1970, among others. More globally, in 1974 the “Sun Protection Factor” rating for sunscreens was introduced, leading to more effective sunscreens and consequent lower rates of UV exposure. The early 1970s even saw widespread acceptance of yogurt consumption (Fabricant, 1976), potentially altering the gut microbiome. Careful analysis of long-term trends in USA NHES, NHANES, and other serial biological data, as well as parallel data from non-Caucasian vitiligo cases from other continents, might provide important clues to environmental changes that underlie delayed autoimmune triggering and the shift of vitiligo onset toward later ages.

Data availability statement
Datasets related to this article can be found at [www.ebi.ac.uk/gwas/search?query=30674883](http://www.ebi.ac.uk/gwas/search?query=30674883), hosted at the NHGRI-EBI GWAS Catalog ([www.ebi.ac.uk/gwas/](http://www.ebi.ac.uk/gwas/)) (www.ebi.ac.uk/gwas/downloads/summary-statistics).

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**TO THE EDITOR**

Alopecia areata (AA) is a hair follicle (HF) disorder, in which the immune system attacks the HF and causes reversible hair loss. Even though AA is not a life-threatening disease, an association with psychosocial diseases and a severe drop in quality of life is common (Pratt et al., 2017). There is, as yet, no approved medicine in the therapy for AA. New potent drugs, such as Jak inhibitors, show promising results but incur severe adverse effects (Wang et al., 2018). For such drugs, targeted delivery to the site of action is essential.

The concept for follicular delivery of drug-loaded nanoparticles (NPs) for treatment of hair disorders shows potential. The key benefits of targeted biodegradable polymeric NP delivery into HFs include (i) protection of the encapsulated drug, (ii) minimization of drug exposure to the skin surface, as well as interfollicular permeation, (iii) maximization of the penetration into the HF compared with the free drug (Mathes et al., 2016), (iv) the possibility of building a drug depot in the upper part of the HF, creating possible protection of the NPs from external influences such as, textile contact, washing (Lademann et al., 2007), and (v) the consistency of a sustained drug release from the depot to reduce the application frequency and enhance patient compliance (Hofmeier and Surber, 2017). Taking this into account, Jak inhibitor-loaded NPs could deposit in the upper part of the HF, release the drug in a controlled manner, which diffuses to the site of action (hair bulb), and be taken up by the follicular epithelial cells and immune cells (Divito and Kupper, 2014), thus reducing adverse effects with less systemic drug and skin exposure.

However, a hypothesized penetration mechanism for NP uptake into human HF postulates that, by the movement of the hair shaft, overlapping cuticle cells serve as a gear pump and push the NPs into the HF (Lademann et al., 2007; Radtke et al., 2017). Additionally, appropriate massage seems to be important in this context (Li et al., 2019). Thus, the application frequency and enhance patient compliance (Hofmeier and Surber, 2017). Taking this into account, Jak inhibitor-loaded NPs could deposit in the upper part of the HF, release the drug in a controlled manner, which diffuses to the site of action (hair bulb), and be taken up by the follicular epithelial cells and immune cells (Divito and Kupper, 2014), thus reducing adverse effects with less systemic drug and skin exposure.

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**Nanoparticle Targeting to Scalp Hair Follicles: New Perspectives for a Topical Therapy for Alopecia Areata**

To the Editor

Alopecia areata (AA) is a hair follicle (HF) disorder, in which the immune system attacks the HF and causes reversible hair loss. Even though AA is not a life-threatening disease, an association with psychosocial diseases and a severe drop in quality of life is common (Pratt et al., 2017). There is, as yet, no approved medicine in the therapy for AA. New potent drugs, such as Jak inhibitors, show promising results but incur severe adverse effects (Wang et al., 2018). For such drugs, targeted delivery to the site of action is essential.

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However, a hypothesized penetration mechanism for NP uptake into human HF postulates that, by the movement of the hair shaft, overlapping cuticle cells serve as a gear pump and push the NPs into the HF (Lademann et al., 2007; Radtke et al., 2017). Additionally, appropriate massage seems to be important in this context (Li et al., 2019). Thus,

**Abbreviations**: AA, alopecia areata; HF, hair follicle; NP, nanoparticle; PLGA, poly (lactic-co-glycolic acid)
Figure 1. Study groups. (a) Overview of study groups included in the NP uptake study in vivo and in body donors: on one application area (1,767 cm²), 15 μl of 50 mg/ml fluorescently covalently labeled poly (lactic-co-glycolic acid)-NP suspension was applied by a 3-minute massage with a gloved fingertip and incubated for 1 hour. Additionally, 15 μl of water was applied on one vehicle blank area per participant. Experimental sets were excluded if they did not meet the mass balance limits of 85–115% as recommended by Scientific Committee on Consumer Safety guidelines (2018). (b) Dermatoscopic documentation of application areas: each application area was documented using a dermatoscope for manual count of hair follicle orifices by ImageJ (results shown as mean ± SD). (brightness, saturation, focus, black and white balance of pictures enhanced by Corel PaintShop Pro X8 [18.2.0.61 x64]). NP, nanoparticle. Scale bar = 5 mm.

