

Safety of the use of gold nanoparticles conjugated with proinsulin peptide and administered by hollow microneedles as an immunotherapy in type 1 diabetes

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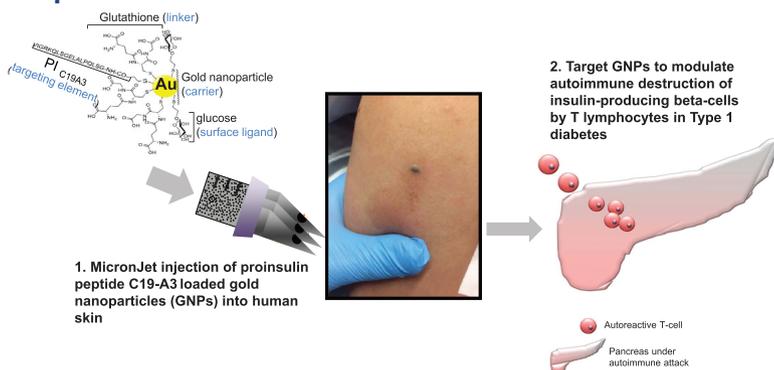
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Abstract

Antigen-specific immunotherapy is an immunomodulatory strategy for autoimmune diseases, such as type 1 diabetes, in which patients are treated with autoantigens to promote immune tolerance, stop autoimmune β -cell destruction and prevent permanent dependence on exogenous insulin. In this study, human proinsulin peptide C19-A3 (known for its positive safety profile) was conjugated to ultrasmall gold nanoparticles (GNPs), an attractive drug delivery platform due to the potential anti-inflammatory properties of gold. We hypothesised that microneedle intradermal delivery of C19-A3 GNP may improve peptide pharmacokinetics and induce tolerogenic immunomodulation and proceeded to evaluate its safety and feasibility in a first-in-human trial. Allowing for the limitation of the small number of participants, intradermal administration of C19-A3 GNP appears safe and well tolerated in participants with type 1 diabetes. The associated prolonged skin retention of C19-A3 GNP after intradermal administration offers a number of possibilities to enhance its tolerogenic potential, which should be explored in future studies

Graphical Abstract



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Keywords: gold nanoparticle, peptide immunotherapy, microneedle, type 1 diabetes, proinsulin

Abbreviations: ACR: Albumin/creatinine ratio; GADA: Anti-glutamic acid decarboxylase antibody; IA-2A: Anti-insulinoma associated antigen 2; ZnT8A: Anti-zinc transporter 8; ASI: Antigen-specific immunotherapy; AUC: Area under the curve; CGM: Continuous glucose monitor; DSMB: Data Safety Monitoring Board; DLS: Dynamic light scattering; GNPs: Gold nanoparticles; ICP-MS: Inductively coupled plasma-mass spectrometry; IDAA1c: Insulin dose-adjusted HbA1c; IMP: Investigational medicinal product; LC-MS: Liquid chromatography-mass spectrometry; MPA: Medical products agency; MHRA: Medicines and healthcare products regulatory Agency; MP-AES: Microwave plasma atomic emission spectroscopy; MMTT: Mixed-meal tolerance test; ¹H-NMR Nuclear magnetic resonance; PES: Poly ether sulfone; RERB: Regional Ethical Review Board; TMAH: Tetramethylammonium hydroxide; TEM: Transmission electron microscopy; T1D: Type 1 diabetes; UCPCR: Urine C-peptide to creatinine ratio.

Introduction

Autoimmune destruction of insulin-producing β -cell by T lymphocytes in type 1 diabetes (T1D) makes affected individuals permanently dependent on exogenous insulin. Despite advances in insulin delivery with glucose sensors and closed-loop systems, in many regions of the world less than 30% of patients achieve glycaemic control sufficient to prevent long-term complications [1]. Immunotherapy aims to stop β -cell destruction, thus preserving the individual's ability to synthesise endogenous insulin. This could lead to better control, or even disease prevention [2, 3]. Antigen-specific immunotherapy (ASI) is an attractive immunomodulatory strategy for antigen-specific autoimmune diseases, such as T1D in which patients are treated with autoantigens to promote immune tolerance. The aim is to boost immunoregulation, by expanding antigen-specific regulatory T-cells, although some ASI appears to act predominantly by deleting or anergising antigen-specific effector cells [4–8]. The advantage of this approach is that it can potentially slow the disease process in a targeted manner, without the need for systemic immunosuppression. Human studies have been conducted in this area [8, 9] and a range of approaches have been used including intramuscular injection of whole antigen alone or with adjuvant [10–14], administration of the antigen via the mucosal route (orally or nasally) [15–18], DNA vaccination [19, 20], and the use of peptide epitopes or altered peptide ligands [21]. These approaches have generally proved safe and very well tolerated but achieving sufficient efficacy to reliably reverse established disease in humans has not yet been demonstrated [8, 14, 16, 17, 22]. A review of the antigen-specific immunotherapy trials to date suggests that the context in which the antigen is presented to the immune system plays an important role in efficacy [23–26].

