Micro RNA Expression after Ingestion of Fucoidan; A Clinical Study

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Abstract: Fucoidans are a class of fucose-rich sulfated polysaccharides derived from brown macroalgae that exert a range of biological activities in vitro and in vivo. To generate an unbiased assessment of pathways and processes affected by fucoidan, a placebo-controlled double-blind pilot study was performed in healthy volunteers. Blood samples were taken immediately before and 24 h after ingestion of a single dose of 1 g of Undaria pinnatifida fucoidan (UPF) or placebo. Levels of isolated miRNAs were analyzed using Taqman Open Array Human MicroRNA panels. Out of 754 miRNAs screened, UPF affected a total of 53 miRNAs. Pathway analysis using the TALOS data analysis tool predicted 29 different pathways and processes that were largely grouped into cell surface receptor signaling, cancer-related pathways, the majority of which were previously associated with fucoidans. However, this analysis also identified nine pathways and processes that have not been associated with fucoidans before. Overall, this study illustrates that even a single dose of fucoidans has the potential to affect the expression of genes related to fundamental cellular processes. Moreover, it confirms previous data that fucoidans influence immunity, cancer cells, inflammation, and neurological function.

Keywords: fucoidan; clinical; microRNA, anti-inflammatory, anti-cancer

1. Introduction

Fucoidans, a class of fucose-rich sulfated polysaccharides, are derived from brown macroalgae and are associated with a large range of biological activities [1,2]. As a naturally occurring part of edible seaweed, it is part of the normal diet in many countries and consequently some fucoidan extracts have obtained ‘generally recognized as safe’ (GRAS) status in the US and received ‘novel foods’ approval in the EU. Fucoidans have long been noted as a selectin blocking compound that inhibits cell–cell interactions. This ability to disrupt cell–cell interactions is likely, at least in part, responsible for the potent anti-inflammatory activity of different fucoidan preparations. Fucoidans can also inhibit the adhesion of proteins and organisms to non-biological surfaces, and may help to inhibit biofouling [3]. Previous data using a yeast deletion library illustrated that fucoidans can interact with more fundamental cellular pathways than previously anticipated [4]. Not only did the
yeast study confirm that cell surface signaling pathways are affected by fucoidan, this study identified many additional fundamental intracellular pathways such as ribosome biogenesis, peroxisome biogenesis, DNA damage repair, cell cycle control and energy metabolism, just to name a few. These results suggested that we have only begun to understand the breadth of interactions that fucoidans can have with biological systems.

Fucoidans and fucoidan-rich foods are widely consumed in different areas of the world, either unintentionally in the form of algae-containing food products or intentionally based on perceived, anecdotal, or experimental evidence of biological effects. However, most intentional consumers of fucoidan products aim to alleviate or prevent very specific health related issues. Together with uncontrolled access to these products, this prevents a broader, unbiased understanding of the different effects that fucoidans can elicit in human consumers, both in terms of therapeutic activities as well as unanticipated effects that might remain unreported. Therefore, the current pilot study set out to provide a first unbiased insight how one particular fucoidan preparation from Undaria pinnatifida (UPF) would affect the plasma microRNA (miRNA) composition in healthy individuals.

miRNAs are small non-coding RNA molecules that are evolutionally conserved and are known to affect gene expression by different mechanisms [5]. Therefore, miRNAs are currently widely studied as biomarkers for a large range of indications including cancer, cardiovascular disease, obesity, inflammation, osteoporosis, and neurodegenerative diseases [6]. Moreover, far from simple biomarkers for some miRNAs, a direct pathological function has been established, which provides the opportunity to address them as therapeutic targets [6]. Nutrients can affect the serum expression of microRNAs, either exogenous or endogenous [7]. Currently there is only scarce information published on the effects of fucoidan on miRNA expression. A single study implicated one specific miRNA in the anti-tumor activity of fucoidan [8]. In this study, Sargassum hemiphyllum fucoidan upregulated miRNA29b in human hepatocarcinoma cells, which was associated with a reduction in cell growth, colony formation, and invasiveness. The authors proposed that miRNA29b was partially responsible for this effect by suppressing its downstream target DNMT3B, which increased the metastasis suppressor MTSS1 and inhibited Epithelial to Mesenchymal Transition (EMT) [8]. In a second study, Sargassum hemiphyllum fucoidan reportedly downregulated miRNA-29c and upregulated miRNA-17-5p, which was also associated with a suppression of EMT in breast cancer cell lines in vitro, while cell survival was reduced by activation of the IP3K/Akt pathway [9]. However, how fucoidan in both studies increased miRNA-29 expression remains unexplained so far. Beyond these in vitro studies, no information is currently available on other fucoidan miRNA interactions. Therefore, the current pilot study provides some information, which circulating miRNAs are affected in the plasma of healthy volunteers by exposure to a single oral dose of UPF. Using pathway analysis of the UPF-induced miRNA changes, our results substantiate the anti-cancer effects of fucoidan and a range of other activities that have been associated with fucoidan previously. In addition, the present study also identified novel pathways not formerly associated with fucoidan.