![Healthy Forearm](28±8) ![Healthy Scalp](144±16) ![Body Donor](142±48) ![Alopecia Areata](95±27)

Figure 2. Follicular deposition of nanoparticles. (a) Amount of NPs in HFs: gray bars, amount of applied NPs (μg) per application area deposited in HFs; white bars, amount of applied NPs (μg) deposited in one single HF. Statistics: one-way analysis of variance with corrections (Tukey) of multiple comparisons (mean of each group with the mean of every other group; GraphPad Prism 7; P = 0.05). n.s. P > 0.05, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. (b) Confocal laser scanning microscopy images of DiD-loaded PLGA-NPs applied on Body Donor scalp: punch biopsy was performed after application of DiD-loaded PLGA-NPs (red; HeNe 633 laser, emission: 665–680 nm) on the skin surface (no skin surface cleaning). Left: longitudinal section 20 μm, counterstained with DAPI (blue; detection: diode 405 laser, emission: 430–450 nm). Right: transversal section 100 μm, transmission light (see Supplementary Materials and Methods and Supplementary Figure S5). NP, nanoparticle. Scale bar = 100 μm.
we questioned if NPs are able to penetrate into AA-affected HFs, where there is no hair present. To fill these critical knowledge gaps, we studied NP deposition in AA-affected HFs with a previously developed quantification method for NP uptake into human forearm HFs, demonstrating an excellent in vivo—in vitro correlation with pig ear skin (Raber et al., 2014). However, there are no quantitative data for human scalp skin. Because hair density and hair type (vellus vs. terminal) differ between the healthy forearm and scalp, such data are critical to assess treatment options.

Therefore, we transferred the quantification method of NP uptake into human forearm HF to human scalp skin of healthy volunteers, subsequent transferred it to AA patients. However, the recruitment of participants for such studies is limited. Accordingly, we assessed the quantification method also in the hairy scalp of body donors as a possible alternative to future in vivo studies (experiments were conducted with the skin attached to the corpus; body donors were not fixated before the experiment and kept at 4°C; maximal post mortem interval was 3 days) (for study group details see Figure 1a). The studies were approved by the Ärztekammer des Saarlandes ethical committee, including informed written consent of participants.

For the model NPs, we continued to use the NPs used in the previously developed quantification method for NP uptake into the HF (Raber et al., 2014). The NPs, size of 150 nm, narrow size distribution and negative zeta potential, consisted of fluorescently covalently labeled poly (lactic-co-glycolic acid) (PLGA), a biodegradable, biocompatible polymer (Weiss et al., 2006; Mittal et al., 2013). For a detailed description of the procedure, see Supplementary Materials and Methods. In brief, differential striping was proceeded 1 hour after applying 15 μl of aqueous fluorescently covalently labeled PLGA-NP suspension (concentration = 50 mg/ml) per application area by a 3-minute massage (Teichmann et al., 2005), which comprises skin surface cleaning by tape stripping and subsequent removal of HF casts by cyanoacrylate biopsies. For mass balance, all samples in contact with the formulation were extracted in organic solvent and analyzed using fluorescence intensity. Stratum corneum recovery after differential striping was monitored by transepidermal water loss measurements (Supplementary Figure S1). Experimental sets were excluded if they did not meet the 85–115% mass balance limits as recommended by the Scientific Committee on Consumer Safety guidelines (2018; Figure 1a).

Comparing percentage of dose (mean ± SD) of applied NPs recovered on the skin surface (61.0 ± 4.1% vs. 56.4 ± 3.7%), the percentage of dose remaining on the glove (28.1 ± 5.5% vs. 24.1 ± 3.7%), and the percentage left on the skin (0.1 ± 0.2% vs. 1.3 ± 1.1%) after cyanoacrylate biopsies, results for forearm and healthy scalp, respectively, showed no significant differences (Supplementary Table S1; Supplementary Figure S2, red and purple bars), demonstrating successful transfer of the method from forearm to scalp skin.