In this study, the HLA-DR4 (DRB1*0401) restricted proinsulin peptide C19-A3 has been selected as the therapeutic immunomodulatory antigen as it is associated with a positive safety profile in Phase 1 clinical studies and is thought to preserve β -cell function by modulating autoreactive CD4 T-cells [27, 28]. In addition, this peptide has been conjugated to gold nanoparticles (GNPs), an attractive drug delivery platform in this context due to the potential anti-inflammatory properties of gold [29], to create a C19-A3 GNP construct. Previous *in vitro* studies conducted in our laboratories administered the C19-A3 GNP construct into the skin using hollow microneedles and exemplified the diffusive properties of the C19-A3 GNP construct, which facilitated its reflux from the point of dermal deposition to the overlying human skin epidermis. These studies demonstrated maintained stability of the construct upon delivery and efficient uptake by antigen-presenting cells in the skin, with the responsible antigen-presenting cells being identified as predominantly Langerhans cells [30], which are considered to have tolerogenic properties by some researchers [31, 32], whilst others favour the tolerogenic potential of other skin dendritic

cells [33, 34]. Importantly, the use of GNP provides the possibility of introducing a second cargo to accompany the peptide to further enhance the tolerogenic properties of the construct. Thus, the microneedle-administered C19-A3 GNP construct may offer a valuable platform for tolerogenic immunomodulation in T1D and, therefore, the aim of this study was to evaluate the safety of the construct in a first-in-human clinical trial.

Materials and methods

Study design

The study was a two-centre, open-label, uncontrolled, single-group first-in-human Phase 1A safety study of C19-A3 GNP peptide in individuals with T1D.

The Investigational medicinal product (IMP) was C19-A3 GNP (Midacore™), which comprises GNPs [30, 35] of a size of less than 5 nm, covalently coupled to an 18-amino acid human peptide, the sequence of which is identical to the residues from position 19 in the C-peptide of proinsulin through to position 3 on the A-chain of the same molecule (GSLQPLALEGSLQKRGIV). The peptide is synthesised with a linker to facilitate binding to the GNPs: 3-mercaptopropionyl-SLQPLALEGSLQKRGIV 2 acetate salt (disulfide bond). The chemical composition of the IMP contained a ratio of 4 C19-A3 peptides: 11 glucose C2: 29 glutathione ligands as determined by ¹H-NMR (proton nuclear magnetic resonance). The nanoparticle suspension was purified using Amicon ultra centrifugal filtration units with a 10 kDa cut-off and sterile pre-filtered through a poly(ether sulfone) (PES) 0.22 μ m pre-sterilised cartridge compliant for use in GMP manufacturing. Liquid chromatography-mass spectrometry (LC-MS) analysis showed that peptide integrity was maintained in the nanoparticles. Stability testing was also performed both on the nanoparticle product stored at 4°C for 23 weeks and at 25°C for 10 weeks. The IMP was manufactured at Midatech Pharma Plc (Derio, Spain) and delivered as a sterilised solution in small vials produced by Baccinex SA (Courroux, Switzerland). Following the filling process of the IMP stability batch (No. F15220), the structure of the peptide was checked both by ¹H-NMR and by LC-MS for the release of the drug product. Both methods confirmed the integrity of C19-A3 peptide. The IMP was stored, QP released, and shipped to the clinical sites by PharmaKorell GmbH (Lörrach, Germany). A typical batch contained: [C19-A3 peptide] = 1.33 mg/ml; [gold] = 5.5 mg/ml; [glucose linker] = 0.6 mg/ml; [glutathione linker] = 1.79 mg/ml. As the drug substance was diluted 1:7 to 1:10, depending on the content of C19-A3 peptide per particle, and as 50 μ l of the diluted solution was administered to the study participants, this corresponded to C19-A3 peptide: 10 μ g; gold: 39 μ g; glucose linker: 4.3 μ g; glutathione: 12.7 μ g. The vials were stored refrigerated at 2–8°C.

C19-A3 GNP was administered intradermally in the deltoid region of the arm via CE-marked 600 μ m length

MicronJet600™ hollow microneedles (NanoPass Technologies Ltd.) attached to a standard luer-lock syringe. The device has been used in over 50 clinical trials, mostly for the delivery of vaccines [36–40], but also for multiple early-phase trials as well as Phase III study in allergy immunotherapy for the purpose of inducing tolerance (<https://clinicaltrials.gov/>).

Three doses were given at 4-weekly intervals (weeks 0, 4, and 8) in alternate arms (2 doses in one arm and 1 dose in the other arm). The single-dose given in 50 µl volume was equivalent to 10 µg of C19-A3 peptide (cumulative dose of 30 µg). Participants were followed up according to the full study protocol for up to 52 weeks in total (Fig. 1).

Inclusion criteria were as follows: age (16–40 years of age), >3 months from a clinical diagnosis of T1D (dated from the day of first insulin injection), commenced on insulin within 1 month of diagnosis, *HLA-DRB1*0401* genotype and 2-h post-meal urine C-peptide to creatinine ratio (UCPCR) >0.2 nmol/mmol or random serum C-peptide >0.06 nmol/l on at least one occasion (maximum three tests on different days). The main exclusion criteria were the use of immunosuppressive or immunomodulatory therapies, immunisation with live or killed vaccinations or allergic desensitisation procedures less than 1 month before the first treatment, raised serum creatinine or abnormal urine albumin/creatinine ratio (ACR), HbA1c >86 mmol/mol, recent participation in other research trials of immunomodulatory agents, pregnancy, and breastfeeding.

Safety laboratory measures of haematological indices, liver function, thyroid-stimulating hormone, urea, creatinine, calcium, lipid levels, immunoglobulin levels, and urine analysis (pH, blood, protein, albumin/creatinine ratio, urine β-2-microglobulin, and cystatin-C) were performed at baseline, 4, 9, 14, 20, and 52 weeks. Serum and urine gold concentrations were measured at baseline, 1 day after the first and third dose, and at 9, 14, 20, and 52 weeks. The planned duration of the study was initially 20 weeks, but this was later extended to 52 weeks to continue safety follow-up of the participants due to a persistent local response to the intradermal injection (as

described below). Skin changes at the injection site were followed up beyond 52 weeks in some individuals in the clinical care setting (12–24 months).

