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Paediatric clinical study of 3D printed personalised medicines for rare metabolic disorders

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Abstract

Rare diseases are infrequent, but together they affect up to 6-10% of the world's population, mainly children. Patients require precise doses and strict adherence to avoid metabolic or cardiac failure in some cases, which cannot be addressed in a reliable way using pharmaceutical compounding. 3D printing (3DP) is a disruptive technology that allows the real-time personalization of the dose and the modulation of the dosage form to adapt the medicine to the therapeutic needs of each patient. 3D printed chewable medicines containing amino acids (citrulline, isoleucine, valine, and isoleucine and valine combinations) were prepared in a hospital setting, and the efficacy and acceptability were evaluated in comparison to conventional compounded medicines in six children. The inclusion of new flavours (lemon, vanilla and peach) to obtain more information on patient preferences and the implementation of a mobile app to obtain patient feedback in real-time was also used. The 3D printed medicines controlled amino acid levels within target levels as well as the conventional medicines. The deviation of citrulline levels was narrower and closer within the target concentration with the chewable formulations. According to participants' responses, the chewable formulations were well accepted and can improve adherence and quality of life. For the first time, 3DP enabled two actives to be combined in the same formulation, reducing the number of administrations. This study demonstrated the benefits of preparing 3D printed personalized treatments for children diagnosed with rare metabolic disorders using a novel technology in the real clinical practice over the current approach.

Keywords: semi-solid extrusion 3D printing, pediatrics, polypills, precision pharmaceuticals, patient acceptability of formulations, additive manufacturing of drug products

1. Introduction

A rare disease has a prevalence of less than 5 cases per 10,000 inhabitants in Europe [1,2]. Paradoxically, rare diseases are relatively common, since together they affect 6-10% of the population. Children affected by hereditary metabolic diseases (12% of all rare diseases) [3] require accurate pharmacological and nutritional treatment with a strict adherence to prevent metabolic decompensation, cardiovascular events or even death [4]. Examples of such disorders include Maple Syrup Urine Disease (MSUD) [5,6], Short-chain Enoyl-CoA Hydratase (ECHS1) deficiency [7], and Ornithine Transcarbamylase (OTC) deficiency [8,9]. Their great impact on patients and families, health services and society [10], confirm that rare diseases are a public health priority [11]. Treatment options are often limited and, in some cases, do not even exist. As the patient population for each disease is small and highly heterogeneous, drug development on an industrial scale faces challenges because medicines need to be adapted to each paediatric patient. Consequently, this leads to therapeutic needs not being adequately met [1,10].

The current treatment for the aforementioned rare metabolic disorders involves the restriction of natural dietary protein intake and nutritional supplementation with the relevant amino acids: isoleucine and valine in MSUD, isoleucine in ECHS1 deficiency, and citrulline in OTC deficiency [7,12,13]. Dosages are personalized based on age, weight, and amino acid blood levels [14], often needing adjustments as the child grows. In current clinical practice, there is a lack of suitable amino acid formulations available on the market and their administration involves the preparation of extemporaneous formulations in hospital settings that are firstly weighed out as a powder [15] to be dispersed in food or drink [14]. Capsules containing the right amount of individual amino acid can be prepared if the child is older (>3 years old). This manual manufacture of medicines according to each patient's needs in a hospital setting is known as 'pharmaceutical compounding'. However, this approach is time-consuming, dosing error-prone, requires human resources, and the final dosage form can be challenging to administer to the child [16]. The paediatric population has been shown to exhibit swallowability issues [17], and although the powder is dispersed in food or drink, children may develop an aversion to the food if the drug exhibits an unpleasant taste.

The personalization of treatments is an ever-growing need [16] and requires flexible and versatile manufacturing approaches, such as the one offered by three-dimensional (3D) printing (3DP) [18-24]. 3DP allows for real-time dose adjustment and modulation of the printlet™ (3D printed tablet) to meet individual patient needs [25-30]. The manufacturing process of the medicine is automatic, avoiding human and dosing errors [31]. The 3D printer can be considered as a tool or equipment that helps to automate, on a small scale (for example, hospital environment), the manufacturing process of small batches of personalized medicines [32-35]. Semi-solid extrusion (SSE) is a material extrusion technology, differing from other 3DP methods through the deposition of a gel or paste at relatively low printing temperatures [36-39]. It is an affordable technique for the potential implementation in a hospital environment, as the preparation of drug loaded gels in the form of 'pharma-ink' is performed in an easy and simple manner inside a disposable syringe [40]. The use of disposable and pre-filled syringes meets the critical quality attributes demanded by regulatory agencies. This enables the syringes to be prepared and filled as per good manufacturing practice (GMP) requirements in pharmaceutical production facilities [40,41]. The dosage forms prepared with SSE can be chewable if the proper excipients are used, so swallowability issues in special populations such as paediatrics, geriatrics and people suffering from dysphagia are overcome and may improve treatment adherence [42-45]. Chewable medicines, particularly vital for paediatric patients, rely on formulation strategies to enhance acceptability and adherence to treatment. Utilizing the proper pharmaceutical excipients, these formulations address sensory characteristics with colouring agents, sweeteners and

flavours, improving palatability [42]. When flavourings alone aren't enough to mask the unpleasant taste of the active ingredient, sweeteners can be added for improvement. Alternatively, for strong unpleasant tastes, cyclodextrin complexation has been investigated for taste-masking purposes [46].

The first aim of this study was to explore the feasibility of 3DP as an alternative method to pharmaceutical compounding, to prepare personalised medicines for paediatrics affected by MSUD, ECHS1 deficiency, and OTC deficiency in a hospital setting. The second aim was to evaluate and compare the efficacy and acceptability of chewable 3D printed medicines containing citrulline, isoleucine, and valine alone or in combination, to the conventional medication (powder or capsules) in children aged 6 - 14.

2. Materials and Methods

2.1 Materials

L-Isoleucine (ILE) (Nutricia, Utrecht, The Netherlands), L-Valine (VAL) (Nutricia, Utrecht, The Netherlands) and L-Citrulline (CIT) (Nutrición Médica, Cantabria Labs, Madrid, Spain) were used as the active ingredients for both the conventional and 3D printed chewable medications. Conventional medication consisted of hard gelatine capsules (Acofarma, Barcelona, Spain) and Avicel® PH 102 microcrystalline cellulose (Acofarma, Barcelona, Spain). Chewable formulation was property of FABRX Ltd. (London, UK). Yellow, green, blue bright and red colorants were purchased in Guinama, (Valencia, Spain). Red colorant for pink colour was purchased in Acofarma (Barcelona, Spain). Strawberry, banana, orange, lemon, peach and vanilla flavours were purchased in Acofarma (Barcelona, Spain).

2.2 Preparation of conventional and 3D printed chewable formulations

All medicines (conventional and 3D printed) were prepared on-site at the hospital. The conventional medicine consisted of weighing out the individual amount of amino acid powder and dispersing it (in water or food) or preparing capsules in the hospital. This involved mixing the amino acid with a standard amount of microcrystalline cellulose for 30 min in an orbital mixer Turbula (WAB-GROUP, Muttenz, Switzerland) and manually filling into hard gelatine capsules.

