Microbiome and Diet Impact in Scalp Disorder: The Example of Alopecia Areata

Rinaldi Fabio, MD1,2,3*, Pinto Daniela1,2,3, Marzani Barbara1,2,3, Giammaria Giuliani2,3 and Sorbellini Elisabetta2,3

1Giuliani S.p.A, Italy
2Human Advanced Microbiome Project-HMAP, Italy
3International Hair Research Foundation (IHRF), Italy

*Corresponding author: Rinaldi Fabio, International Hair Research Foundation (IHRF), Milan, Italy, Tel: ++39-2-76006089

Abstract
Alopecia areata (AA) is a potentially reversible auto-immune non-scarring baldness on the scalp, which can be extended to the entire body. The impact of diet on hair growth disorder is well established as the influence of diet on gut microbiome. Poor information is still available as regards the link between microbiome, especially scalp microbiome and hair diseases. Here we reported a two case-reports study on patients affected by AA, with and without lactose intolerance, respectively, with the aim to underline how diet could emphasize microbiome changing related to scalp disease. Subjects were asked to fill out a 7-day dietary survey and scalp and oral swabs were collected. Data from the dietary survey, qRT-PCR on main bacterial strains inhabiting the scalp and 16S sequencing of the scalp and oral microbiome were matched and compared each other and with healthy and general AA population. Beyond diet well-known impact on general human health, our results highlighted the role of diet in modifying oral and scalp microbiome, which in turn seems to have an impact on AA evolution. Our results provide the first evidence of strict intercorrelation between microbial dysbiosis on the scalp of patients with AA and dietary habits.

Keywords
Alopecia areata, Hair disorders, Dietary therapy, Microbiome, Dysbiosis

Introduction
Alopecia areata (AA) is a potentially reversible auto-immune disease affecting the scalp [1,2]. Its typical manifestations occur in the form of non-scarring baldness on the scalp, which can be possibly extended to the entire body [3]. When affected by non-scarring alopecia, a kind of disorder in hair follicle cycling has been observed [4], leading to the arrest of anagen phase, hair loss and, consequently, annular or patchy bald lesions [5,6]. In AA, in particular, this disorder has been reported to be strictly linked to immunity and inflammation [7-9]. As the second most common type of hair loss disorder (incidence higher than 2%) [10], AA has been extensively studied as regards causes [1] and clinical management options [11]. A novel innovative approach also includes the use of Platelet-rich plasma (PRP) as inflammatory cytokines suppressor [12].

Hair follicle cells have a high turnover and a very active metabolism so they require a good intake of nutrients and energy from the diet. The impact of diet on hair growth disorder is well established, especially as regards nutritional deficiencies [13,14]. Also, the influence of diet on shaping the gut microbiome and its implications for human health has been largely studied [15,16]. Changing in diet regimen can induce large, reversible microbial alterations in less than one day [15]. This is especially true when we speak about gut but it is also true, for example, for the skin [17,18].

Poor information is still available as regards the link between microbiome and s diseases and they are mostly related to gut microbiome [19,20]. But, poor knowledge is currently available about the impact of changing in scalp microbial community in hair disorders [21,22]. In a recently published work [23] we reported, for the
first time, evidence about a microbial shift in hair loss disorder, such as Alopecia androgenetica, AA and Lichen planopilaris. In the present work, we reported a two case-reports study on patients affected by Alopecia areata, with and without lactose intolerance, respectively, with the aim to underline how diet could emphasize microbiome changing related to scalp disease (Figure 1).

Case Presentation

Case report 1

A 17-year-old male (Milan, Italy), affected by Alopecia universalis (Figure 2). The patient presented to the dermatology clinic with a history of rapidly progressing total body hair loss. There was no history of similar illness in family members and also no history of drug intake and trauma. Previously treatment includes stem cell therapy. No therapy for at least 3 months at the time of inclusion. The clinical evaluation reported 100% hair loss of the scalp based on the Severity of Alopecia Tool (SALT) Score [24] with no signs of erythema or scaling. Eyebrows, eyelashes, and body hair were also completely absent. The patient demographics include being Caucasian, weight 72 kg, height 1.80 m, and BMI 22.2 kg/m².