Owing to higher HF density on a healthy scalp, we hypothesized a higher amount of NP deposition in scalp HFs than in forearm HFs. Indeed, the amount of NPs deposited in the HFs per application area of healthy scalp (8.5 ± 3.6% equals 59.79 ± 24.46 μg/HFs per area) was significantly higher than for forearm (2.5 ± 0.9% equals 19.05 ± 12.78 μg/HFs per area) (P < 0.001; Figure 2a, gray bars). To assess the influence of HF density, we documented each application area using a dermatoscope (handyscope, FotoFinder, Bad Birnbach, Germany) (Figure 1b) and determined the number of HF orifices visible on the skin surface of each individual application area manually using ImageJ (Image) 1.51β (NIH). After normalizing the NPs deposited per application area for the different HF orifice densities on the skin surface of each individual application area manually using ImageJ (Image) 1.51β (NIH), the differences were no longer statistically significant (Figure 2b), that is, the amount of NPs deposited per HF was about the same, regardless of the body site. Given that this study is limited by its number of participants, the results need to be considered carefully. Nevertheless, this is an interesting result, because vellus and terminal HF show fundamental morphometric differences (Vogt et al., 2007). Also, the HF densities and infundibular surfaces significantly differ between different vellus HF covered body sites (Otberg et al., 2004). This study takes only human forearm HF into account.

There was no significant difference when comparing the data of a healthy scalp with a body donation scalp regarding the amount of follicular deposition per application area (59.79 ± 24.46 μg vs. 41.83 ± 18.53 μg) (Figure 2b) or the deposition per single HF (0.42 ± 0.16 μg vs. 0.33 ± 0.21 μg), healthy scalp and body donor scalp, respectively. This implies that body donors may be used as models to develop future follicle-targeted drug carriers for dermatological applications.

Finally, we investigated the NP uptake in vivo into AA-affected hairless scalp HFs. Despite the postulated NP uptake mechanism in HF (Lademann et al., 2007), we demonstrated NP deposition in human HF in healthy (8.5 ± 3.6% equals 59.79 ± 24.46 μg) and AA-affected HFs (2.7 ± 0.4% equals 20.49 ± 2.90 μg); the difference of NP deposition per application area (Figure 2b, white bars) was no longer significant (P ≤ 0.01). Interestingly, after correction for the number of HF orifices per application area, the NP deposition per single HF (Figure 2b, white bars) was no longer significantly different (P > 0.05; 0.42 ± 0.16 μg vs. 0.23 ± 0.07 μg, healthy vs. AA scalp, respectively).

In conclusion, we were able to demonstrate that topically applied NPs do penetrate into human scalp HFs. This phenomenon appeared to be of the same scale as the previously reported follicular penetration in human forearm HFs (Raber et al., 2014) and thus to be independent of the HF type. Moreover, and most importantly, NPs were found to penetrate into HFs also in the absence of a hair shaft. This opens possibilities for the treatment of inflammatory hair loss, most importantly AA, by designing appropriate nanomedicines capable of penetrating and delivering their cargo directly to the affected HFs.

Data availability statement
Datasets related to this article can be found at https://doi.org/10.17632/fn6gg34py.1, hosted at Mendeley Data.
Glyburide, a NLRP3 Inhibitor, Decreases Inflammatory Response and Is a Candidate to Reduce Pathology in Leishmania braziliensis Infection

TO THE EDITOR
Cutaneous leishmaniasis (CL) is caused by *Leishmania braziliensis* and is characterized by an exaggerated inflammatory response that leads to parasite control, but it is also the cause of tissue damage and ulcer formation (Carvalho et al., 2012). The NLRP3 inflammasome is an intracellular protein complex that is activated by pathogen-associated molecular patterns or danger-associated molecular patterns, promoting activation of caspase 1 and release of active IL-1β (Latz et al., 2013). In C57BL/6 mice infected with a non-healing *L. major* strain, NLRP3 inflammasome and IL-1β were associated with severe pathology (Charmoy et al., 2016), and IL-1β caused severe disease in BALB/C mice infected with *L. major* (Voronov et al., 2010). Recently, we showed that CD8+ T cells induce immunopathology by NLRP3

Abbreviations: CL, cutaneous leishmaniasis; TNF, tumor necrosis factor

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NLRP3 Inhibitor in Leishmaniasis

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**CONFLICT OF INTEREST**
The authors declare no conflict of interest.

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**AUTHOR CONTRIBUTIONS**
Conceptualization: TV, UFS, CML; Funding Acquisition: TV, CML; Investigation: RC, CT, NJ; Methodology: ASR, UFS, RC, CT; Project Administration: BL, TV, Resources: TT, CT, TV, RC; Supervision: BL, TV; Resources: TT, CT, TV, RC; Investigation: RC, CT, NJ; Methodology: ASR, UFS, BL, TT, TV, CML.