Chewable formulations before the 3D printing process or pharma-inks of citrulline, isoleucine, valine, and combinations of isoleucine-valine were previously optimized and characterized before the study in the laboratory (Section 2.5). After that, optimized pharma-inks were made following the standard operating protocol at the hospital (Table 1).

Table 1. Pharma-ink compositions for each patient and the temperature used to print the formulations*. ILE: Isoleucine; VAL: Valine; CIT: Citrulline.*

Pharma-ink code	Patient	Isoleucine (% w/w)	Valine (% w/w)	Citrulline (% w/w)	Printing temperature (°C)
ILE	3 and 4	40	-	-	60
VAL	3	-	40	-	55

CIT	5 and 6	-	-	30	40
ILEVAL1	1	20	20	-	60
ILEVAL2	2	22.5	17.5	-	60

Pharma-inks were property of FABRX Ltd. (London, UK) and the excipients used to prepare them included sucrose, pectin (gelling agent), maltodextrin, water, maltitol, flavourings, colourants, and citric acid. Briefly, water was added to a metal container and the corresponding amount of solid excipients were added, little by little, under mechanical stirring (HEI-TORQUE 200, Heidolph Instruments, Schwabach, Germany) to prevent the formation of pectin lumps. Maltitol was added, under stirring, after the solid excipients were mixed. The formulation was heated to 80°C under stirring for 10 minutes. After this, 65°C was selected on the heating plate to prevent the formulation from burning. The amount of amino acid was added according to Table 1 and any flavourings and colourants were also added. Finally, citric acid was added to gel the pectin. Syringes were filled manually using a spatula as the final pharma-ink had a gel-like consistency. The syringes were then stored at room temperature for 1 day before printing to ensure gelation.

Six types of chewable formulations based on different flavours and colours were provided to each patient every 15 days. The flavour and colour schemes were as follows: strawberry-red; orange-green; lemon-yellow; vanilla-blue; banana-bright yellow; peach-pink.

3D printed medicines were manufactured using the pharmaceutical 3D printer M3DIMAKER™ (FABRX Ltd., London, UK) with the SSE printhead function. The printing process included inserting the pharma-ink loaded disposable and sterile luer lock 20 mL syringe (B.Braun, Melsungen, Germany) into the printer. The syringe was then heated to different temperatures depending on the formulation (Table 1) to reach a viscosity suitable to produce the 3D printed chewable medicines by mechanical extrusion. The 3D computer model used to print the formulations was a cylinder (10 mm diameter x 5 mm height) and was designed with Tinkercad (Autodesk, San Francisco, USA). The 3D model was loaded onto the software M3DIMAKER Studio™ (FABRX Ltd., London, UK) which controls the printer. Based on the selected printing parameters, the software sent instructions to the M3DIMAKER printer to print a batch of various printlets arranged in different rows, with varying size and weights per row. The weight of each printlet was then measured using a balance (KERN PEJ model, KERN & SOHN, Balinge, Germany) and the mean value was calculated per row and submitted into the software. The software employed internal algorithms to establish a relationship between the size, weight, dose (considering the amino acid loading), and the selected printing parameters. Once the correlation was established, the printing parameters of the pharma-ink were successfully validated, enabling the initiation of a new print. As a result, the original cylinder model could be scaled to reach the target dose (the higher the dose, the larger the printlet) after the validation process. The slicing configuration was generated using the open-access Slic3r software (version 1.3.1) and the main parameters were: first layer height (1.1 mm), layer height (1.4 mm), two perimeters, external perimeter speed (50%), rectilinear infill pattern, infill speed (25%) and filament diameter (8 mm). 14 Ga tips (Ellsworth Adhesives, Germantown, Wisconsin, USA) were used during the printing

process to print the 3D chewable medication. A post-printing step included weighing of the individual chewable formulations and placing them in Class B X-Large amber PVC blisters (Health Care Logistics, Circleville, USA) after mass uniformity testing.

2.3 Mass uniformity testing of printlets

Uniformity of mass testing was performed according to the European Pharmacopoeia (Ph. Eur.) specifications for tablets [47]. This involved weighing all the printlets from the prepared batch during Stage 2 of the study (**Figure 1**) to confirm if any deviate from that value by a predetermined percentage, using an analytical balance located at the hospital (AUW 120 model, Cobos Precision, Barcelona, Spain). According to the Ph. Eur. standards (section corresponding to mass uniformity for tablets with weights ≥ 250 mg), the deviation of the individual mass that is accepted is 5% with respect to the declared mass. The printlet weight must fall within the accepted limits (± 5% of the expected weight). If a printlet weight deviated outside these accepted limits, the dosage form was rejected and removed from the final batch.

2.4 Printlet characterization

2.4.1 Amino acid content

Printlets (n = 10) were placed in a beaker (1000 mL) with phosphate buffer solution (pH = 7.4) for isoleucine and valine formulations, and 0.03 mM phosphoric acid (pH = 2.5) for citrulline formulations. After being subjected to magnetic stirring (300 rpm) overnight, solution samples were then filtered through hydrophilic PTFE 0.22 µm filters (Millipore Ltd., Dublin, Ireland) and the concentration of drug was determined using high performance liquid chromatography-ultraviolet (HPLC-UV) (Agilent Technologies, Santa Clara, USA). The injection volume was 30 µL.

For isoleucine and valine content determination, the samples were injected using a mobile phase of methanol and phosphate buffer solution (pH = 7.4) (1:99 v/v) through a Novapak 4 µm C18, 300 mm x 3.9 mm column (Waters, Milford, Massachusetts) maintained at 30 °C. The mobile phase was pumped at a flow rate of 1.0 mL/min and the eluent was screened at a wavelength of 225 nm. Measurements were made for 10 printlets prepared at the lowest and highest doses: 200 mg isoleucine (Patient 2), 650 mg isoleucine (Patient 4), 200 mg valine (Patient 2), and 500 mg valine (Patient 5). The retention time was 2.2 min for valine and 3.7 min for isoleucine, and the concentration range was 20 – 240 µg/mL.

For citrulline determination, the mobile phase consisted of 0.03 mM phosphoric acid in water (100 % v/v) and the column used was Spherisorb 3 µm ODS2, 150 mm x 4.6 mm (Waters, Milford, Massachusetts) maintained at 30 °C. The mobile phase was pumped at a flow rate of 1.0 mL/min and the eluent was screened at a wavelength of 225 nm. Measurements were made for 10 printlets prepared for the lowest and highest doses of citrulline: 600 mg (Patient 5) and 950 mg (Patient 6). The retention time was 3.7 min, and the concentration range was 20 – 240 µg/mL.