All subjects were observed for 6 h after their first dose/injection. The first subject was dosed >24 h before subsequent subjects as per sentinel dosing strategy. If no serious adverse events were observed after the first dose, all subjects were observed for a minimum of 1 h after each subsequent injection, with the plan to extend the observation period if there were any signs of hypersensitivity. All local and systemic reactions were documented. Safety data were under regular review by an independent Data Safety Monitoring Board (DSMB).

The primary end-point was an assessment of the safety of C19-A3 GNP administration; secondary end-points were assessments of changes in (i) C-peptide secretion at weeks 14 and 52, compared to baseline, assessed by a mixed-meal tolerance test (MMTT) and a stimulated urine C-peptide test, (ii) glycaemic control assessed by glucose profiles, insulin requirements, and HbA1c at weeks 14 and 52, compared with baseline, (iii) level or quality of lymphocyte biomarkers of β-cell-specific immune response, and (iv) level or quality of islet cell autoantibody biomarkers of β-cell-specific immune response.

Ethics statement

This study was carried out with the approval of the UK Research Ethics Service, Swedish Regional Ethical Review Board (RERB), UK Medicines and Healthcare products Regulatory Agency (MHRA) for Clinical Trial Authorisation, and Swedish Medical Products Agency (MPA). Written informed consent was obtained from all participants. The trial was conducted in compliance with the principles of the Declaration of Helsinki (1996) and the principles of Good Clinical Practice and in accordance with all applicable regulatory requirements including but not limited to the Research Governance Framework and the Medicines for Human Use (Clinical Trial) Regulations 2004, as amended in 2006. Further details are available at <https://clinicaltrials.gov/>.

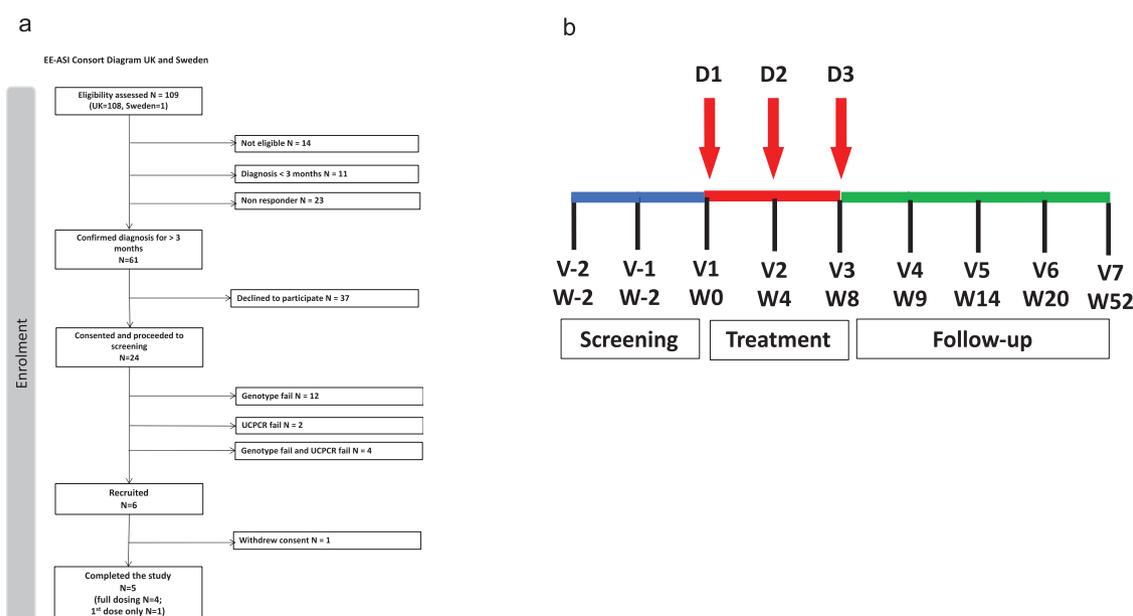


Figure 1. Study recruitment and visit schedule. (a) Consort diagram and (b) schedule of the study visits. D, dose; V, visit; W, week.

Assays

Ensure Plus® [Abbott Nutrition, Maidenhead, UK; 6 ml/kg (max 360 ml)] was used as a mixed-meal stimulant of β -cell production, in both the standard MMTT and the assessment of stimulated urine C-peptide/creatinine ratio as described previously [41, 42]. MMTTs were carried out after an overnight fast at 0, 14, and 52 weeks. Serum samples for C-peptide and glucose were collected at -10, 0, 15, 30, 60, 90, and 120 min. Urine samples were collected from the second void in the morning (before MMTT) and 120 min after the MMTT (mixed-meal-urine C-peptide/creatinine ratio) with no urine loss in between.

Urine samples were collected in boric acid containers (Sterilin; Thermo Scientific, Newport, UK) and transported to a laboratory at ambient temperature within 72 h. If not assayed within 72 h of collection, they were stored at -80°C for up to 14 days. Serum samples were stored at -20°C and transported on dry ice in batches. Urine C-peptide level was measured in samples diluted 1:10, using an enzyme-linked immunosorbent assay (10-1136-01; Mercodia, Uppsala, Sweden). The detection limit for the C-peptide assay was 0.025 nmol/l, with intra- and inter-assay coefficients of variation of <5% and <5%, respectively. Urine creatinine was assayed using a colorimetric method (Jaffe reaction; CR8316; Randox Ltd, London, UK). The detection limit, and intra- and inter-assay coefficients of variation were 100 $\mu\text{mol/l}$, <4% and <6%, respectively. Results were expressed as urine C-peptide/creatinine ratio (nmol/mmol). Serum C-peptide was measured using an immunochemiluminometric assay (IV2-004, Invitron, Monmouth, UK). The detection limit, and intra- and inter-assay coefficients of variation were 0.005 nmol/l, <5% and <8%, respectively.