2. Results

In total, 754 miRNAs were screened for this analysis. When human plasma miRNAs were compared between baseline (0 h) and 24 h post-treatment, a total of 63 miRNAs were found to be differentially expressed in the placebo-treated individuals (19 up-regulated, 44 down-regulated). In comparison, in the UPF-treated individuals, 53 miRNA were identified to be differentially regulated (15 up-regulated, 38 down-regulated) in plasma (Table 1). The miRNAs that were differentially expressed between the placebo and UPF groups were also assessed. For the significantly upregulated miRNAs, only one miRNA (hsa-miR-34b) was common to both the placebo and UPF groups, while for the significantly downregulated miRNAs, 5 (hsa-miR-369-3p, hsa-miR-500, hsa-miR-548a, hsa-miR-548d-5p, hsa-miR-886-3p) were common to both the placebo and UPF groups. The remaining miRNAs that were either upregulated or downregulated were unique to each of the groups.

Using TALOS (v2) software, several cellular pathways were identified that could likely be affected by these miRNAs (Table 2). For the plasma of placebo-treated individuals, 13 pathways were identified, while the analysis of the plasma miRNAs of UPF-treated individuals identified 39
potentially affected pathways. Since some pathways overlapped between the placebo- and UPF-treated samples, these were excluded to highlight the 31 pathways that were selectively associated with UPF-exposure (Table 3). While the pathway analysis predicted several pathways that have been associated with fucoidans before, it also predicted pathways and processes not formerly associated with fucoidans (Table 3).