Case report 2

A 36-year-old female (Figure 3) came to the clinic...
reporting a history of strong hair loss since one month. Other reported symptoms are severe itching, psoriasis on the scalp, birch and pauliary allergy, insomnia and high sensation of fatigue. The patient is also intolerant to lactose. Clinical evaluation showed strong hair loss but no signal of miniaturization. Alopecia areata was confirmed by histological examination. No therapy for at least 3 months at the time of inclusion. The patient demographics include being Caucasian, weight 48 kg, height 1.62 m, and BMI 18.3 kg/m².

**Nutrient intake**

Both subjects were asked to fill out a 7-day dietary survey at the time of enrollment, following being instructed by a dietician on how to record the food and beverages consumed. The food surveys were analyzed by Winfood software (Winfood 2.7 Medimatica Srl, Colonnella, Italy) in order to estimate the energy intake and the percentage of macronutrients and micronutrients. Data collected were compared to the tables of food consumption and recommended dietary intakes of the Italian National Institute of Nutrition and Food Composition Database in Italy.

**Samples collection**

Before sampling patients had to avoid the use of antibiotics in the last 30 days no probiotics, the use of probiotics in the last 15 days, to perform the last shampoo 48h before sampling. They also did not have to undergo to anti-tumor, immunosuppressant or radiation therapy in the last 3 months and also topical or hormonal therapy on the scalp. The study was under the approval of the Ethical Independent Committee for Clinical, not pharmacological investigation in Genoa (Italy) and in accordance with the ethical standards of the 1964 Declaration of Helsinki. All of the volunteers signed the informed consent. Microbiome samples were collected from the scalp (minimum area sampled of 16 cm²) and oral mucosa with a sterile cotton swab, previously soaked in ST solution (NaCl 0.15 M and 0.1 % Tween 20) for at least 30s [25,26]. Samples from the same subjects were collected together and stored at 4 °C until DNA extraction. Sterile cotton swabs placed in ST solution have been used as negative controls.

**DNA extraction and 16S amplicon generation and sequencing**

Genomic DNA from scalp swabs was extracted by mean of QIAamp UCP Pathogen Mini Kit (Qiagen) according to manufacturer protocol, with minor modifications [27]. After extraction, bacterial DNA was suspended in DNAse free water and quantified by the QIAexpert system (Qiagen) before sequencing and qRT-PCR.

For sequencing, variable region V3-V4 was amplified by mean of the following universal primers: 341 F CTGNCAGCMGCCGCGGTAA [28,29] and 806bR GGACTACNVGGGTWTCTAAT [30-32]. Library preparation and Illumina MiSeq V3-V4 sequencing were carried out at StarSEQ GmbH, Mainz, Germany, according to Caporaso, et al. [25] and Kozich, et al. [26] methods, with minor modifications. Real-Time Analysis software (RTA) v. 1.16.18 and 1.17.22, MiSeq Control Software (MCS) v. 2.0.5 and 2.1.13 were using.

**qRT-PCR of main bacterial species**

Main bacterial species (Propionibacterium acnes, Staphylococcus epidermidis and Staphylococcus aureus) on the scalp were quantified by real-time quantitative PCR (RT qPCR), using Microbial PCR assay kit (Qiagen). Samples were mixed with 12.5 μL of Microbial qPCR Mastermix, 1 μL of Microbial DNA qPCR Assay, 5 ng of genomic DNA sample and Microbial-DNA-free water up to a final volume of 25 μL. Positive PCR Control, No Template Control, and Microbial DNA Positive Control were also included. Pan-bacteria assays are also included as positive controls for the presence of bacterial DNA, as human GAPDH and HBB1 for the determination of proper sample collection. Following thermal cycling conditions were used: 95 °C for 10 min, 40 cycles of 95 °C for 15 sec, 60 °C for 2 min. Each PCR reaction was performed in duplicate using an MX3000p PCR machine (Stratagene, La Jolla, CA). Relative abundance in the expression of each strain was calculated using the ΔΔCt.
method [33], normalizing fold-change against PanBacteria, using MX3000p software (v.3; Stratagene).