2.4.2 *In vitro* amino acid release studies

Isoleucine, valine, and citrulline release profiles from the printlets containing the lowest and highest doses were evaluated using an SR8-Plus Dissolution Test Station (Hanson Research, Chatsworth, CA, USA) with USP-II apparatus. The paddle speed was set to 100 rpm with a temperature of 37 ± 0.5 °C. The printlets were placed in a 900 mL vessel of 0.1 M HCl (pH = 1.2) until complete dissolution to simulate gastric conditions. 5 mL of sample was manually withdrawn from each vessel and immediately replaced with fresh

media, filtered through hydrophilic PTFE 0.22 µm filters (Millipore Ltd., Dublin, Ireland), and analysed using HPLC to determine the final amount of amino acid released. Tests were conducted under sink conditions. Data were reported throughout as mean ± standard deviation (n = 3).

2.4.3 Amino acid stability

A stability test was carried out to obtain information on the stability period of isoleucine, valine and citrulline in the chewable printlets stored in Class B X-Large amber PVC blisters. As the chewable formulations were intended for extemporaneous use, a shelf life of 1 month was established. To determine the test conditions, the harmonized documents for stability tests of the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) were consulted [48]. According to the World Health Organization (WHO) document "*Stability testing of active pharmaceutical ingredients and finished pharmaceutical products. Stability conditions for WHO Member States by Region*" which establishes the climatic zones of the different countries and the conditions of temperature and relative humidity (RH) at which the stability tests must be carried out, Spain belongs to climatic zone II [49].

Stability testing was conducted on 10 printlets, containing the lowest and highest dose of amino acids: 200 mg and 650 mg isoleucine, 200 mg and 500 mg valine, 600 mg and 950 mg citrulline. Printlets were stored in Class B X-Large amber PVC blisters and stored in a climatic chamber (CCSR 0150 model, Ineltec, Barcelona, Spain) for 1 month. The temperature and relative humidity conditions were 30°C and 65%, respectively. After 1 month, the individual content of each printlet was determined following the HPLC method previously described.

2.5 Study design and participants

The study design was a prospective, single-centre, crossover, observational study during the administration of two types of formulations in outpatients with MSUD, OTC deficiency and ECHS1 deficiency during 6 months. The study was conducted at the Clinic University Hospital in Santiago de Compostela. The study was approved by the Galician Ethics Committee of Drug Research (Comité de Ética da Investigación con medicamentos de Galicia, CEIm-G) and was carried out in accordance with Declaration of Helsinki. Reference number: 2020/623. Informed consent was obtained from all parents.

The study comprised of six patients (Table 2) diagnosed and treated with MSUD, ECHS1 deficiency, and OTC deficiency in Galicia (Spain) who voluntarily took part in the study after parental signing of the informed consent form. Exclusion criteria included hypersensitivity to any of the components of the new formulation or severe metabolic decompensation. A participant information sheet stating that the data obtained during the study was to be used for research purposes was given to parents and patients aged >12 years. A written consent was also obtained from parents or legal guardians. Additionally, a member of the research team verbally explained the purpose of the study and what it entails. Prior to the study, the recruited patients were prescribed different doses of amino acids with individual prescribing instructions based on their amino acid blood levels (Table 2).

Table 2. Amino acid supplement dose and patient information prior to study. The number of printlets per batch refers to the number of chewable formulations printed for each patient for 15 days. On the 15th day, patients received the new colour and flavour chewable printlet to assess acceptability. *ECHS1: Short-chain Enoyl-CoA Hydratase;*

F: female; h: hour; M: male; MSUD: Maple Syrup Urine Disease; OTC: Ornithine Transcarcamylase.

Patient	Gender	Age	Disease	Dose	Prescribing instructions	No. of printlets per batch
1	M	9	MSUD	200 mg isoleucine and 200 mg valine	Daily	14
2	M	6	MSUD	450 mg isoleucine and 350 mg valine	Daily	14
3	F	8	MSUD	600 mg isoleucine and 500 mg valine	Daily	14 ILE and 14 VAL
4	M	7	ECHS1 deficiency	1300 mg isoleucine	Daily	28
5	F	14	OTC deficiency	1200 mg citrulline	Per 12h, daily	56
6	F	6	OTC deficiency	1900 mg citrulline	Per 8h, daily	84

Firstly, patients received the conventional formulation (powder or capsules) followed by the 3D printed chewable formulation. Each type of medication was administered during a period of 3 months, after which there was a crossover. The study was divided into two stages (Figure 1). During Stage 2, the flavour and colour of the 3D printed chewable formulations was modified every 15 days to assess the acceptability and to evaluate the preferences of each patient.

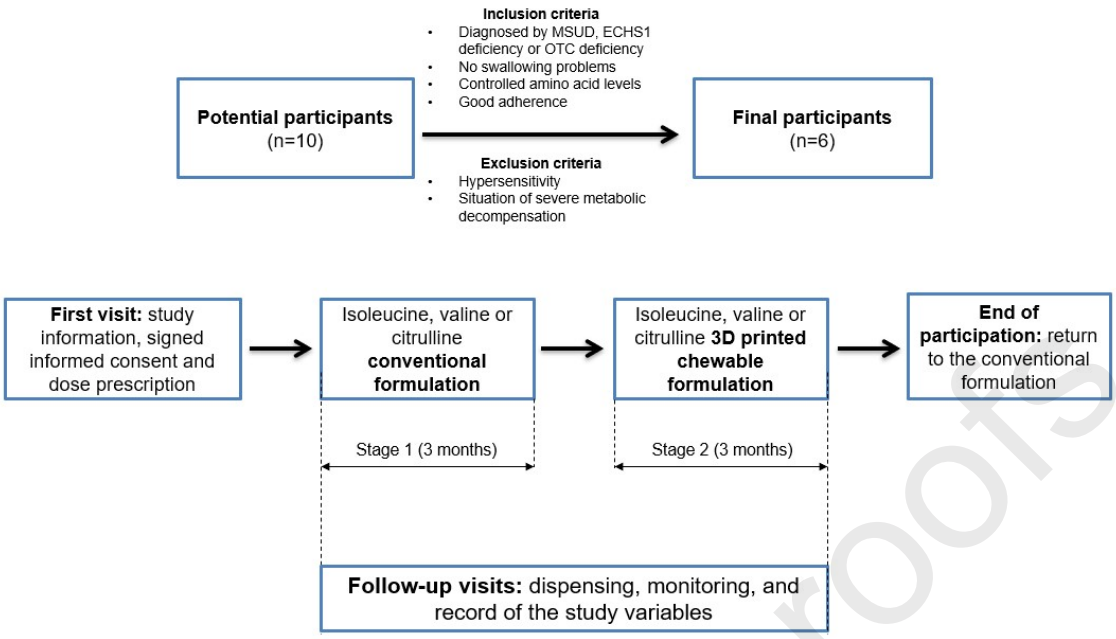

Figure 1. Study design conducted in patients diagnosed with MSUD, ECHS1 deficiency and OTC deficiency during 6 months.

Treatment was performed according to the Spanish and international guidelines for MSUD and OTC Deficiency management [50,51]. For MSUD, the objective was to maintain isoleucine and valine levels above 200 µmol/L. For ECHS1 deficiency, the objective was to maintain isoleucine levels within the normal range according to the patient's age (approximately 15 – 58 µmol/L). For OTC deficiency, the recommended doses of citrulline supplementation were 150 - 200 mg/kg/day to maintain citrulline concentrations between 17 - 50 µmol/L.