Anti-glutamic acid decarboxylase antibody (GADA), anti-insulinoma-associated antigen 2 (IA-2A), and anti-Zinc transporter 8 (ZnT8A) were measured by enzyme-linked immunosorbent assay (GDE/96, IAE/96/2, ZnT8/96; RSR Ltd., Cardiff, UK) according to the manufacturer's instructions. Positive cut-off values were ≥ 5 , ≥ 7.5 , and ≥ 15 U/ml for GADA, IA-2A, and ZnT8A, respectively. The detection limit for the GADA was 0.57 U/ml, for IA-2A was 1.25 U/ml, and for ZnT8A was 1.2 U/ml.

The gold concentration of the IMP was measured by Midatech Pharma Plc using microwave plasma atomic emission spectroscopy (MP-AES) and inductively coupled plasma-mass spectrometry (ICP-MS). Ligand ratios and purity of the IMP were measured by nuclear magnetic resonance ($^1\text{H-NMR}$). Quantification of peptide bound to the GNP was performed using liquid chromatography-mass spectrometry (LC-MS). Particle hydrodynamic size and ζ potential were measured by dynamic light scattering (DLS) and particle diameter by transmission electron microscopy (TEM).

The concentration of gold in serum and urine samples was measured by ICP-MS, using a methodology developed and validated by Midatech Pharma Plc. The ICP-MS was a Perkin Elmer NEXION 300x instrument, equipped with the software NEXION (version 1.4). Serum and urine samples were digested with tetramethylammonium hydroxide (TMAH) solution, using iridium as an internal standard. Standards and control standards were prepared in the same way as the samples, using foetal bovine serum as an organic matrix. Samples were bracketed by control standards at low and high gold concentration. The concentration of gold in

serum and urine was calculated interpolating in a simple-linear through-zero calibration curve (ranging from 0.1 to 100 ng/ml). Detection limit was 0.2 ng/ml and quantitation limit was 0.6 ng/ml.

Glucose variability was assessed by using a Dexcom Platinum G4 continuous glucose monitor (CGM). Recordings were taken for at least 72 h before study visits at weeks 0, 14, and 52.

Punch skin biopsies of the local area were performed under aseptic conditions and under local anaesthetic (Lidocaine Hydrochloride Injection BP 2%, w/v), using a 6-mm sterile disposable biopsy punch. Following the biopsy, samples were immediately placed in 10% formalin and transported to the laboratory.

Automatic tissue processing (Excelsior AS) and staining (Leica Autostainer) produced Haematoxylin & Eosin-stained tissue sections for assessment. Immunohistochemistry for CD2, CD3, CD4, CD8, CD20, CD79a, CD68, CD1a, and Ki67 was performed (Ventana Benchmark Ultra stainer).

The distribution of the gold was assessed using light microscopy of skin sections by catalytic deposition of silver. Dewaxed and rehydrated sections were incubated in Newman and Jasani's physical developer [43]. Sections were then counterstained with Nuclear fast red + picro-methyl blue/light green [44]. The presence of elemental gold was confirmed on reprocessed samples using a transmission electron microscope (TEM; Philips CM12) fitted with an energy dispersive X-ray spectrometer (EDAX).

Gold hypersensitivity was assessed by using an epicutaneous patch test. Aqueous gold sodium thiosulphate (concentration range 2.0–0.0002%, w/v) and gold sodium thiosulphate in petrolatum 2% w/w were applied by using Finn Chambers (Chemotechnique Diagnostics, Sweden) to the skin on the participant's back. The patches were removed after 48 h and the test was read after a further 48 h.

Details of immunological assays will be described in a separate publication (Hanna S.J. and are not part of this manuscript.

Statistical analysis

Data are expressed as mean \pm SD and median and interquartile range. A Wilcoxon signed-rank test was used to test the significance of the change in relation to the baseline value. Differences were considered significant if the *P*-value was <0.05. GraphPad PRISM version 9.0 for Macintosh was used for the analysis.

The area under the curve (AUC) was calculated using the trapezoidal method, not adjusted for baseline C-peptide but normalised for the 120-min period of the standard MMTT using the serum C-peptide value at each time point.

The insulin dose-adjusted HbA1c (IDAA1c) was used as a surrogate measure of β -cell function. It examines the combined impact of changes in HbA1c and insulin usage on metabolic control. Levels ≤ 9 are considered favourable. It was calculated according to the formula: $\text{HbA1c (\%)} + [4 \times \text{insulin dose (units per kg per 24 h)}]$ [45].

Safety assessments (i.e. safety blood and urine tests and IMP-related adverse events during the course of the study) were counted in terms of both the number of events and the number of participants. Injection site reactions were described verbally and photographed.

Results

Study enrolment and study participants

Twenty-four participants attended a screening visit. Participants who did not have either the *HLA-DRB1*0401* genotype ($N = 12$), stimulated serum/urine C-peptide below the defined threshold ($N = 2$) or both ($N = 4$) were excluded (Fig. 1a).

A total of six participants were enrolled in the study and received the first injection. Four participants received all three doses as planned. Two participants received only the first dose. One of them withdrew from further assessments due to competing time commitments and was withdrawn from further analysis, whilst another did not receive the second and third doses because of the decision to halt further drug administration. The latter participant remained in the study and attended all planned visits and assessments. A total of five participants were included in the final analysis.

The study was initially temporarily halted to obtain further information and expert advice about a delayed local reaction at the injection site that was observed in all participants (as described below). The study was subsequently terminated before recruitment of the planned eight participants was completed because the study was considered to have had achieved its primary aim, i.e. to assess the safety and tolerability of the IMP.