**Table 1.** Significantly up- or down-regulated miRNAs (basal vs. 24 h post treatment).

| Placebo | UPF |
|---------|-----|
| **Up-regulated** | **Down-regulated** | **Up-regulated** | **Down-regulated** |
| Name | fold* | Name | fold* | Name | fold* | Name | fold* |
| hsa-miR-584 | 14.068 | hsa-miR-193b# | 13.219 | hsa-miR-1247 | 13.767 | hsa-miR-183# | 17.262 |
| hsa-miR-9 | 13.72 | hsa-miR-500 | 13.17 | hsa-miR-200b | 12.888 | hsa-miR-107 | 16.182 |
| hsa-miR-656 | 13.528 | hsa-miR-199a | 13.12 | hsa-miR-1180 | 12.714 | hsa-miR-339-5p | 15.277 |
| hsa-miR-485-3p | 12.983 | hsa-miR-326 | 12.827 | hsa-miR-623 | 11.927 | hsa-miR-335# | 13.787 |
| hsa-miR-34b | 11.862 | hsa-miR-502 | 12.712 | hsa-miR-135a | 11.879 | hsa-miR-299-5p | 12.97 |
| hsa-miR-605 | 11.565 | hsa-miR-381 | 12.058 | hsa-miR-1303 | 11.094 | hsa-miR-199b | 12.227 |
| hsa-miR-29c# | 10.092 | hsa-miR-213 | 11.713 | hsa-miR-380-5p | 9.369 | hsa-miR-369-3p | 11.453 |
| hsa-miR-99a# | 10.85 | hsa-miR-369-3p | 11.527 | hsa-miR-518e | 7.745 | hsa-miR-29b-2# | 11.401 |
| hsa-miR-361-3p | 10.017 | hsa-miR-1 | 11.215 | hsa-miR-454# | 6.642 | hsa-miR-27a# | 11.007 |
| hsa-miR-30d# | 9.587 | hsa-miR-886-3p | 11.196 | hsa-miR-34b | 3.802 | hsa-miR-520h | 10.762 |
| hsa-miR-196b | 8.419 | hsa-miR-424 | 10.948 | hsa-miR-638 | 3.667 | hsa-miR-500 | 10.741 |
| hsa-miR-651 | 7.135 | hsa-miR-363# | 10.611 | hsa-miR-517c | 3.016 | hsa-miR-9# | 10.691 |
| hsa-miR-18a# | 5.996 | hsa-miR-301b | 10.506 | hsa-miR-502-3p | 2.934 | hsa-miR-886-3p | 10.258 |
| hsa-miR-9f# | 5.242 | hsa-miR-337-5p | 10.262 | hsa-miR-662 | 2.427 | hsa-miR-487a | 10.243 |
| hsa-miR-452 | 5.228 | hsa-miR-362-3p | 10.232 | hsa-miR-512-3p | 2.411 | hsa-miR-20a# | 9.263 |
| hsa-miR-324-3p | 5.154 | hsa-miR-184 | 10.091 | hsa-miR-542-5p | 9.079 |
| hsa-miR-365 | 3.769 | hsa-miR-876-5p | 10.011 | hsa-miR-548a | 7.654 |
| hsa-miR-454 | 3.625 | hsa-miR-376b | 9.925 | hsa-miR-548d-5p | 7.262 |
| hsa-miR-494 | 2.905 | hsa-miR-548d-5p | 9.678 | hsa-miR-520f | 6.821 |
| hsa-miR-450a | 9.491 | hsa-miR-486-3p | 5.978 |
| hsa-miR-362 | 8.547 | hsa-miR-590-3p | 3.606 |
| hsa-miR-1285 | 8.325 | hsa-miR-296 | 3.308 |
| hsa-miR-545# | 8.313 | hsa-miR-509-5p | 3.284 |
| hsa-miR-517c | 8.044 | hsa-miR-125a-5p | 3.194 |
| hsa-miR-517b | 7.977 | hsa-miR-28-3p | 3.083 |
| hsa-miR-454# | 7.972 | hsa-miR-342-5p | 3.071 |
| hsa-miR-214# | 7.444 | hsa-miR-489 | 2.897 |
| hsa-miR-519e# | 7.273 | hsa-miR-17df# | 2.741 |
| hsa-miR-579 | 3.677 | hsa-miR-34a | 2.505 |
| hsa-miR-135a | 3.402 | hsa-miR-203 | 2.487 |
| hsa-miR-501-3p | 3.177 | mmu-miR-374-5p | 2.410 |
| hsa-miR-29c | 3.123 | hsa-miR-652 | 2.388 |
| hsa-miR-218 | 2.999 | hsa-miR-339-3p | 2.197 |
| hsa-miR-512-3p | 2.908 | hsa-miR-1249 | 2.193 |
| hsa-miR-520e | 2.54 | hsa-miR-31 | 2.174 |
| hsa-miR-1180 | 2.488 | hsa-miR-551b | 2.114 |
| hsa-miR-522 | 2.46 | hsa-miR-145# | 2.029 |
| hsa-miR-133a | 2.456 | hsa-miR-411 | 2.024 |
| hsa-miR-548a | 2.439 |
| hsa-miR-425# | 2.438 |
| hsa-miR-664 | 2.403 |
| hsa-miR-93# | 2.173 |
| mmu-miR-491 | 2.145 |
| hsa-miR-625# | 2.074 |

* Only fold changes >2 were considered in the analysis.
Table 2. Predicted pathways (basal vs. 24 h post treatment).