2.6 Efficacy evaluation

3D printed chewable medicines were compared to the conventional ones in terms of efficacy of maintaining amino acid blood levels of the patients. Blood samples were collected every 15 days in patients 2 and 3, and every 15 days for the first time in patients 1,4,5, and 6 followed by every 25 days after. Isoleucine and valine levels were determined by ion-exchange chromatography (IEC) in dried-blood samples (DBS) in MSUD and ECHS1 deficiency patients, including a preparative step of elution and deproteinization with 3% trichloroacetic acid [6]. Citrulline levels were measured in liquid blood samples from OTC deficiency patients by ion-exchange chromatography after deproteinization of the plasma samples with 5-sulfosalicylic acid. In both procedures, L-Norleucine was used as an internal standard and spectrophotometric detection with a post-column reaction with ninhydrin [52].

2.7 Acceptability testing

Acceptability data of the 3D printed chewable medicines obtained by the patients were collected via participant and parent reported outcome measures using a mobile phone application (M3DIFEEDBACK, FABRX AI, Currelos, Spain), installed in patient or parent mobile phones (Figure 2). A personal code was given to the users for application access.

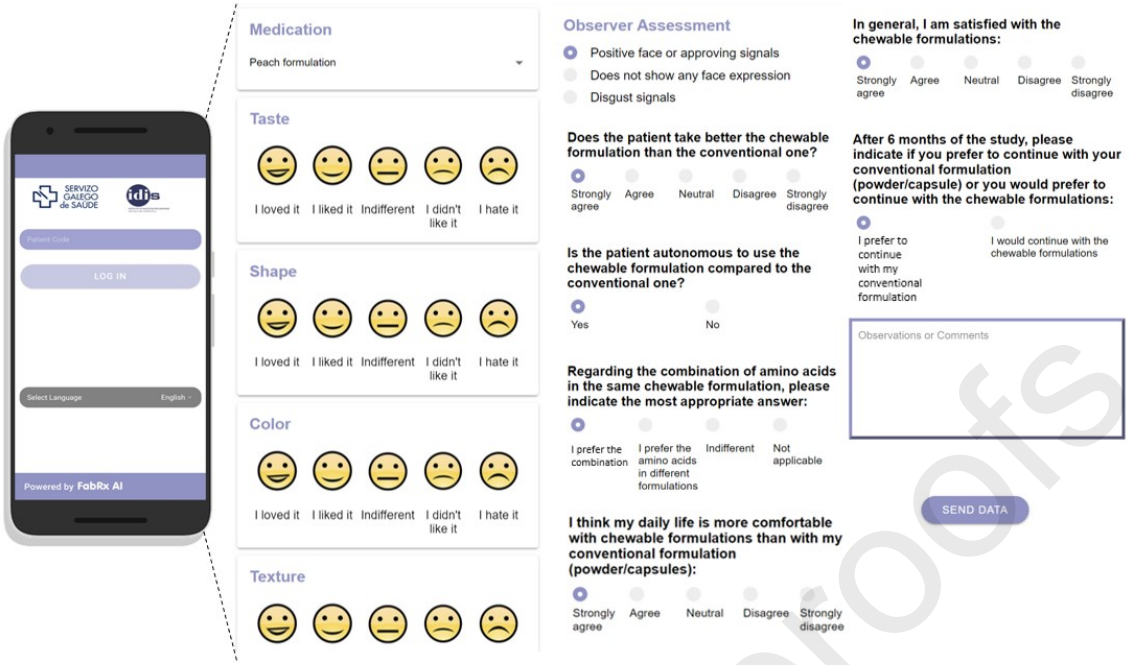

Figure 2. Images of the different screens of the M3DIFEEDBACK app, which was used by the patients/parents to complete the hedonic facial scale and answer the questionnaire.

2.8 Statistical analysis

The number of tests to determine amino acid levels usually differs for each patient and for each formulation. Median blood levels of isoleucine, valine, and citrulline achieved following treatment with conventional and 3D printed chewable medicines were respectively calculated. To compare the outcome of treatment with conventional and 3D printed medicines, the Wilcoxon signed-rank test was used ($P < 0.05$).

Flavours, colours, shape, and texture were evaluated using the five-point facial hedonic scale, characterised with descriptions ranging from 5 = I loved it to 1 = I hated it (Figure 2). A parent or guardian was present to observe the facial expression of the child when taking each chewable or conventional formulation and scored it on a scale ranging from 1 point (signs of disgust) to 3 points (signs of approval). To compare the flavour and colour acceptability of the 3D printed chewable and conventional medicines, the collected scores from the hedonic facial scale were analysed using Kruskal-Wallis ANOVA ($P < 0.05$). Scores were analysed using Wilcoxon signed-rank test ($P < 0.05$) to compare the acceptability of the shape and texture of 3D printed chewable and conventional medicines.

To obtain more information regarding the acceptability of the new formulation and to assess the impact on daily life, answers to the questionnaire evaluating the subjects' or their parents' preference for the chewable or conventional medicine were expressed in percentages (Figure 2). All statistical analyses were performed using GraphPad Prism (v9.0.2, Dotmatics, Boston, USA).

3. Results and discussion

3.1 3D printing process and printlet characterization

This study explored the feasibility of preparing personalized chewable medicines using SSE 3DP in a hospital setting, for children diagnosed with rare metabolic disorders. For the first time, two active ingredients were combined in the same formulation to reduce the number of administrations and ease the therapeutic regimen, which is not possible with the current manufacturing method of pharmaceutical compounding. The 3D chewable medicines were successfully manufactured in the hospital at different temperatures, depending on the amino acid and its loading in the pharma-ink (Figure 3). As shown in Table 1, CIT pharma-ink was printed at 40°C and the amino acid proportion was the lowest (30% w/w). However, when the amino acid loading was increased, the printing temperature also rose (for instance, ILE or ILEVAL1 pharma-inks). Notably, the active ingredient played an important role in the printing temperature since VAL pharma-ink had the same amino acid proportion as ILE, but the printing temperature was slightly lower (55°C). The amino acids were dispersed within the printlets due to the high content of each amino acid in the pharma-ink.

Six different formulations containing personalized doses were prepared in different flavours and colours (Figure 3).

