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**Tissue and circulating microRNA co-expression analysis reveals potential involvement of
miRNAs in the pathobiology of frontal fibrosing alopecia**

Christos Tziotziou¹, Chrysanthi Ainali¹, Susan Holmes², Fiona Cunningham², Su M. Lwin¹,
Ioulios Palamaras³, Kapil Bhargava¹, Janice Rymer⁴, Catherine Stefanato⁵, Niall Kirkpatrick⁶,
Sergio Vano-Galvan⁷, Christos Petridis⁸, David A. Fenton¹, Michael A. Simpson⁸, Alexandros
Onoufriadis¹, John A. McGrath¹

¹St John's Institute of Dermatology, Division of Genetics and Molecular Medicine, King's
College London, London, United Kingdom; ²Alan Lyell Centre for Dermatology, Queen
Elizabeth University Hospital, Glasgow, United Kingdom; ³Department of Dermatology,
Barnet General Hospital, Royal Free Foundation Trust, London, United Kingdom; ⁴King's
College London School of Medicine, King's College London, London, United Kingdom;
⁵Department of Dermatopathology, St. John's Institute of Dermatology, St. Thomas' Hospital,
London SE1 7EH, United Kingdom; ⁶Department of Plastic Surgery, Guy's Hospital, London
SE1 9RT, United Kingdom; ⁷Ramon y Cajal Hospital, Carretera de Colmenar, KM 9.100,
Madrid, Spain; ⁸Department of Genetics, Division of Genetics and Molecular Medicine,
King's College London, Guy's Hospital, London SE1 7RT, United Kingdom

Corresponding author: John A. McGrath, Dermatology Research Labs, Floor 9 Tower Wing,
Guy's Hospital, Great Maze Pond, London SE1 9RT, United Kingdom. Tel: 44-2071886409;
Fax: 44-2071888050; E-mail: john.mcgrath@kcl.ac.uk

Short title: microRNAs in frontal fibrosing alopecia

Abbreviations used: FFA, frontal fibrosing alopecia; PCA, primary cicatricial alopecia; LPP, lichen planopilaris; eHFSC, epithelial hair follicle stem cells

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TO THE EDITOR,

Frontal fibrosing alopecia (FFA) is a predominantly postmenopausal, primary lymphocytic cicatricial alopecia (Kossard, 1994), thought to be a clinical subvariant of lichen planopilaris (Kossard et al., 1997). Somewhat of a misnomer, the descriptor FFA remains widely used for what is a generalized cutaneous lichenoid and fibrosing disorder, invariably associated with widespread body hair loss (Chew et al., 2010), extending beyond the frontovertex, especially if untreated (Figure 1a). Histopathologically, FFA features peri-isthmic lymphocytes (Figure 1b), and immune privilege collapse at the bulge may underpin inflammation-driven epithelial hair follicle stem cell loss and scarring (Tziotziou et al., 2016).

MicroRNAs (miRNAs) are small, non-coding ribonucleic acid molecules (RNAs) that may impact protean biological functions (Lau et al., 2001). They have emerged as potential candidates of pathobiologic, diagnostic and therapeutic interest in chronic inflammatory, fibrotic and autoimmune diseases (Hwang et al., 2006; Galasso et al., 2010; Zhang and Farwell, 2008; Pauley et al., 2009; Patel and Nouredine, 2012). MicroRNAs can be detected in plasma, wherein they circulate in a stable form (Yang et al., 2015; Gilad et al., 2008; Chen et al., 2008; Mitchell et al., 2008; 2008; Valadi et al., 2007; Guo et al., 2017). We sought to characterize miRNAs in FFA and probe disease relevance by undertaking tissue and circulating miRNA co-expression analysis in FFA cases vs matched controls. Neither deep transcriptomic analysis nor miRNA signature exploration has previously been explored for their bearing in FFA, to our knowledge.

Seven newly-diagnosed, treatment-naïve FFA cases and seven matched controls were ascertained for tissue miRNA analysis (Table S1). After written informed consent and institutional ethics approval, temporal scalp skin biopsy was obtained from cases and

controls. Each case was evaluated clinically and histologically to confirm active disease. Part of each biopsy was immediately immersed in RNAlater; total RNA was isolated using RNeasy Plus Universal kit (Qiagen, Valencia, USA) and miRNAs retained according to the manufacturer's protocol (see Supplementary section). All samples underwent microarray analysis on Affymetrix GeneChip miRNA 4.0 arrays. Next, to explore whether circulating miRNAs pertinent to fibrosis are relevant to FFA pathogenesis, venous blood was obtained from a separate cohort of 10 biopsy-proven treatment-naïve FFA cases and 10 matched controls (Table S2). Plasma was isolated by centrifuging blood at 2,000g for 20 minutes at 4°C and stored at -80°C until analysis. MicroRNAs were isolated using Qiagen miRNeasy Serum/Plasma Kit, according to the manufacturer's protocol. Expression analysis was undertaken using the Human Fibrosis miRNA PCR Array (Qiagen) (see Supplementary section).