| Source   | Name                                           | Placebo E   | p-value       |
|----------|------------------------------------------------|-------------|---------------|
| kegg     | Signaling pathways regulating pluripotency    | 1.770       | 9.1 × 10⁻⁴   |
| wp       | Nuclear Receptors                             | 2.871       | 1.5 × 10⁻⁴   |
| kegg     | Axon guidance                                 | 1.761       | 1.9 × 10⁻⁴   |
| wp       | TGF Beta Signaling Pathway                    | 2.331       | 2.1 × 10⁻³   |
| wp       | Mesodermal Commitment Pathway                 | 1.657       | 2.4 × 10⁻³   |
| wp       | BMP Signalling and Regulation                 | 6.252       | 4.5 × 10⁻³   |
| wp       | Leptin signaling pathway                      | 1.875       | 9.1 × 10⁻³   |
| wp       | Endoderm Differentiation                      | 1.576       | 9.4 × 10⁻³   |
| kegg     | Thyroid cancer                                | 2.566       | 1.6 × 10⁻²   |
| kegg     | Pancreatic cancer                             | 1.849       | 1.7 × 10⁻²   |
| wp       | Wnt Signaling Pathway and Pluripotency        | 1.613       | 2.2 × 10⁻²   |
| wp       | Serotonin Receptor 4-6-7 and NR3C Signaling   | 2.865       | 3.4 × 10⁻²   |
| kegg     | Non-small cell lung cancer                    | 1.808       | 3.6 × 10⁻²   |

| Source   | Name                                           | UPF-treated E | p-value       |
|----------|------------------------------------------------|---------------|---------------|
| wp       | BDNF signaling pathway                         | 2.481         | 1.0 × 10⁻³   |
| wp       | EGF-EGFR Signaling Pathway                     | 2.271         | 1.6 × 10⁻³   |
| kegg     | Axon guidance                                  | 2.208         | 2.2 × 10⁻⁴   |
| kegg     | ErB signaling pathway                          | 2.568         | 4.7 × 10⁻⁴   |
| kegg     | Endocytosis                                    | 1.671         | 1.1 × 10⁻³   |
| wp       | Insulin Signaling                              | 1.812         | 1.2 × 10⁻³   |
| kegg     | Signaling pathways regulating pluripotency    | 1.837         | 1.9 × 10⁻³   |
| wp       | Endochondral Ossification                      | 2.699         | 2.0 × 10⁻³   |
| kegg     | Focal adhesion                                 | 1.605         | 2.8 × 10⁻³   |
| kegg     | Prostate cancer                                | 2.150         | 3.0 × 10⁻³   |
| wp       | ErbB Signaling Pathway                         | 2.801         | 3.3 × 10⁻³   |
| wp       | TSH signaling pathway                          | 2.491         | 3.6 × 10⁻³   |
| kegg     | Renal cell carcinoma                           | 2.312         | 6.3 × 10⁻³   |
| wp       | TGF beta Signaling Pathway                     | 1.728         | 6.8 × 10⁻³   |
| kegg     | Glioma                                         | 2.267         | 7.9 × 10⁻³   |
| kegg     | Rapl signaling pathway                         | 1.500         | 9.1 × 10⁻⁵   |
| wp       | Leptin signaling pathway                       | 2.087         | 9.4 × 10⁻⁵   |
| kegg     | Adherens junction                              | 2.050         | 1.2 × 10⁻²   |
| wp       | MAPK Cascade                                   | 3.885         | 1.2 × 10⁻²   |
| kegg     | Chronic myeloid leukemia                       | 2.050         | 1.2 × 10⁻²   |
| wp       | Wnt Signaling Pathway and Pluripotency         | 1.797         | 1.2 × 10⁻²   |
| wp       | Regulation of Microtubule Cytoskeleton         | 2.557         | 1.4 × 10⁻²   |
| kegg     | Proteoglycans in cancer                        | 1.462         | 1.6 × 10⁻²   |
| wp       | Mesodermal Commitment Pathway                  | 1.558         | 1.6 × 10⁻²   |
| wp       | Oncostatin M Signaling Pathway                 | 2.074         | 1.6 × 10⁻²   |
| wp       | Signaling Pathways in Glioblastoma            | 1.868         | 1.6 × 10⁻²   |
| wp       | Angiogenesis                                   | 5.592         | 1.8 × 10⁻²   |
| kegg     | Circadian rhythm                               | 3.232         | 1.9 × 10⁻²   |
| kegg     | Neurotrophin signaling pathway                 | 1.642         | 1.9 × 10⁻²   |
| kegg     | FoxO signaling pathway                         | 1.574         | 2.2 × 10⁻²   |
| kegg     | Acute myeloid leukemia                         | 2.105         | 2.3 × 10⁻²   |
| kegg     | Pathways in cancer                             | 1.273         | 2.9 × 10⁻²   |
| kegg     | TGF-beta signaling pathway                     | 1.748         | 3.5 × 10⁻²   |
| wp       | Androgen receptor signaling pathway            | 1.685         | 3.6 × 10⁻²   |
| kegg     | Hippo signaling pathway                        | 1.462         | 3.8 × 10⁻²   |
| kegg     | Long-term potentiation                         | 1.829         | 4.0 × 10⁻²   |
| kegg     | Estrogen signaling pathway                     | 1.600         | 4.3 × 10⁻²   |
| wp       | Physiological and Pathological Hypertrophy of  | 3.106         | 4.9 × 10⁻²   |
| wp       | the Heart                                      | 1.793         | 4.9 × 10⁻²   |
Table 3. Predicted pathways selective for UPF-treatment (basal vs. 24 h post treatment).