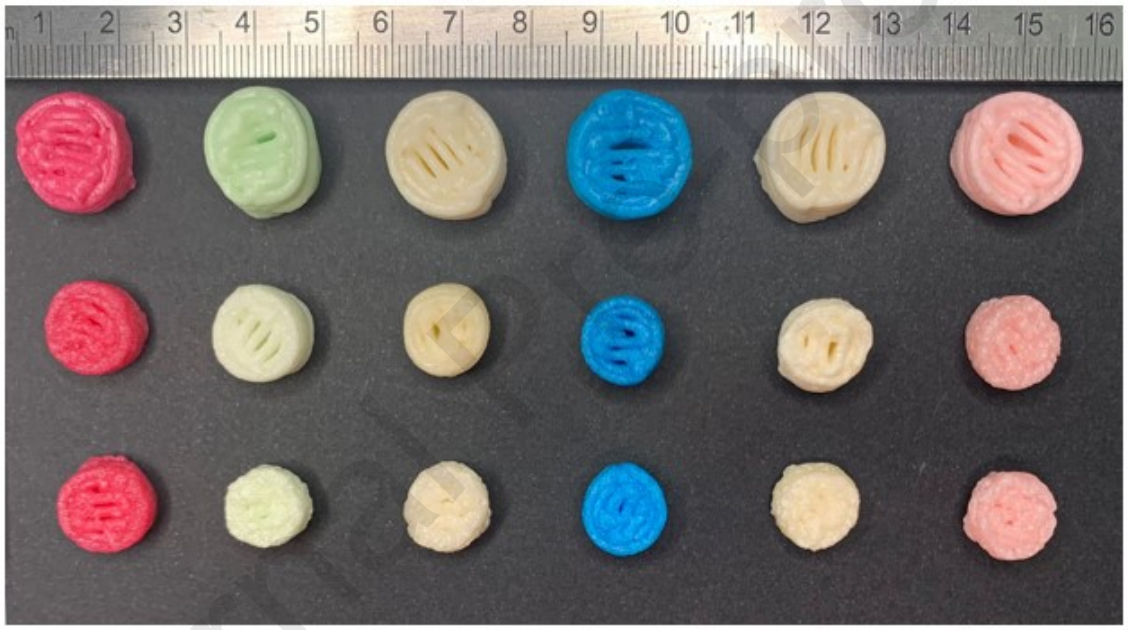

Figure 3. Image of the 3D chewable medicines printed in different colours and flavours during the study. The first row (bottom) corresponds to CIT formulations for patient 6. The second row (middle) corresponds to VAL formulations for patient 3. The third row (top) corresponds to the combined ILEVAL1 formulation for patient 1. Scale is in cm.

Prior to patient enrolment, initial and after 1 month of storage amino acid content was determined for the lowest and highest doses to ensure the correct dose was achieved and to assess stability (Table 3). The Ph. Eur. states that each individual content must be between 85% and 115% of the average content [53,54]. The batch does not comply with the assay if more than one individual content is outside these limits or if one individual content is between 75% – 125% of the mean content. All the individual % contents were within the accepted limits. This ensured that all the 3D printed chewable formulations contained the declared dose of each amino acid and that there was no amino acid degradation during the printing process or after 1 month of storage at 30 °C. As the printlets are formulated for extemporaneous use and packed in PVC blisters, microbiological stability should not be an issue.

Table 3. The lowest and highest amino acid supplement dose in each printlet alongside the initial amino acid content recovery and amino acid content recovery after 1 month. Results are shown as mean ± standard deviation (n = 10).

Theoretical printlet dose	Recovery (%)	Recovery after 1 month (%)
200 mg isoleucine	100.41 ± 1.03	100.26 ± 1.22
650 mg isoleucine	100.11 ± 1.10	100.28 ± 0.88
200 mg valine	100.61 ± 1.09	100.35 ± 1.24
500 mg valine	100.80 ± 1.10	100.40 ± 0.72
600 mg citrulline	100.53 ± 1.47	100.59 ± 1.07
950 mg citrulline	100.04 ± 1.24	100.41 ± 1.03

In vitro release profiles for the lowest and highest dose of each amino acid are shown in Figure 4. The dissolution mechanism was erosion, and all amino acids were progressively released from the dosage form as it eroded. The release from the lowest doses was slightly faster, because of a smaller dosage form size and therefore larger surface area to volume ratio. Each printlet has a different surface area to volume ratio (200 mg ILE and 200 mg VAL – 0.162 cm^{-1}; 650 mg ILE – 0.120 cm^{-1}; 500 mg VAL – 0.127 cm^{-1}; 600 mg CIT – 0.117 cm^{-1}; and 950 mg CIT – 0.111 cm^{-1}), resulting in a different amino acid release rate. Approximately 69% (200 mg ILE), 71% (200 mg VAL), 50% (650 mg ILE), 68% (500 mg VAL), 90% (600 mg CIT) and 51% (950 mg CIT) of the amino acids were released within 15 min. It can be observed in Figure 4 that 100% of the valine and citrulline were released after 30 min (independent of the surface area to volume ratio). In contrast, 97% of isoleucine was released after 45 min. The Ph. Eur. states that conventional immediate release dosage forms must release, at least, 75% of the active substance within a specified time, typically 45 min or less [55]. Approximately 100% of amino acid was released within 45 mins regardless of the dosage form size, therefore, the chewable printlets can be considered as immediate release dosage forms.