Mean participant age on entry was 28.00 ± 7.92 years [18–37]. Their age at diagnosis of T1D was 27.00 ± 10.12 years with a mean diabetes duration of 32.20 ± 35.39 months (Supplementary Table S1). Two out of the five participants were female, and all were of white ethnicity.

All participants were autoantibody-positive defined by being positive to at least one of GADA, IA-2A, or ZnT8A.

Evaluating changes in C-peptide levels

Median serum C-peptide AUC at baseline was 0.88 (0.56–1.30) nmol h/l. There was no significant difference in median serum C-peptide AUC at week 14 (0.70 (0.44–0.80) nmol h/l) and week 52 (0.40 (0.26–0.95) nmol h/l) in comparison to

baseline. The serum C-peptide remained stable in two out of five participants (Fig. 2a, Supplementary Table S3).

Median UCPCR at baseline was 1.56 (0.86–3.18) nmol/mmol. There was no significant difference in median UCPCR at week 14 (1.84 (0.69–1.91) nmol/mmol) and week 52 (0.82 (0.31–1.08) nmol/mmol) in comparison to baseline. It remained stable in three out of five participants (Fig. 2b, Supplementary Table S3).

Evaluating glycaemic control

Median HbA1c at baseline was 48.0 (38.5–65.0) mmol/mol. There was no significant difference at week 4 (52.0 (38.5–67.5) mmol/mol), week 9 (49.0 (40.5–68.5) mmol/mol), week 14 (45.0 (43.5–79.0) mmol/mol), and week 52 (45.0 (42.0–68.5) mmol/mol) in comparison to baseline (Fig. 2c, Supplementary Table S3).

Median insulin dose at baseline was 0.43 (0.24–0.63) units/kg. It remained at similar levels at weeks 4, 9, and 14 (0.46 (0.19–0.60) units/kg, 0.52 (0.18–0.61) units/kg, and 0.45 (0.27–0.61) units/kg, respectively). The observed increase to 0.58 (0.30–0.80) units/kg at week 20 and to 0.54 (0.33–0.81) units/kg at week 52 was not statistically significant in comparison to baseline (Fig. 2d, Supplementary Table S3).

Median IDAA1c at baseline was 7.72 (7.10–10.34). It remained unchanged at weeks 4 and 9 (7.73 (7.31–9.74) and (7.94 (7.17–10.13)), respectively). The observed increase to 8.00 (7.27–11.84) at week 14 and to 8.34 (7.37–11.68) at week 52 was not statistically significant in comparison to baseline (Fig. 2e, Supplementary Table S3).

At the start of the study, glucose levels were in range (glucose between 4 and 10 mmol/l; measured by CGM) 80.80% (52.80–86.50%) of the recorded period (at least 72 h). This did not change significantly when assessed at 14 and 52 weeks (57.50 (49.50–82.00)% and 69.70 (60.00–76.25)%, respectively) – Fig. 3a, Supplementary Table S3.

There was no significant difference in time spent above range (glucose >10 mmol/l) at week 14 (39.10 (13.50–48.70) %) or

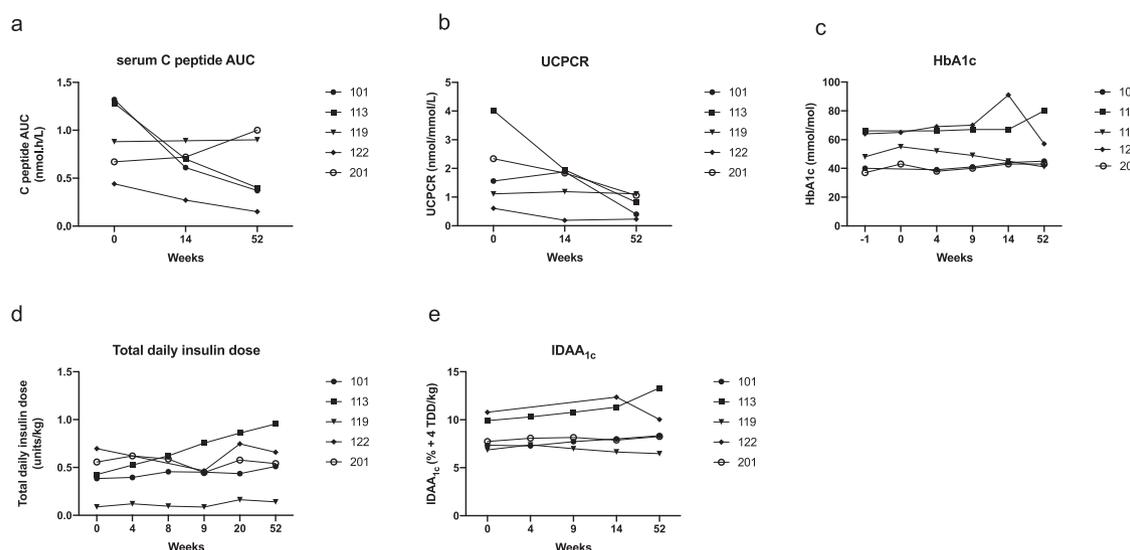


Figure 2. Effect of C19-A3 GNP on β -cell function and metabolic parameters. (a) Serum C-peptide expressed as area under the curve (AUC) over 120 min after mixed-meal challenge; (b) urine C-peptide to creatinine ratio (UCPCR) 2 h after mixed meal; (c) HbA1c; (d) median total daily insulin dose recorded over 3 days before the study visit and normalised for body weight; (e) insulin dose-adjusted A1c (IDAA1c) calculated according to the formula: $\text{HbA1c (\%)} + [4 \times \text{insulin dose (units per kg per 24 h)}]$. Each line represents an individual participant with an assigned study number as shown in the legend.

week 52 (25.90 (15.00–36.80) %) in comparison to baseline (16.00 (10.55–47.00) %) – Fig. 3b, Supplementary Table S3.