| Source | Signaling Pathways | Prior evidence | References | p-value |
|--------|--------------------|----------------|------------|---------|
| wp     | BDNF signaling pathway | Yes | Reid and Lee [10,11] | $1.0 \times 10^{-5}$ |
| wp     | EGF-EGFR Signaling Pathway | Yes | Wang [12] | $1.6 \times 10^{-5}$ |
| kegg   | ErbB signaling pathway | Yes | Thakur [13] | $4.7 \times 10^{-4}$ |
| wp     | Insulin Signaling | Yes | Wang, Sim [14,15], Wright [16], Hernandez [17] | $1.2 \times 10^{-3}$ |
| wp     | TSH signaling pathway | No | - | $3.6 \times 10^{-3}$ |
| kegg   | Rap1 signaling pathway | No | - | $9.1 \times 10^{-3}$ |
| wp     | MAPK Cascade | Yes | Sharma [18] Sun [19] Che [20] | $1.2 \times 10^{-2}$ |
| wp     | Oncostatin M Signaling Pathway | No | - | $1.6 \times 10^{-2}$ |
| kegg   | Neurotrophin signaling pathway | No | - | $1.9 \times 10^{-2}$ |
| kegg   | FoxO signaling pathway | No | - | $2.2 \times 10^{-2}$ |
| kegg   | Hippo signaling pathway | No | - | $3.8 \times 10^{-2}$ |
| kegg   | Estrogen signaling pathway | Yes | Zhang [21] | $4.3 \times 10^{-2}$ |

**Cellular Processes**

| Source | Signaling Pathways | Prior evidence | References | p-value |
|--------|--------------------|----------------|------------|---------|
| kegg   | Endocytosis | Yes | Zhang [22], Wu [23] | $1.1 \times 10^{-3}$ |
| wp     | Endochondral Ossification | Yes | Carson [24] | $2.0 \times 10^{-3}$ |
| kegg   | Focal adhesion | Yes | Zhou [25] | $2.8 \times 10^{-3}$ |
| kegg   | Adherens junction | No | - | $1.2 \times 10^{-2}$ |
| wp     | Regulation of Microtubule Cytoskeleton | Yes | Park (query) [26] | $1.4 \times 10^{-2}$ |
| wp     | Angiogenesis | Yes | Ustuyuzhanina [27] | $1.8 \times 10^{-2}$ |
| kegg   | Circadian rhythm | No | - | $1.9 \times 10^{-2}$ |
| kegg   | Long-term potentiation | No | - | $4.0 \times 10^{-2}$ |

**Cancer related pathway**

| Source | Signaling Pathways | Prior evidence | References | p-value |
|--------|--------------------|----------------|------------|---------|
| kegg   | Prostate cancer | Yes | Boo [28] and Choo [29] | $3.0 \times 10^{-3}$ |
| kegg   | Renal cell carcinoma | No | - | $6.3 \times 10^{-3}$ |
| kegg   | Glioma | Yes | Ko [30] | $7.9 \times 10^{-3}$ |
| kegg   | Chronic myeloid leukemia | Yes | Jin [31], Astashrazm [32] | $1.2 \times 10^{-2}$ |
| wp     | Signaling Pathways in Glioblastoma | Yes | Ko [30], Iv [33] | $1.6 \times 10^{-2}$ |
| kegg   | Proteoglycans in cancer | Yes | Liu [34] | $1.6 \times 10^{-2}$ |
| kegg   | Acute myeloid leukemia | Yes | Jin [31], Astashrazm [32] | $2.3 \times 10^{-2}$ |
| kegg   | Pathways in cancer | Yes | Corban [4], Van Weelden [35] | $2.9 \times 10^{-2}$ |