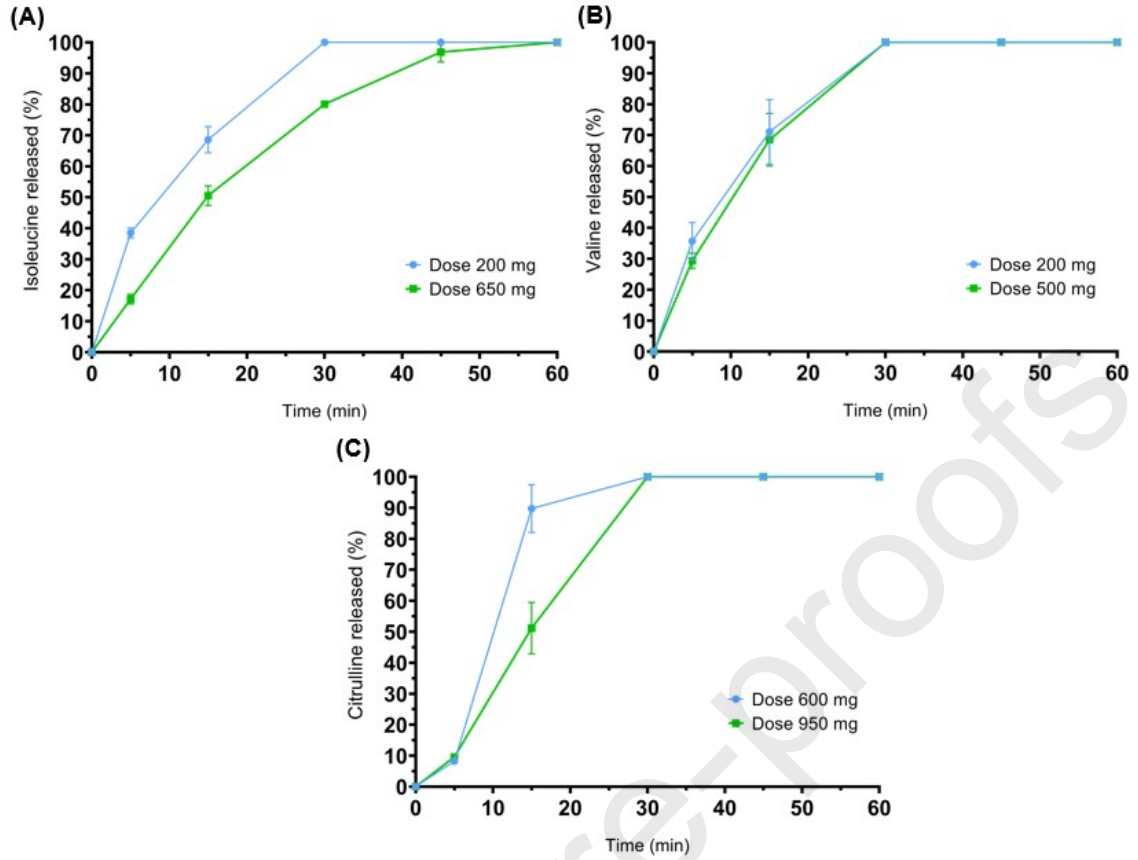

Figure 4. Release profile from: A) ILE; B) VAL; and C) CIT chewable printlets. The blue line represents the lowest dose, and the green line the highest dose for each amino acid (n = 3).

During the clinical study, batches of chewable printlets were successfully printed to comply with the prescribing instructions for each patient for 15 days (Table 2). No clogging of the nozzle occurred during the printing process. The pharma-ink rapidly solidified when deposited during the fabrication without requiring additional cooling, and the resulting printlets exhibited satisfactory handling properties. The time taken to print each printlet was less than 1 minute.

3.3 Clinical study design and mass uniformity testing

Patients 1 and 2 received the combination of isoleucine and valine in the same chewable formulation, to reduce the number of administrations and improve adherence. Patient 3 received isoleucine (600 mg) and valine (500 mg) separately due to the high dose of each amino acid. Patient 4 received his chewable medication as two separate formulations containing 650 mg of isoleucine each, to comply with his prescribing instructions (total isoleucine dose = 1300 mg). Patients 5 and 6 received their chewable medications also divided into two chewable formulations, containing 600 mg of citrulline and 950 mg of citrulline, respectively, to comply with their prescribing instructions (1200 mg total dose for patient 5 and 1900 mg total dose for patient 6). This was because a single formulation with such a high dose (Table 2) was too large to fit inside the blister packing.

After the 3DP process of the batch, each printlet was manually weighed to ensure that there were no deviations according to the accepted limits of Ph. Eur (± 5%). If the printlet passed the mass uniformity test, it was immediately stored in the blister. The number of chewable formulations required for each patient for 15 days differed due to their

prescribing instructions (Table 2). Six batches for each patient were prepared and weighted during Stage 2 of the study (Table 4).

Table 4. Weight results of each printed batch. Results are shown as mean ± standard deviation. B is referred to as batch (for each batch: n = 14 for patient 1, n = 14 for patient 2, n = 14 for patient 3, n = 28 for patient 4, n = 56 for patient 5 and n = 84 for patient 6).

Patient	B1 (mg)	B2 (mg)	B3 (mg)	B4 (mg)	B5 (mg)	B6 (mg)
1	1002.6 ± 14.3	990.2 ± 18.0	1009.1 ± 15.3	1005.2 ± 24.6	1003.9 ± 20.2	1009.2 ± 17.8
2	1992.0 ± 26.7	1999.3 ± 18.8	1990.7 ± 31.3	2012.3 ± 21.9	2000.6 ± 20.7	2023.5 ± 18.5
3	1489.5 ± 24.2 ILE 1271.7 ± 23.3 VAL	1483.4 ± 24.2 ILE 1276.5 ± 34.1 VAL	1496.5 ± 32.7 ILE 1266.9 ± 35.0 VAL	1495.1 ± 32.2 ILE 1257.9 ± 24.8 VAL	1768.1 ± 30.4 ILE 1270.8 ± 26.0 VAL	1773.5 ± 28.6 ILE 1243.3 ± 18.2 VAL
4	1501.9 ± 29.2	1508.7 ± 28.7	1503.7 ± 26.2	1506.0 ± 30.8	1499.9 ± 30.7	1496.4 ± 29.6
5	2045.6 ± 37.6	1994.1 ± 39.4	2014.1 ± 37.6	2015.3 ± 39.4	2025.8 ± 38.2	2032.6 ± 38.6
6	3200.2 ± 53.2	3188.1 ± 42.0	3234.0 ± 46.4	3193.6 ± 42.36	3190.4 ± 61.2	3196.3 ± 42.3

According to the Ph. Eur., it is necessary to individually weigh 20 solid dosage forms. In this study, the flavour and colour were changed every 15 days to evaluate patient acceptability and preferences. Therefore, all the printed chewable formulations were weighed as a requirement for small batches of 3D printed medicines. Table 4 shows that all the batches were within the accepted limits (± 5% declared weight). Batches 5 and 6 for patient 3 were different from batches 1, 2 and 3 due to changed prescribing instructions, accommodating a higher dose. The new dose was prepared using the same pharma-ink in the M3DIMAKER Studio software. Besides printing time, the time required to weigh all the printlets can also be time-consuming. Recently, an in-line analytical balance was implemented inside a pharmaceutical 3D printer using the SSE printhead, with a specialised software-controlled weighing system for the automated mass uniformity testing of the entire printed batch [56]. The integrated balance was compared with an external balance and no significant differences were found. The integration of this system into pharmaceutical 3D printers could potentially save time when more clinical studies are carried out in the future.

3.5 Efficacy and acceptability of the printlets

All six patients maintained controlled amino acid levels regardless of medication type. Patients 1, 4, 5 and 6 required four amino acid assessments, while patients 2 and 3 had six, as per standard practice. Five patients received the same dose throughout the study, with patient 3 requiring an increase in dose from 600 mg to 700 mg of isoleucine due to disease progression. It was simple to prepare the new dose due to the use of M3DIMAKER Studio software after validating printing parameters (Section 2.2), where it's possible to print any dose since the software scaled the initial 3D model and established correlations between the size, weight, dose, and printing parameters using internal algorithms. The pharmacist could add the target dose and number of dosage forms required in the software, which will select the necessary scaled file from the generated library and the medication would be prepared with the new dose. Both conventional and chewable medicines were administered before meals, with the conventional formulation dispersed in water or yogurt. Isoleucine, valine, and citrulline levels for each patient during the study are depicted in Figures 5A, 5B and 5C, respectively.