There was also no significant difference in time spent below range (glucose <4 mmol/l) at week 14 (3.40 (1.80–4.50) %) and 52 (2.60 (0.85–12.00)) in comparison to baseline (0.40 (0–4.55) %) – Fig. 3c, Supplementary Table S3.

Evaluating gold serum concentration and excretion

Serum gold concentrations increased in all participants after the first and third dose of C19-A3 GNP peptide. The decline in the gold serum concentrations was observed as early as week 9 (1 week after the third dose), although detectable levels were present in all participants at that point in time, with the exception of the participant who received the first dose only. A reduction in gold serum concentration became more evident at weeks 14 and 20, when only three and one participant(s), respectively, had detectable levels. Serum gold levels were undetectable at week 52 in all participants – Fig. 4a, Supplementary Table S4.

Measurements of gold in the urine showed a less consistent pattern. An increase in urinary gold concentration after dosing was recorded in two of the five participants 1 day after the first dose, one of four participants 1 day after the third dose, and in two of the four participants 1 week after the third dose. Urinary gold urine levels were undetectable at week 52 in all participants – Fig. 4b, Supplementary Table S5.

Safety assessments in blood and urine tests

There were no clinically significant abnormalities in safety blood and urine tests and no IMP-related serious

adverse events were reported during the course of the study. There was one serious adverse event requiring hospital admission, which was secondary to *Campylobacter* infection, which was considered unrelated to the IMP (Supplementary Table S6).

Participants also reported headache, mild upper respiratory tract infection symptoms, and nausea and vomiting during the study. None of the reported adverse events were considered to be directly related to the IMP (Supplementary Table S6).

Injection site reactions and gold hypersensitivity

All participants developed an immediate localised skin reaction after all IMP injections. It consisted of an asymptomatic GNP-derived dark-coloured central area (3–4 mm) surrounded by a circular erythematous area (diameter of 2–3 cm), as illustrated in Fig. 5a–g. Erythema disappeared after 45–60 min of close observation, but the dark central area persisted and reduced in size to 1–2 mm by the following day.

Subsequently, delayed skin changes over the injection site were observed. The change consisted of a small central area of GNP-derived pigmentation surrounded by an asymptomatic circular erythematous induration. Induration initially reached 2–3 cm in diameter that reduced to approximately 1 cm during the follow-up period. These delayed reactions appeared 17.4 ± 2.3 days after the first injection, but much sooner, i.e. 1–3 days after the second and third injection. This was consistent in all participants. The skin changes significantly faded over the course of the study, although they were still visible at the end of the observation period (12–24 months depending on the participant) – Fig. 5a–g.

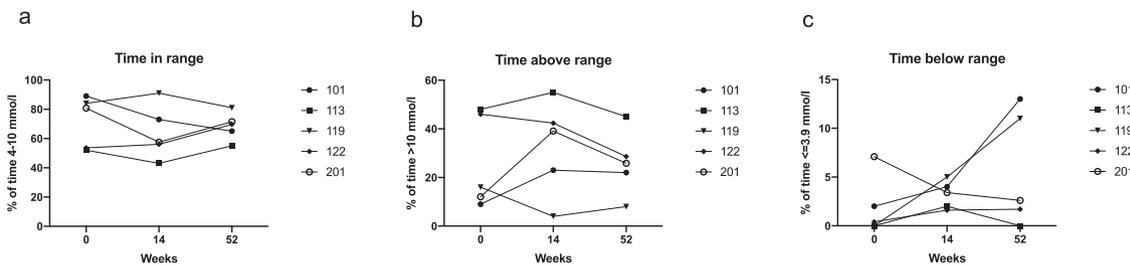


Figure 3. Effect of C19-A3 GNP on glucose variability. (a) Time in range (the % of time participants recorded a blood glucose level of 4–10 mmol/l); (b) time above range (the % of time participants recorded a blood glucose level of >10 mmol/l); (c) time below range (the % of time participants recorded a blood glucose level of <4 mmol/l). Recordings were taken for at least 72 h before study visits. Each line represents an individual participant with an assigned study number as shown in the legend.



Figure 4. Serum and urine gold concentration following administration of C19-A3 GNP. (a) Serum gold concentration; (b) urine gold concentration. Blood and urine samples for measurement of gold concentration were taken at baseline (week 0), one day after 1st dose (week 0 + 1 day) and one day after 3rd dose (week 8 + 1 day). Detection limit was 0.2 ng/ml and quantitation limit 0.6 ng/ml. Levels below detection and quantitation limit are presented as zero on the graph. Each line represents an individual participant with an assigned study number as shown in the legend.

Four participants who received the IMP injection agreed to have gold hypersensitivity testing. All had positive reactions with epicutaneous patch testing to gold sodium thiosulphate 2% in petrolatum.

A skin biopsy of the injection site was performed on two participants. Haematoxylin & eosin-stained tissue sections showed a normal epidermis with perivascular inflammation in the upper- and mid-dermis. The inflammatory infiltrate was composed of lymphocytes and histiocytes, but no granulomas were present. Immunohistochemistry showed the infiltrate to be mainly CD3-positive T-cell lymphocytes (equal distribution

of CD4+ and CD8+) with small numbers of CD20-positive B-cell lymphocytes. There was also evidence of intradermal gold retention – Fig. 6a-f.