**Other disease states**

| Source | Signaling Pathways | Prior evidence | References | p-value |
|--------|--------------------|----------------|------------|---------|
| wp     | Physiological and Pathological Hypertrophy of the Heart | No | - | $4.9 \times 10^{-2}$ |

3. Discussion

All living organisms have acquired the ability to rapidly respond to environmental stimuli including food. These responses are typically associated by altered gene expression to adequately ensure homeostasis of a range of metabolic, hormonal and physiological functions. Over the last 10 years, the regulation of gene expression by micro RNAs (miRNAs) has gained significant attention and multiple miRNAs have been identified that can either be used as biomarkers of physiological functions or are causative for different states of disease [6]. For example, food, such as milk, can influence serum miRNA expression both by delivery of exogenous miRNAs and influencing endogenous miRNAs, as recently reviewed by Cui et al [7].

This study, for the first time, illustrates that a single dose of Undaria pinnatifida fucoidan (UPF) is able to affect miRNA composition in the plasma of healthy individuals, which suggests that fucoidan actively can alter gene expression in individuals that consume fucoidan.

Out of 754 tested miRNAs, 53 were differentially regulated by UPF. The subsequent pathway analysis identified 31 pathways that are predicted to be selectively influenced by UPF. Since it is well described that fucoidans directly affect signaling pathways associated with cell surface receptors, it
was reassuring to observe that major membrane receptor pathways for growth factors such as BDNF, EGFR/ErbB, and insulin receptor were predicted by the pathway analysis, as well as the associated downstream signaling components such as MAPK. This surface-activity of fucoidan is also reflected by the prediction of fucoidan effects on focal adhesion in the present study, which supports previous observations that low molecular weight fucoidan can attenuate aortic aneurism [15]. Additionally, research into fucoidan effects via MAPK cascade includes curative effects on leishmaniosis [18], affecting M2 type macrophages, inhibiting cancer growth by modulating immune responses [19], and reducing cerebral reperfusion injury [20]. Fucoidan interferes with the binding of cancer cells to extracellular matrix [34].

It is acknowledged that direct fucoidan-receptor interactions are different from affecting a signaling pathway by altering the associated gene expression. Nevertheless, in most reports, no direct interaction but rather an indirect modulation of signaling was reported. For example, fermented Laminaria japonica, which contains fucoidan, increased serum BDNF levels in elderly subjects over a six week period [10]. Whether this effect translated to increased BDNF-signaling in those individuals was not reported. In an animal model of depression, chronic delivery of (Fucus vesiculosus) fucoidan inhibited clinical signs, blocked the increase in tyrosine hydroxylase expression in the localized areas of the brain and inhibited the decrease in BDNF mRNA expression in the hippocampus [11]. Whilst correlated, it is not known at present whether these changes led to a restored BDNF signaling.

This suggests that our data needs to be interpreted cautiously and does not necessarily represent direct fucoidan-receptor interactions. Most likely, the actual interactions are significantly more complex. This is illustrated by several related studies. While fucoidan was reported to restore insulin stimulated glucose-uptake in adipocytes in vitro [15], no fucoidan-effects on insulin or glucose control were observed in healthy overweight subjects [16] and in one clinical trial fucoidan even increased insulin resistance [17].

It was previously reported that Sargassum hemiphyllum fucoidan up-regulated miRNA29b and down-regulated miRNA29c in different cancer cell lines, which was proposed by the authors as the reason for the anti-cancer activity of this particular fucoidan [8,9]. Although the current study did not detect any selective modulation of any of these miRNAs by UPF, this was not unexpected given the different test systems (cancer cell lines versus healthy individuals) and different sources for fucoidan (Sargassum hemiphyllum versus Undaria pinnatifida). In this context it is also important to note that our prior research illustrates that cancer cells can respond very differently compared to non-immortalized cells. While UPF effectively induced DNA damage in colorectal cancer cells, it had no effect on non-immortalized primary human skin fibroblasts [4], which could also account for the different miRNA responses observed by the different studies.