### Statistical analysis

Data are expressed as Relative abundance % ± SEM for qRT-PCR analysis. Results were checked for normal distribution using the D’Agostino & Pearson normality test before further analyses. Statistically significant differences in the bacterial community were determined by Student’s t with Welch’s correction. Analyses were performed with GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA). P-values equal to or less than 0.05 were considered significant (Flow chart of Materials and Methods).

### Results and Discussion

In the present work, we investigated diet impact on microbial dysbiosis caused by the presence of AA which in turn could impact on disorder evolution and manifestations.

The preliminary analysis of macronutrients % intake (data not shown) suggested a Mediterranean diet framework [34] for both case reports included in the present study. Case report 1 diet included processed food, red meat and low intake of fruits and vegetables. Case report 2 diet included vegetables, fruit, peas, and beans (legumes) and grains. When analyzed more deeply as regards the type of proteins, lipids and carbohydrates ingested, diet from case-report 1 is better classified as High fat-diet (more processed food, sugars, and few fibers). The second case reports can be considered, instead, as following the Mediterranean like diet (lower fruits intake compared to normal Mediterranean regimen).

Table 1 and Table 2 show the intake of macronutrients and micronutrients in both case reports, compared to Recommended (LARN) values in Italy.

The daily amount of total calories was significantly different among case reports (p < 0.01) (1,331.58 ± 189.61 and 693.09 ± 143.48, respectively) (Table 1). Food diary from both subjects also reported a very small intake of fiber (16.59 ± 13.23 and 7.44 ± 1.33, respectively). Therefore, a lower percentage of saturated fatty acid intake was reported for case report 2 (3.12 ± 1.00).

### Table 1: Daily reported energy and nutrient intake of studied case reports, assessed by a 7-day weighed food record.

<table>
<thead>
<tr>
<th>Daily Intake</th>
<th>Recommended (LARN)</th>
<th>Case report 1</th>
<th>Case report 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total calories (kcal/day)</td>
<td>M: 2000-2400a</td>
<td>1,331.58 ± 189.61b</td>
<td>693.09 ± 143.48c</td>
</tr>
<tr>
<td></td>
<td>F: 1800-2300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total proteins (g/day)</td>
<td>75a</td>
<td>60.93 ± 15.19a</td>
<td>37.11 ± 16.01b</td>
</tr>
<tr>
<td></td>
<td>40a</td>
<td>72.44 ± 10.14b</td>
<td>47.33 ± 2.01a</td>
</tr>
<tr>
<td>Animal proteins (% of total proteins)</td>
<td>40a</td>
<td>27.56 ± 11.22b</td>
<td>52.67 ± 4.05a</td>
</tr>
<tr>
<td>Vegetal proteins (% of total proteins)</td>
<td>60a</td>
<td>27.56 ± 11.22b</td>
<td>52.67 ± 4.05a</td>
</tr>
<tr>
<td>Total lipids (g/day)</td>
<td>65a</td>
<td>50.58 ± 18.81a</td>
<td>29.11 ± 4.64b</td>
</tr>
<tr>
<td>Total carbohydrates (g/day)</td>
<td>290a</td>
<td>165.80 ± 22.15b</td>
<td>75.54 ± 18.88c</td>
</tr>
<tr>
<td>Amide (g/day)</td>
<td>220a</td>
<td>95.15 ± 35.35b</td>
<td>27.72 ± 28.77b</td>
</tr>
<tr>
<td>Fiber (g/day)</td>
<td>23a</td>
<td>16.59 ± 13.23a</td>
<td>7.44 ± 1.33b</td>
</tr>
<tr>
<td>Cholesterol (mg/day)</td>
<td>255a</td>
<td>126.50 ± 81.79a</td>
<td>51.76 ± 39.61b</td>
</tr>
<tr>
<td>Saturated fatty acids (% of total)</td>
<td>7a</td>
<td>10.06 ± 6.31a</td>
<td>3.12 ± 1.00b</td>
</tr>
<tr>
<td>Polyunsaturated fatty acids (% of total)</td>
<td>18a</td>
<td>6.45 ± 1.31b</td>
<td>6.36 ± 1.82b</td>
</tr>
<tr>
<td>Monounsaturated fatty acids (% of total)</td>
<td>4a</td>
<td>11.74 ± 5.01b</td>
<td>15.06 ± 5.24b</td>
</tr>
</tbody>
</table>