We first constructed a co-expression network of tissue miRNAs to investigate their molecular importance in FFA. The network was generated by assessing pairwise similarity of miRNAs expressed in each group (controls and FFA), calculated by the Pearson correlation coefficient (PCC) (see Supplementary section and Figure 2a). A co-expression network establishes an association among miRNAs that demonstrate a coordinated expression pattern across a group of samples. It has been postulated that conserved co-expression confers a selective advantage and therefore such genes might be functionally related (Piro et al., 2011; Stuart et al. 2003); such networks can therefore provide insight into how cells accomplish their functions. Only miRNA pairs that correlated above the 0.95 PCC threshold value were represented for each tissue network to capture the strongest relationship for miRNA expression regulation. The resulting FFA network displayed 2,089 co-expressed miRNAs (nodes) with 3,009 non-duplicate interactions (edges), while for controls there were

2,100 co-expressed miRNAs (nodes) with 2,934 non-duplicate interactions (edges). Both tissue networks were used as molecular reference datasets to identify communities of miRNAs sharing common functions. For this analysis, we used the Affinity Propagation Clustering algorithm (see Supplementary section). We generated a list of communities for the two tissue networks and automatically identified cluster centers (exemplars) as representative miRNAs of each community (Table S4).

Next, differential expression analysis was applied in the plasma dataset to calculate the changes in expression between control and FFA samples and to identify up- or down-regulated circulating miRNAs (Figures 2b and S1). Fifty-five miRNAs were found to be up-regulated in FFA while 11 miRNAs were down-regulated. For those circulating miRNAs, we further tested: (i) their predictive value; and (ii) if any of them mapped within the communities of tissue networks. To evaluate their predictive value, we used Random Forest with CARET package in R, which ranks all data elements representing the most influential predictors (Table S5 and Figure S2). To further evaluate circulating miRNAs that were found to be differentially expressed between controls and cases, we mapped them within the tissue-specific co-expression networks. Twenty exemplars in the control tissue miRNA network were significantly enriched in the plasma dataset compared to 27 exemplars, which were significantly enriched in the FFA tissue miRNA network. Amongst these exemplars, there were 17 miRNAs common in both networks (cases and controls): three of these were specific to controls whilst nine were representative of FFA. Of those nine circulating miRNAs, four were found to be highly predictive of disease status. Those miRNAs were, in order of importance, hsa-let-7d-5p, hsa-miR-18a-5p, hsa-miR-20a-5p and hsa-miR-19a-3p, which are also co-targeting similar sets of genes. While the latter three are part of the same cluster, hsa-let-7d-5p is independent of the cluster (Figure 2c) and the commonality in co-targeting

similar genes is of particular interest. As previously suggested, if different miRNAs co-target similar sets of genes, these might regulate the same functions (Tsang et al., 2010; Su et al., 2011). We applied GAGE analysis to infer pathways associated with FFA and found MAPK signaling, Endocytosis and Focal Adhesion pathways to be down-regulated and enriched in the networks of co-targeting genes across these four miRNAs (Table S6).

Our study identified circulating miRNAs hsa-let-7d-5p, hsa-miR-18a-5p, has-miR-20a-5p and hsa-miR-19a-3p as being highly predictive of disease status in FFA by using a skin-specific miRNA network as a validating molecular mapping dataset. Functional validation is needed in future to determine mechanistic relevance, while any additional circulating miRNAs that did not map to the skin network, despite having been found to be predictive in our analysis of plasma, could be further explored in larger cohorts.

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Figure legends

Figure 1. Clinical and histologic features of FFA. (a) Scarring alopecia with frontal and marginal scalp involvement as well as eyebrow loss; (b) Horizontal section of the isthmic area showing perifollicular fibrosis, follicular tufting, and a moderately dense perifollicular lymphoid cell infiltrate. A fragment of a naked hair shaft is also seen. (Hematoxylin-eosin stain; scale bar=75 μ m). Patient permission to publish these clinical photographs has been received.

Figure 2. Circulating miRNAs within tissue-specific co-expression network establishes evident differences between FFA and controls. (a) miRNA expression matrix displays the expression values of the two different phenotypes: FFA (gray color) and Controls (white color) in relation to different miRNAs in tissue-specific dataset. Pairwise similarity in miRNA expression vectors was expressed by the Pearson Correlation Coefficient (PCC). Nodes correspond to miRNAs while edges correspond to the pairwise expression similarity. Tissue co-expression networks in control (left) and FFA (right) were generated. Communities of miRNAs were further detected in the two networks after applying APCluster algorithm. (b) Plasma-specific expression profiles used to identify differentially expressed circulating miRNAs in the skin of patients with FFA, compared to controls. Plasma-specific miRNA signature was further mapped into the cluster centers identified in the two networks after applying APCluster algorithm. Over-expressed miRNAs are colored red while under-expressed miRNAs are depicted in green; (c) Predicted circulating miRNAs co-targeting similar sets of genes.

a

b