Van Weelden et al. recently reviewed the different cancer pathways that are affected by fucoidan [35] and a range of these pathways were also predicted by our analysis. Our results specifically identified prostate cancer-related pathways, which supports the previous studies by Boo [28] and Choo [29]. Both studies reported inhibitory effects of Undaria fucoidan on prostate cancer cells in vitro. Fucoidan was also reported to affect estrogen signaling, a mechanism that was proposed to induce cancer cell apoptosis [21]. Fucoidan was reported to suppress the growth pro-myeloid cancer cells [32], which supports the results of the present study that predicted effects of UPF on chronic and acute myeloid leukemias. Fucoidan has also been shown to affect the growth of glioma cells [30] and inhibit glioma cell-induced angiogenesis [33]. Fucoidans can act as both anti-angiogenic and pro-angiogenic agents, dependent mostly on their molecular weight [27], which was also identified by the present study.

In addition, our analysis identified the process of endocytosis as a potential target for fucoidan. While cellular uptake of fucoidan might rely on clathrin-dependent endocytosis [22], previous studies reported that fucoidan can inhibit endocytosis in HeLa cells [23]. Fucoidan suppressed Ca²⁺-dependent endocytosis, potentially by inhibition of agonist-induced Ca²⁺ responses [23]. Whilst the present study identified endochondral ossification, fucoidan has been associated with bone cell differentiation [24], but not specifically with endochondral ossification, an interaction that remains to be verified.
Interestingly, our analysis also predicted fucoidan-regulated pathways that have not previously been reported in the literature such as circadian rhythm and long-term potentiation. If these interactions can be confirmed by future studies in more detail, it might identify novel unexpected applications of dietary fucoidan products. The results of the current study justify future trials that will assess detailed pharmacokinetic parameters, as well as dose–effect relationships, to provide a rational approach towards optimized fucoidan dosing.

Ultimately, the molecular mechanism by which miRNA levels are altered in healthy individuals are unknown at present and will require systematic studies in the future. It can be speculated that these effects could be mediated directly via systemic uptake of small concentrations of fucoidan, or perhaps by perturbations of the microbiome that could cause indirect systemic effects.

4. Materials and Methods

4.1. Materials

If not otherwise stated, all chemicals were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). *Undaria pinnatifida* fucoidan (UPF) was obtained from Marinova (Cambridge, TAS, Australia). This material was provided with a quoted fucoidan purity of 85.1% (dry weight). The calculation of fucoidan purity requires several inputs that are determined using spectrophotometric assays. The total carbohydrate content of a hydrolyzed sample was determined using the phenol-sulfuric method of Dubois [36], while the uronic acid content was determined by spectrophotometric analysis of the hydrolyzed compound in the presence of 3-phenylphenol, based on a method described by Filisetti-Cozzi and Carpita [37]. Sulfate content was analyzed spectrophotometrically using a BaSO4 precipitation method (BaCl2 in gelatin), based on the work of Dodgson [38], and found to be 24.6%. The molecular weight profile was determined via gel permeation chromatography using a size-exclusion column and reported relative to Dextran standards, with peak molecular weight found at 47.7 kDa.

4.2. Test compounds

Test compounds (Placebo and experimental) were hand-filled by a pharmacist into gelatin capsules (size 1, opaque, pink, *The Capsule Guy*, Adelaide SA, Australia) without excipients and individually weighed. Placebo consisted of 250 mg ± 3 mg/capsule of microcrystalline cellulose powder, while experimental contained 500 mg ± 3 mg/capsule of *Undaria pinnatifida* extract (Marinova, Cambridge, Australia). Capsules were stored in bottles marked “A” and “B” respectively before transported to the test site. Apart from the pharmacist, all other members of the study (investigators and test subjects) remained blinded to the treatment until the analysis of data had been finalized.