LARN: Nutrition and Energy Reference Assuming Levels. a-cValues with different superscript letters, in the same row, differ significantly (P < 0.05).
As regards micronutrients intake (Table 2) we noticed a significant lower intake of iron (5.50 ± 1.66 and 4.26 ± 1.91 vs. recommended) (p < 0.001) and folic acid (149.25 ± 43.89 and 67.33 ± 24.08 vs. recommended) (p < 0.01), riboflavin (0.45 ± 0.11 and 0.46 ± 0.25 vs recommended) (p < 0.01) and vitamin D (0.78 ± 1.48 and 4.58 ± 6.55 vs. recommended) (p < 0.01) for case report 1 and case report 2, respectively. Case report 1 also has a significant (p < 0.01) lower intake of niacin (7.79 ± 3.45) and vitamin E (3.21 ± 2.57) and higher (p < 0.001) intake of vitamin A.

Hypocaloric regimen or scarcity of proteins, minerals, amino acids, vitamins and essential fatty acids derived from an unbalanced diet can lead to structural changes in the hair follicle and, eventually, to hair loss [35].

Micronutrients have been implicating in affecting chronic telogen effluvium, androgenetic alopecia (AGA), female pattern hair loss (FPHL), and AA [36-38]. Indeed, many of the above micronutrients are reported to affect the hair follicle as regards restoration of hair

<table>
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<tr>
<th>Daily Intake</th>
<th>Recommended (LARN)</th>
<th>Case report 1</th>
<th>Case report 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (mg)</td>
<td>M: 1200&lt;sup&gt;a&lt;/sup&gt;</td>
<td>419.06 ± 406.77&lt;sup&gt;b&lt;/sup&gt;</td>
<td>141.05 ± 47.31&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>F: 1500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iron (mg)</td>
<td>18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.50 ± 1.66&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.26 ± 1.91&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Zinc (mg)</td>
<td>7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.33 ± 2.59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.03 ± 1.30&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Folic acid (µg)</td>
<td>200&lt;sup&gt;a&lt;/sup&gt;</td>
<td>149.25 ± 43.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>67.33 ± 24.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Niacin (mg)</td>
<td>14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.79 ± 3.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.05 ± 4.22&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Riboflavin (mg)</td>
<td>1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.45 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.46 ± 0.25&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Thiamine (mg)</td>
<td>0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.65 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.48 ± 0.30&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin A (µg)</td>
<td>600&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1,147.00 ± 141.88&lt;sup&gt;b&lt;/sup&gt;</td>
<td>499.68 ± 351.71&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin B6 (mg)</td>
<td>1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.99 ± 0.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.70 ± 0.29&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>70&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42.33 ± 25.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.57 ± 29.15&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin D (µg)</td>
<td>10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.78 ± 1.48&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.58 ± 6.55&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin E (mg)</td>
<td>8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.21 ± 2.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.25 ± 3.81&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

LARN: Nutrition and Energy Reference Assuming Levels. *<sup>b</sup>-Values with different superscript letters, in the same row, differ significantly (P < 0.05).

Figure 4: Distribution of main bacterial strains in healthy subjects and AA subjects.
growth, cell division, cycling [13].

Recent evidence [15] also strongly highlighted the ability of diet to impact on gut and also, more recently, oral [39] microbiome. Poor knowledge is still available about the influence of diet and microbiome dysbiosis on skin disorders [17,40]. Evidence are mainly linked to acne vulgaris [18,41-43], atopic dermatitis [44] and psoriasis [45]. In our previous work [23] we highlighted the microbial unbalancing in the normal resident microbial community of the scalp of patients affected by different hair growth-related conditions, included AA.