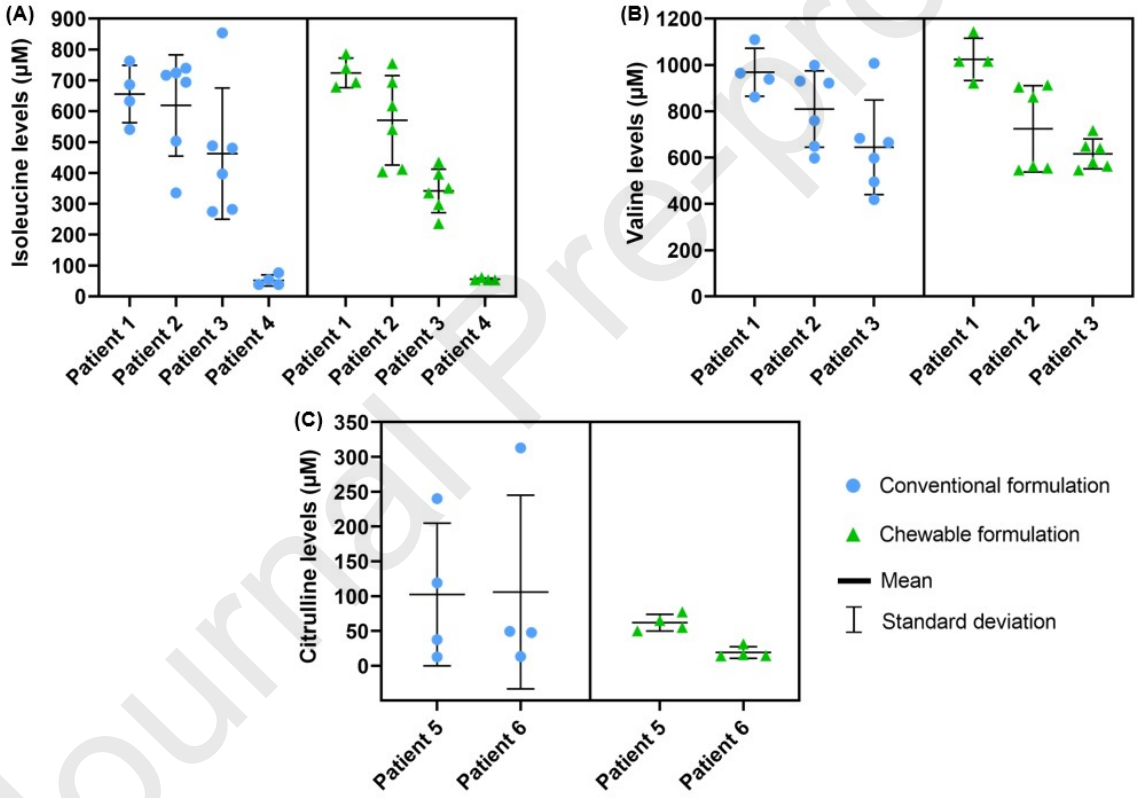

Figure 5. Representation of individual amino acid levels in the blood for the conventional formulation (blue circle) and chewable formulation (green triangle) for each patient: A) Isoleucine levels for patients 1, 2, 3, and 4; B) Valine levels for patients 1, 2, and 3; and C) Citrulline levels for patients 5 and 6. Measurements are expressed as mean ± standard deviation (SD).

The chewable 3D printed medicines effectively maintaine isoleucine, valine, and citrulline blood levels within the targets, similar to conventional medicines, with no significant difference observed ($P < 0.05$) (Figure 5). However, the small sample size precluded any statistically significant differences. Patient 4 showed significantly lower isoleucine levels compared to patients 1, 2, and 3 for both formulations, attributed to differing pathologies (Figure 5A). Patient 4 had ECHS1, while others had MSUD, resulting in isoleucine levels

within recommended ranges for each condition (> 200 µM for MSUD and 15 – 58 µM for ECHS1) for all four patients. A remarkable finding of the present study is that the standard deviation in amino acid levels is lower for the chewable formulation (Figures 5A-C). This may be attributed to the fact that the chewable formulation was taken directly by mouth, whereas the conventional medication was dispersed in water or yogurt. If the child does not finish all of the yogurt or the powder isn't dispersed in the water properly, a loss in amino acid dose may occur.

The blood levels of individual amino acids in all subjects were pooled together to investigate differences in the deviation of amino acid blood levels achieved by the chewable 3D printed medicine and conventional medicine (Figures 6A-C).

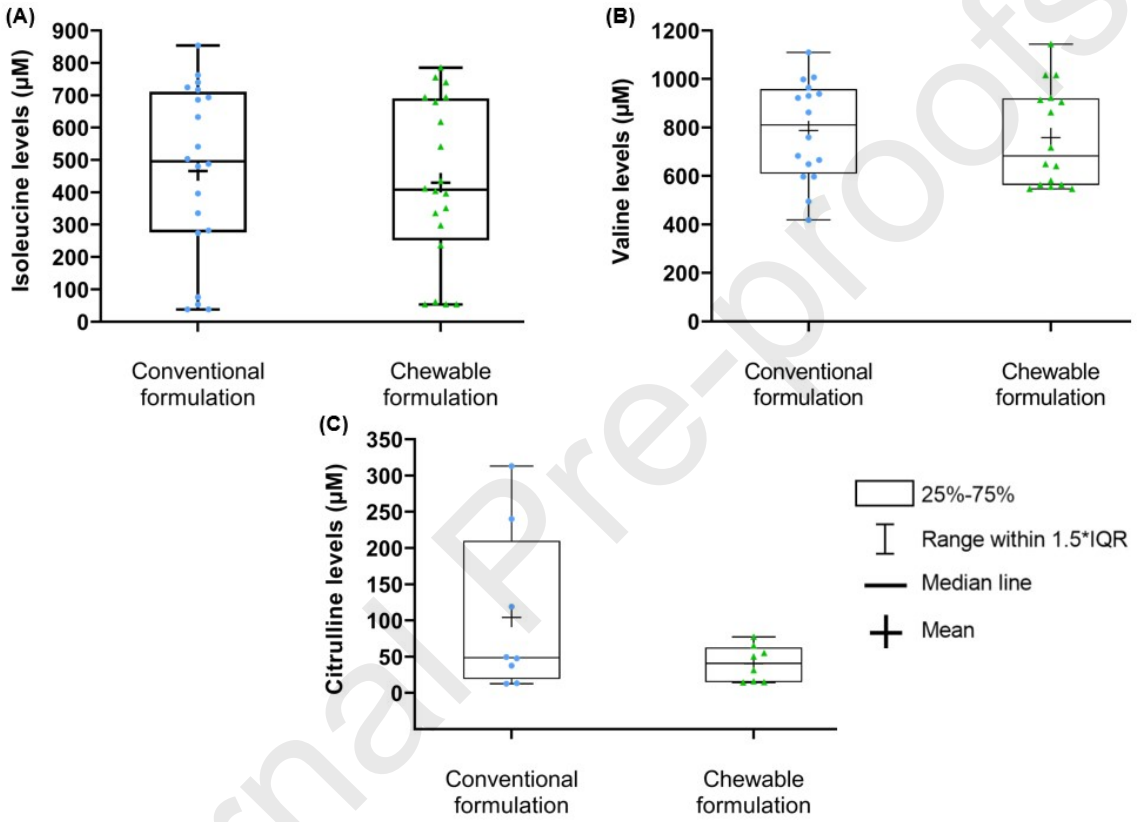

Figure 6. Representation of: A) Isoleucine levels, B) Valine levels, and C) Citrulline levels for both conventional and chewable formulations during the 6-month study.