4.3. Trial design

The study was a double-blind randomised placebo control study. All protocols and procedures were approved by the University’s Human Ethics committee (H0015183). Healthy males, 25–65 of years, with no health conditions were recruited by a university newsletter. This study was designed as a pilot study with low participant numbers. Therefore, to avoid hormone-induced fluctuations of miRNA levels that would have required a much larger number of participants, the inclusion criteria were restricted to males. Exclusion criteria also included regular endurance sport, frequent recreational drug use, impaired digestive function, including occasional or regular use of laxatives, infection, drug related diarrhea, or regular use of food supplements (vitamins, herbs, plant extracts, especially aloe vera, seaweed/extracts, mushroom, spirulina, and spirulina juice). All subjects were informed about potential risks, read, and signed informed consent. Subjects ingested two capsules (size 1) with 500 mg fucoidan extract/capsule or placebo. Blood samples were taken immediately before the study capsules were ingested and 24 h later. Participants were randomised by a person not directly involved in recruitment, data collection or data analysis and was performed in blocks of 2; stratification was performed based on age and body surface area. Blood samples were collected from
9 participants for placebo and 8 for fucoidan in EDTA-blood collection tubes (BD). Plasma was prepared by centrifugation and stored at −80 °C until further processing.

4.4. miRNA Quantification

**RNA Isolation:** Individual plasma samples were extracted using a Norgen total RNA purification kit (Cat #17200, Norgen Biotek Corp, ON Canada) according to the manufacturer’s instructions. Briefly, 200 µL of plasma from baseline (0 h) and treatment (24 h) groups were extracted and eluted in 50 µL elution buffer and quantified using the Qubit RNA HS (high sensitivity) and microRNA assay kits (Life Technologies), aliquoted and stored at −80 °C.

**TaqMan OpenArray:** The expression levels of 754 miRNAs were profiled using the Taqman OpenArray Human MicroRNA panels (PN: 4470189; Life Technologies Forster City, CA, USA) on a QuantStudio 12K Flex instrument. For all experimental groups, 3 µL (~10 ng) of total RNA was used for reverse transcription (RT) reactions using MegaPlex RT Primers Human Pool Set v3.0 (PN: 4444745; Pool A v2.1 and Pool B v3.0) according to the manufacture’s application note (Optimised protocol with low sample input for profiling human microRNA using the OpenArray platform) using a BioRad c1000 Touch thermal cycler. No-template controls were also included. Pre-amplification of RT products was performed using 5 µL RT reaction combined with the matching MegaPlex PreAmp Primer Pool A v2.1 or B v3.0 and amplified using the BioRad thermal cycler. The pre-amplified products were diluted 1:40 in 0.1x TE pH 8. For each experimental set 10 µL of the diluted products were combined to give a total of 40 µL pooled sample. For both Pool A and Pool B groups, 22.5 µL of the pooled products were combined with an equivalent volume of TaqMan OpenArray Real-Time Master Mix and aliquoted into a 96 well plate. Then, 5 µL from each well were then transferred into a 384 well plate for loading onto OpenArray plates using an AccuFill robotic system. The OpenArray plates were run on a QuantStudio 12K Flex instrument (Life Technologies) and the raw data files were imported and analysed using the DataAssist software (Life Technologies). Failed reactions were excluded from analysis and undetermined Cr values for samples sets determined to have good amplifications were assigned a threshold value of 40, defining low abundance or absence of miRNA expression. Global mean normalisation was used to calculate relative fold change for miRNA expression.

4.5. Pathway Analysis

Before unblinding the treatment groups, pathway analysis was performed using miTALOS online software (v2; http://mips.helmholtz-muenchen.de/mitalos/#/search) [39]. TALOS associates up- or down-regulated miRNAs with biological processes that are derived from three major pathway databases KEGG, WikiPathways and Reactome [39]. The software default settings for human samples and Target Scan as prediction tool were employed. Therefore, three miRNAs listed in Table 1 (mmu-miR-29c#, mmu-let-7d#, and mmu-miR-374-5p) had to be excluded based on their nomenclature. No cell lines or tissues were pre-selected. A p-value of <0.05 was considered significant. The final data (Table 3) represents the differently represented pathways in the UPF-treated cohort compared to the placebo-treated cohort. Pathways identified in both treatments were excluded from Table 3.

5. Conclusion

The modulation of serum miRNA expression after acute administration of a single dose of Undaria-derived fucoidan indicates potential activity in several biological pathways, some of which have not previously been identified.

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