameters compared to previous reports (Gopaul et al., 2012; Lephart, 2013, 2018a) and extend its positive impact on ECM integrity biomarkers such as serpin peptidase inhibitor H1 (SERPINH1) and versican (VCAN) (Carrino, Sorrell, & Caplan, 2000; Widmer et al., 2012). Whereas, at the same time, the equol treatment significantly inhibited many of the ECM breakdown biomarkers (i.e., F2FL1/PAP2, KLK 5 and 7, SERPINB3 and SPINK5) compared to the astaxanthin treatment, which known to have a negative impact on ECM components/structural proteins (Briot et al., 2009; Eissa & Diamandis, 2008; Hou et al., 1998; Morizane et al., 2012; Rothmeier, 2012; Sivaprasad et al., 2015; Yamaski, 2016).

Also, it is well established that skin aging is caused by ROS/oxidative stress and environmental factors like air pollution (Lephart, 2016, 2018a; Park, Byun, Lee, Kim, & Kim, 2018). For the inflammation and immune biomarkers the equol treatment significantly inhibited 6 genes, while astaxanthin inhibited 2 genes [defensin, beta 1, (DEFB1) and cyclooxygenase 2 (COX 2)] that are known for their proinflammatory actions (Lee, Mukthar, Bickers, Koplovich, & Athar, 2003; Van Kilsdonk et al., 2017). In general, the inhibition of gene expression by the equol treatment of interleukin 1 alpha (IL1A), interleukin-6 (IL-6), cyclooxygenase 1(COX1) and tumor necrosis factor alpha (TNF) in the present study was similar to other reported findings (Bashir, Sharma, & Werth, 2009; Gopaul et al., 2012; Lephart, 2013). Additional new findings include the pro-inflammatory and ECM modulator, colony stimulating factor 2 (CSF2/GM-CSF) and toll-like receptor 2 (TLR2) that can modulate MMP-3 and MMP-13 expression, which were significantly inhibited by the equol treatment (Hamilton, 2008; Hari, Flach, Shi, & Regine Mydlarski, 2010; Mascia et al., 2010; Miller, 2008; Scholz et al., 2017; Stamatas, Morello, & Mays, 2013; Yao et al., 2017). Again, it must be pointed out that, while astaxanthin is a good anti-inflammatory agent, as reported by others (Chou et al., 2016; Davinelli et al., 2018; Volmer et al., 2018), equol's inhibitory influence on the parameters was greater as far as the number and degree of impact of the genes that were influenced.

Finally, the bioavailability of topically applied racemic equol has been reported in detail in human skin percutaneous absorption studies, where it has an epidermal 'reservoir" delivery mechanism that provides a sustained release into the dermis up to 28 h after a single topical application (Lephart, 2013). In fact, it was determined that approximately 14 nM of racemic equol was delivered after a single dose *in vitro* into the keratinocytes compared to cell culture results that showed exposure to 10 nM of racemic equol in primary human fibroblasts significantly stimulated collagen and elastin and at the same time inhibited MMPs (Lephart, 2016). In confirmation with the above findings a recent clinical study by Magnet et al. (2017) showed that topically applied racemic equol after 8 weeks improved structural and molecular skin parameters in women (for: roughness, texture, smoothness, firmness, elasticity & decreased methylation and teleomere length in skin cells). Also, in this clinical study, the women did not show a significant difference in topically applied equol verses micro-encapsulated equol, suggesting the delivery was not enhanced by microencapsulation (Magnet et al., 2017). Unfortunately, "limited literature evidence devoted to showing improvements in astaxanthin bioavailability reveals that the enhancement of astaxanthin bioavailability has not gained significant attention, especially for skin tissue." (Davinelli et al., 2018). Therefore, there is not comparable data for the characteristics of astaxanthin bioavailability in human skin.

5. Conclusions

Human skin fibroblasts secrete up to 998 proteins, and recent data suggests that skin fibroblast genomic biomarkers can be a useful tool for predicting biological age in humans (Fleischer et al., 2018; Waldera Lupa et al., 2015). Using this perspective, this study demonstrates that astaxanthin and equol are skin anti-aging phytochemical compounds by altering the expression of human dermal-related genes via microarray analysis. However, in the analysis of 63 biomarkers across nine different skin function categories in 39 genes the equol treatment significantly altered (increased or decreased) the parameters in a positive manner compared to astaxanthin (see Table 4). Conversely, the astaxanthin treatment significantly influenced 6 dermal genes compared to equol-treated skin cultures, while 18 of the skin biomarkers were not significantly different between the two treatment groups. Specifically, the obtained results revealed significantly greater effects of equol compared to astaxanthin for the antioxidants, growth factors, extracellular integrity and extracellular breakdown, and the inflammatory biomarkers. These findings indicate that equol's efficacy is greater than astaxanthin for various skin biomarkers and suggest that equol may be incorporated into topical and oral applications to improve skin health and reduce photo-aging.

6. Ethics statement

My research did not include any human subjects and animal experiments.

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Declaration of interest

The author declares no corporate/business, funding or founder sponsor conflict of interest in the decision of the data/research presented in this report and regarding the publication of this manuscript. The author is an inventor on equol and resveratrol analog patents (U.S. and worldwide) on various human health applications.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2019.03.025.

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