Figure 4 reports the % of the distribution of main bacterial strains in case report 1 and case report 2 compared with data from 15 healthy subjects and 15 AA subjects in our database.

Data from case report 1 showed an increase in *P. acnes* population and parallel decrease both of *S. epidermidis* and *S. aureus* species. These data are in line with data from the panel of AA subjects and clearly evidence the presence of bacterial dysbiosis compared to healthy control.

On the contrary, the percentage of distribution of main bacterial species in case report 2 resulted more similar to the healthy population (Figure 4). Even if an interindividual difference has to be considered, the analysis of food diary of the panel of fifteen AA subjects and case report 1 and 2 strongly suggested the impact of diet in shaping scalp microbiome.

Data from oral bacterial DNA sequencing corroborated these findings (Figure 5). Also, in this case, data from case report 1 and 2 were compared to data from our internal database of healthy and AA subjects, previously collected. The analysis of sequence at the phylum level highlighted a slow decrease of *Firmicutes* both for case report 1 and 1 (Figure 6) and these results are in line with our previous findings in AA subjects and results found in another autoimmune disease [46]. In both case reports, an increase of *Proteobacteria* (33.28% and 27.71%, respectively) has been reported. Most interesting, analysis of sequences from case report 1 bacterial oral DNA showed a decrease in *Bifidobacteria*. A link between a high-fat diet and this phylum decrease was previously reported [47] thus confirming the role of diet in influencing oral bacterial composition. A significant reduction of *Bacteroidetes* has been found in case report 2 compared to healthy control subjects. Since the high intake of n-6 PUFA by case report 2, a link between diet and this microbial unbalance could be hypothesized as also suggested by some authors [48].

Results on bacterial composition of scalp microbiome confirm our previous findings on microbial shift on the scalp in patients affected by AA [23]. In the present work, we investigated if different dietary habits can re-modulate this microbial dysbiosis with the aim to highlight the strict intercorrelation between diet and oral but especially scalp microbiome of these subjects.

Data from the present study are just a limited representation of a larger set of data we have accumulated in our clinical practice. Indeed, we have noticed, for example, how gluten-free diet could strongly affect AA evolution in patients affected by non-celiac gluten sensitivity (NCGS), in which AA manifestations systematically recurred following a non-gluten free diet. An explicative photographic example was reported in Figure 6. Most interesting this modulation reflects also in the microbi-
al composition of scalp microbiome (data not shown) enhancing the existence of a link between diet and skin bacterial communities scalp microbiome.

More and more evidence accumulated as regards the link between gut and hair disorders [19,20]. In autoimmune disease, among which AA, immune response leads to tissue damage and loss of function of the intestinal barrier [48]. Therefore, the permeability of the epithelial lining may be compromised; antigens, toxins, and bacteria migrated from the lumen to bloodstream leading to a syndrome known as “leaky gut” [48]. Modulating the gut microbiome also by mean of diet represents a valid approach for regulating and restore such damage leading to an improvement of the autoimmune disease. Data from the present study add to knowledge to this evidence also highlighting that not only gut but also oral and scalp microbiome could be modulated by dietary habits.

Nowadays, the study of human microbiome represents a novel diagnostic and therapeutic approach to treat many human conditions, also including that strictly related to skin and scalp. Beyond diet well-known impact on general human health, our results highlighted the role of diet in modifying oral and scalp microbiome, which in turn seems to have an impact on Alopecia areata evolution. Therefore, even not conclusive, our data also open to new diet and microbiome based adjuvant approaches in the management of hair disorders such as AA.

Larger studies are still needed to better investigate the role of the microbiome in scalp diseases and different drivers involved in this process.

Acknowledgments

This study was supported by Giuliani S.p.A.

Conflict of Interest

RF and SE serve as a consultant for Giuliani S.p.A. P.D. and M.B. are employed by Giuliani S.p.A.

References


Materials and Methods Flow-Chart

Enrollment of subjects

- Collection of scalp and oral mucosa swabs
- 7 days dietary surveys

- Analysis on nutrient intake by Winfood software
- 16S Amplicon Generation and Sequencing
- qRT-PCR of main bacterial species (on the scalp)

- Statistical analysis