Discussion

This study has explored the concept of enhanced antigen-specific immunotherapy by conjugating proinsulin peptide C19-A3 which has a known favourable immunomodulating profile [28] to very small gold nanoparticles to target antigen delivery to potentially tolerogenic DCs in the skin.

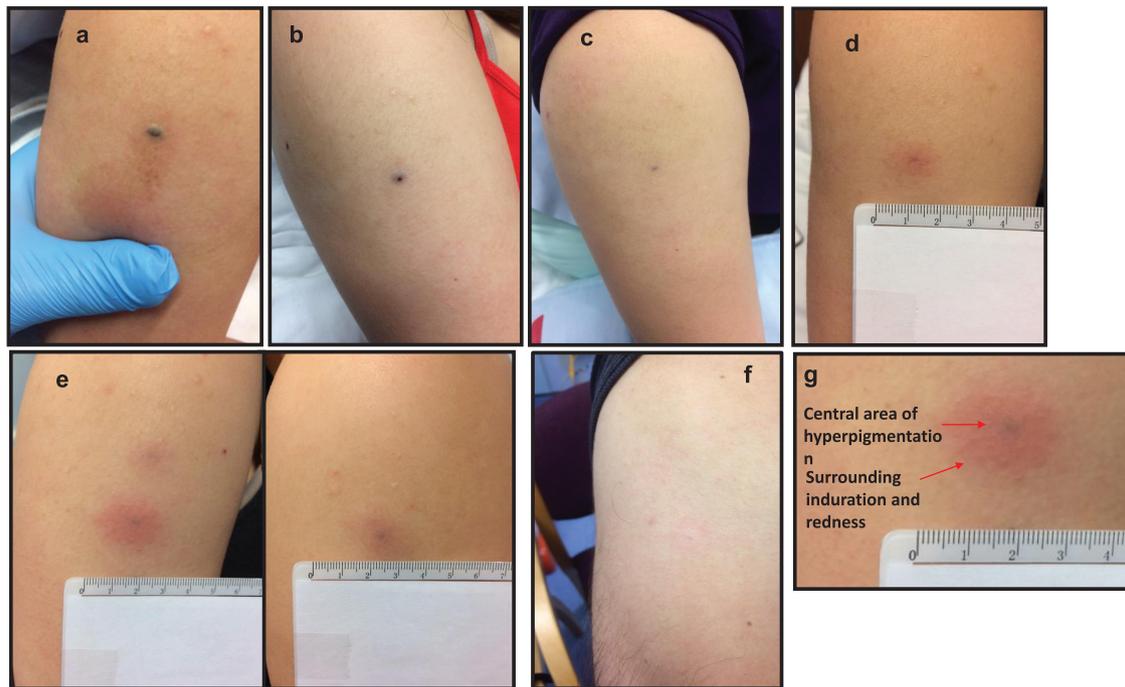


Figure 5. Skin changes after intradermal injection of C19-A3 GNP. (a) Immediately after injection; (b) 5 min after injection; (c) 24 h after injection; (d) 30 days after injection; (e) 2 months after injection (upper left), 1 month after injection (right) and 7 days after injection (lower left); (f) 20 months after injection; (g) close-up of the injection site showing central area of hyperpigmentation and surrounding induration and redness.

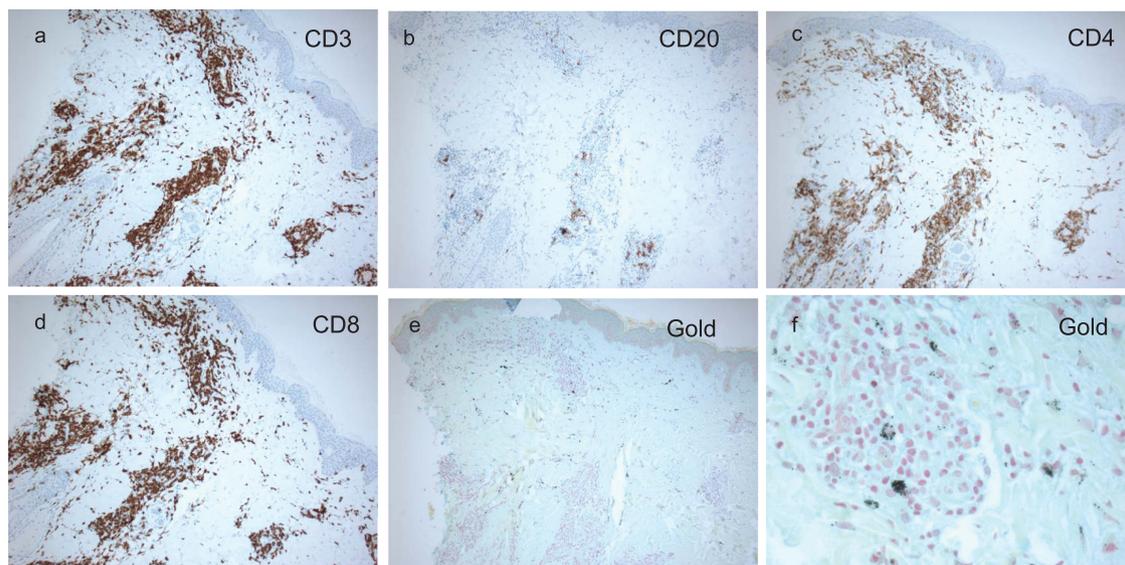


Figure 6. Histopathology and immunohistochemistry staining of the punch biopsies of the injection sites. (a) Anti-CD3 staining; (b) anti-CD20 staining; (c) anti-CD4 staining; (d) anti-CD8 staining; (e) and (f) gold staining.