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Li BS, Cary JH, Maibach Hl. Should we instruct patients to rub topical agents into skin?: the evidence. J Dermatol Treat 2019;30:328–32.

**SUPPLEMENTARY MATERIAL**
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2019.05.028.

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Weiss et al., 2006) was applied per
acid) (PLGA) NPs (Mittal et al., 2013;
lently labeled poly (lactic-co-glycolic
mV. On blank areas (one per partici-
son with a gloved fingertip (Mathes
Newton) was applied by a trained per-
was documented using a dermatoscope
(cyanoacrylate (5
minutes), the tape strip was removed with a forceps in one swoop. This was
done twice. It has been shown by Raber
ectively covalently labeled PLGA-NPs were chosen for
penetration inside human scalp
Hair follicles
Aqueous nanoparticle suspension
nanoparticles (NP) and had a

suspension calibration curve was
calculated according to the FDA Bio-
analytical Method Validation
guideline (FDA/CDER, 2018). Values lower than
quantification were excluded. Experimental sets were
excluded if they did not meet
50% as the recommended range
of mass balance by the Scientific
Committee on Consumer Safety guide-
lines (2018). Count of HF orifices on
dermatoscopic pictures was done
manually by ImageJ (ImageJ 1.51j8,
NIH) for each application area indi-
vidually. By correction of the follicular
deposition of NPs per application area
to the number of HF orifices counted,
the amount of NPs (in µg) deposited into
one single HF was determined.
Recovery of skin barrier function
was monitored by transepidermal
water-loss measurements according to
the ethical committee (Supplementary
Figure S1).

Visualization of nanoparticle penetration inside human scalp hair follicles

DiD-loaded PLGA-NPs were chosen for the visualization of NPs inside the HF, because fluorescently covalently labeled-PLGA-NPs in conjugation with skin and HFs are difficult to differentiate, because of the high auto fluorescence of the organs (Na et al., 2000) and the terminated fluorescence intensity of Fluorescein. DiD-loaded PLGA-NPs were prepared as described previously (Mittal et al., 2013) with addition of Vybrant DiD cell-labeling solution (Vybrant Multicolor Cell-Labeling Kit, Invitrogen, Waltham, MA) to the organic polymer solution. The free dye was separated from NPs by dialysis in purified water (Spectra/Por Float-a-Lyzer, 100 kDa, Spectrum Europe B.V., Breda, The Netherlands) for 29 hours. NPs were 124.0 ± 0.9 nm, and had a polydispersity index of 0.07 ± 0.02 and a zeta potential of −21.8 ± 0.2 mV. To visualize the DiD-loaded PLGA-NP deposition inside HFs (before the scalp hair was shortened to 1 mm) per application area (1,767 cm²), 20 µl of the NP formulation (250 mg PLGA/ml) was applied by massage. After the incubation time, no further procedure for skin cleaning (no tape stripping) or hair removal was applied; the applied NP formulation remained on the skin surface and in the HFs. An 8-mm punch biopsy was taken. Blank application areas (Control without NP) were treated the same without application of NP formulation. Subsequently, the skin tissue was imbedded in Tissue-Tek O.C.T. Compound (Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands) and cut by cryostat (SLEE, Mainz, Germany) (Supplementary Figure S3). Images were taken with a confocal laser scanning microscope (CLSM) (Leica TCS SP8, Leica, Mannheim, Germany) and processed with Leica Application Suite X (LAS X) software (Leica, Mannheim, Germany). Transversal sections to 100 µm were cut (Supplementary Figure S3c, S3d). On the first section, the amount of NPs on the skin surface was too high for a sufficient differentiation between NPs remaining on the skin surface and deposited in the HF. Therefore, the second 100-µm section is shown in Supplementary Figure S3c, demonstrating the DiD-loaded PLGA-NP (red fluorescence) inside the HF (hair shaft is present) (overlay with transmission light image). On the third 100-µm section, no NPs were detectable (not shown). The second transversal 100-µm section of the blank biopsy is shown in Supplementary Figure S3d (control without NP [HF with hair shaft] [overlay transmission

www.jidonline.org 246.e1
light image and red fluorescence channel). Results of longitudinal tissue sections of 20 μm are shown in Supplementary Figure S3a and b. DiD-loaded PLGA-NPs (red fluorescence) can be detected only on the skin surface and inside the HF (hair shaft is present), counterstaining with DAPI (Sigma, Darmstadt, Germany) (blue fluorescence) (Supplementary Figure S3a, NP in HF). In the blank tissue sections, no strong red fluorescence was detectable (Supplementary Figure S3b, control without NP [HF with hair shaft]) (blue fluorescence: DAPI). Visualization of NPs inside the scalp HFs can only be shown by Body Donors, because ethical reasons do not allow invasive procedures in living participants. In addition, because of ethical reasons, only fluorescently covalently labeled -PLGA-NP for the human in vivo studies could be used.

**SUPPLEMENTARY REFERENCES**


Supplementary Figure S2. Overview of differential stripping results of study groups. Percentage of dose of applied NPs per 1,767 cm² recovered on skin surface, hair follicles, skin rest, glove, and calculated mass balance are shown as mean ± SD. The limits of 85–115% for desired mass balance range recommended by the Scientific Committee on Consumer Safety guidelines (2018) are marked, experimental sets were excluded if they did not meet this criteria.
Supplementary Figure S3. Confocal laser scanning microscope (CLSM) images of DiD-loaded PLGA-NPs applied on the scalp of a Body Donor. (a, b) 20-μm longitudinal sections, counterstained with DAPI; left-hand images, no transmission light overlay; right-hand images, with transmission light overlay. (a) CLSM image of DiD-loaded PLGA-NPs (red) in HF (with hair shaft); DiD detection: HeNe 633 laser, emission: 665–680 nm, gain 40; DAPI detection: diode 405 laser, emission: 430–450 nm, gain 60; magnification ×25; pixel size/voxel size: x = 0.455 μm, y = 0.455 μm, z = 2.664 μm; 15 frames. Scale bars = 100 μm. (b) CLSM image of Control without NP in HF (with hair shaft); DiD detection: HeNe 633 laser, emission: 665–680 nm, gain 100; DAPI detection: diode 405 laser, emission: 430–450 nm, gain 60; magnification ×10; pixel size: x = 0.001 mm, y = 0.001 mm; 1 frame. Scale bars = 200 μm. (c, d) 100-μm transversal sections, overlay with transmission light channel. (c) CLSM image of DiD-loaded PLGA-NPs (red) in HF (with hair shaft); DiD detection: HeNe 633 laser, emission: 665–680 nm, gain 40; magnification ×25; pixel size/voxel size: x = 0.104 μm, y = 0.104 μm, z = −0.568 μm; 137 frames. Scale bar = 100 μm. (d) CLSM image of Control without NP in HF (with hair shaft); DiD detection: HeNe 633 laser, emission: 665–680 nm, gain 40; magnification ×25; pixel size/voxel size: x = 0.455 μm, y = 0.455 μm, z = 3.582 μm; 25 frames. Scale bar = 100 μm.
Supplementary Table S1. Differential stripping data (Mean ± SD) of healthy forearm and healthy scalp, Body Donor scalp and alopecia areata-affected scalp

<table>
<thead>
<tr>
<th>% of dose of applied NP per 1,767 cm² application area</th>
<th>Forearm</th>
<th>Scalp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin surface</td>
<td>Healthy</td>
<td>Healthy</td>
</tr>
<tr>
<td></td>
<td>No. of experimental sets</td>
<td>13</td>
</tr>
<tr>
<td>Skin rest</td>
<td>0.1±0.2</td>
<td>1.3 ± 1.1</td>
</tr>
<tr>
<td>Glove</td>
<td>28.1 ± 5.5</td>
<td>24.1 ± 3.7</td>
</tr>
<tr>
<td>HF</td>
<td>2.5 ± 0.9</td>
<td>8.5 ± 3.6</td>
</tr>
<tr>
<td>Mass balance</td>
<td>91.7 ± 3.2</td>
<td>90.3 ± 3.4</td>
</tr>
</tbody>
</table>

| NP deposited in HFs per application area (µg) | Forearm | Scalp |
|                                             | No. of experimental sets | 13 | 7 | 6 | 5 |
| HF                                          | 19.05 ± 12.78 | 59.79 ± 24.46 | 41.83 ± 18.53 | 20.49 ± 2.90 |

| NP deposited per HF (µg) | Forearm | Scalp |
|                         | No. of experimental sets | 13 | 7 | 6 | 5 |
| HF                      | 0.75 ± 0.57 | 0.42 ± 0.16 | 0.33 ± 0.21 | 0.23 ± 0.07 |

Boldface indicates deposition in HFs.
For statistical analysis, refer to Figure 2b.
Abbreviations: HF, hair follicle; NP, nanoparticle.