This first-in-human study was designed to assess the safety and tolerability of the new construct. In general, dosing was well tolerated by patients. The study was not powered to assess effects on β -cell preservation, although there was no evidence of accelerated β -cell function decline in the small number of participants. Participants were not recruited immediately after diagnosis of T1D as these patients are often recruited to other immunomodulatory trials in T1D. However, the requirement for a participant to have a threshold C-peptide value ensured they had a residual β -cell function at the time of their enrolment in the study. Two participants maintained stable levels of stimulated C-peptide and three had an IDAAC1 level in the favourable range (<9) for the duration of the trial [45]. However, in at least one participant stable C-peptide levels can be explained by the stage of their diabetes, i.e. a slow natural decline in C-peptide in that phase of the disease was expected. Others showed moderate decline in endogenous insulin production, which again may be consistent with the natural progression of T1D during the early years of the disease (three out of five participants), [Supplementary Table S2](#) [46]. Importantly, insulin requirements of most participants remained low (<0.8 units/kg of body weight) throughout the study and there was reasonably good glycaemic control with median HbA1c of 45.00 (42.0–68.5) mmol/mol and glucose levels, recorded at the time of the last visit, that was maintained within expected range for 69.7% (60.0–76.2%) of the time ([Figs. 2 and 3](#)).

There was no evidence of systemic hypersensitivity and no IMP-related serious adverse effects were observed during the study. In addition, there was no evidence of systemic gold retention or effect as evidenced by normalisation of serum and urine gold concentration ([Fig. 4](#), [Supplementary Tables S4 and S5](#)) and normal safety tests including renal and liver function tests.

All participants developed a delayed local skin reaction at the site of each injection ([Fig. 5](#)). It appeared 1–3 weeks after the first injection, 1–3 days after the second and third injection, and persisted, gradually fading over 12–24 months. Histopathological findings indicated retention of gold in the dermis ([Fig. 6e and f](#)) with B- and T-cell infiltrate in distribution and quantity atypical to normal skin, but not consistent with foreign body granuloma ([Fig. 6](#)). Immunological analysis of this infiltrate and its antigen specificity (gold vs. C19-A3 peptide) will be reported separately (Hanna S.J.). However, the observation of the prolonged retention of the GNP (and possibly the whole construct, including peptide) in the skin is a unique outcome of this study that opens a number of therapeutic approaches, including the creation of a depot with the potential to create a sustained immunomodulating environment in the skin. In this example, the addition of tolerogenic cargo could facilitate a favourable effect on autoimmune processes and β -cell function.

To assess the possibility of induced gold hypersensitivity, participants had a skin patch hypersensitivity test to gold thiosulphate which came back positive in all tested subjects. Gold thiosulphate was chosen as it is the antigen routinely used in standard clinical practice for patch testing to detect gold hypersensitivity. Contact allergy can occur in routinely patch-tested patients, but the clinical relevance of this is unclear and often not demonstrated, as a systemic hypersensitivity rarely, if ever, develops [47, 48]. There is a potential

cosmetic drawback of permanent or semi-permanent skin changes. These could be practically overcome in future studies by choosing less exposed areas for injection sites and they may be more or less visible in different skin types. The occurrence of injection site reactions was reviewed by the DSMB who considered that events did not adversely change the risk/benefit ratio and did not constitute a safety issue.

We are of the opinion that benefits of this approach (well-tolerated, minimally invasive intradermal administration that could be modified to self-administration in the future; potential for creating a sustained tolerogenic immunomodulating environment in the skin; no systemic gold retention or effects) outweigh the risks (possible induction of gold hypersensitivity with unknown clinical significance and no evidence of systemic hypersensitivity; unfavourable cosmetic effect).

In summary, allowing for the limitation of the small number of participants, intradermal administration of C19-A3 GNP constructs appears safe and well tolerated in people with T1D. The associated prolonged skin retention after intradermal administration offers a number of possibilities to enhance its tolerogenic potential in T1D, which should be explored in future studies.

Supplementary material

Supplementary data are available at *Immunotherapy Advances* online.

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Author contributions

D.T.: conception and design of work; acquisition, analysis, and interpretation of data; drafting and revising the manuscript. M.A.Mc.A.: conception and design of work; acquisition, analysis, and interpretation of data; revising the manuscript. J.B.: acquisition, analysis, and interpretation of data; revising the work. A.B.: acquisition, analysis, and interpretation of data; revising the manuscript. K.R.T.: acquisition, analysis, and interpretation of data; revising the manuscript. I.P.: acquisition, analysis, and interpretation of data; revising the manuscript. E.K.: interpretation of data; revising the manuscript. Y.L.: interpretation of data; revising the manuscript. M.D.: acquisition, analysis, and interpretation of data; revising the manuscript. S.A.C.: conception and design of work; analysis and interpretation of data; revising the manuscript. J.C.B.: conception and design of work; analysis and interpretation of data; revising the manuscript. C.v.R.: acquisition, analysis, and interpretation of data; revising the manuscript. A.H.: acquisition and interpretation of data; revising the manuscript. R.S.: acquisition and interpretation of data; revising the manuscript. M.A.A.: conception and

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Conflict of interest

J.R.I. is Editor-in-Chief of the British Journal of Dermatology. He is a Consultant for UCB Pharma, Novartis, Boehringer Ingelheim and ChemoCentryx and participated in Advisory Boards for Kymera Therapeutics and Viela Bio, all in the field of hidradenitis suppurativa.

Y.L. is the CEO of NanoPass Technologies Ltd. EK is the Medical Director of NanoPass Technologies Ltd. M.M.U.C. is clinical lead for Dermatology e-learning for Health (HEE) for British Association of Dermatologists.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

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