Mean and median values for all patients with the conventional formulation were: 465.81 µM and 495.54 µM for ILE, 787.45 µM and 810.83 µM for VAL, and 104.05 µM and 48.46 µM for CIT. With the chewable formulation, mean and median values for all patients were: 429.60 µM and 407.87 µM for ILE, 758.64 µM and 682.89 µM for VAL, and 40.40 µM and 40.65 µM for CIT. For isoleucine and valine supplementation, the interquartile range (IQR) of ILE (434.5 µM) and VAL (347.9 µM) levels achieved with conventional medicines was slightly narrower than the chewable 3D printed medicines (ILE: 438.2 µM, VAL: 356.4 µM) (Figures 6A and B, respectively). However, the IQR for citrulline levels achieved using the conventional medicines was wider (190.45 µM) than that attained with the chewable medicines (47.6 µM) (Figure 6C). Lower variability observed with chewable 3D printed medicines suggests better citrulline level control compared to conventional medicines. This is supported by mean citrulline levels with the chewable formulation being closer to the desired range (17 – 50 µM) compared to the conventional

formulation [50,51] (Figures 5C and 6C). The larger deviation observed with conventional formulations is not well understood but may be partly attributed to the influence of food or water in which the citrulline powder was dispersed. Chewable formulations, designed to be taken without food or water, may reduce variability in citrulline absorption.

Results from the hedonic facial scale suggested that the chewable 3D printed medicines were more acceptable than the conventional medicines (Figures 7A-D).

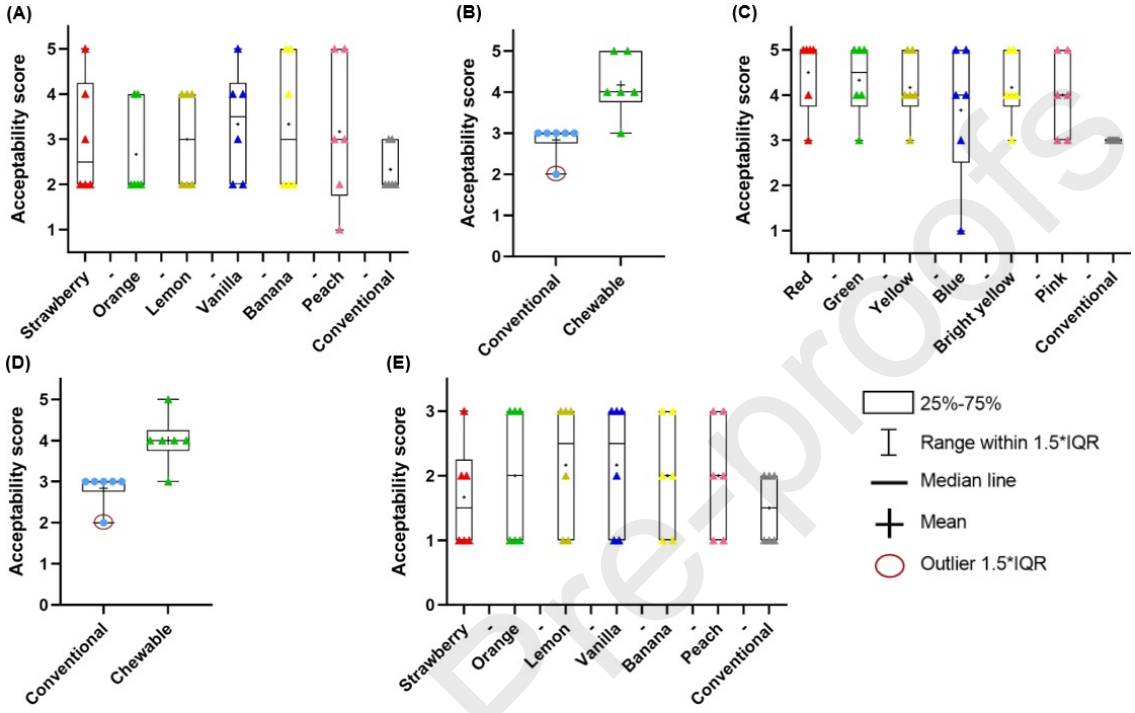

Figure 7. Representation of acceptability score of: A) Flavour, B) Shape, C) Colour, D) Texture and E) Observations of facial expressions.

Scores for different chewable 3D printed medicines indicated higher acceptance for vanilla (median = 3.5), banana (median = 3.0), lemon (median = 3.0), and peach (median = 3.0) flavours. All colours used were highly acceptable (median score 4.0 - 5.0), with red (median = 5.0) and green (median = 4.5) being most preferred. Chewable medicine shape (median = 4.0) was preferred over conventional medicine (median = 3.0) as was texture (median = 4.0 vs. 3.0). Individual preferences resulted in no statistical differences between chewable and conventional medicines in flavour, shape, colour, and texture ($P < 0.05$). The findings highlight that medicine personalisation according to patient preferences is critical to obtain better adherence to treatment and an improved therapeutic outcome.

Scores attained from parent observations of their child's reaction to different flavours of chewable 3D printed medicines also showed a general preference for chewable medicines over conventional medicines (Figure 7E). Vanilla (median = 2.5), lemon (median = 2.5), orange (median = 2.0), banana (median = 2.0) and peach (median = 2.0) were the most accepted flavours. Strawberry flavour scored the least and similarly to conventional medicines (median = 1.5).

Apart from the equivalent therapeutic performance of the chewable 3D printed medicines, this study also demonstrated the design flexibility that the manufacturing technology offers to adapt to dose modifications and improve patient adherence. Using the hedonic facial scale, we identified vanilla, banana, lemon, and peach as the most

preferred flavours (Figure 7A). These results differ with our previous study, where orange was favoured [57]. However, there were noticeable differences in taste preferences among subjects in this study. Patient 1 (9 years old) favoured strawberry, lemon, orange, and vanilla flavours, while patient 2 (6 years old) only responded positively to the orange flavour. Moreover, patient 2 was keen on continuing with the chewable medicine post-study, as the inclusion of both isoleucine and valine in the same medicine made administration more convenient and improved their quality of life. Patient 3 (8 years old) only liked the vanilla flavour and expressed intent to continue with it post-study. Patient 4 (7 years old) favoured banana, peach, vanilla and strawberry flavours, but also liked the lemon and orange flavours. Patient 5 (14 years old) preferred peach and banana flavours, while patient 6 (6 years old) favoured citrus based flavours (orange and lemon). Despite the small sample size, age-related differences in flavour preferences were apparent, with younger patients (< 9 years old) exhibiting more selective preferences. These results underscore the significance of patient-specific flavour preferences in medication adherence. Therefore, to enhance the palatability of medicines to improve patient adherence, a flexible manufacturing technology such as 3D printing is needed to tailor medicines to each patient's unique preference and therapeutic need.

The parent or subject responses to the questionnaire regarding the impact of the chewable medicines on their daily life suggested that the 3D printed medicines were preferred over the conventional medicines, and polypills (combination of isoleucine and valine in the same formulation) were preferred over single amino acid medicines (Figure 8).

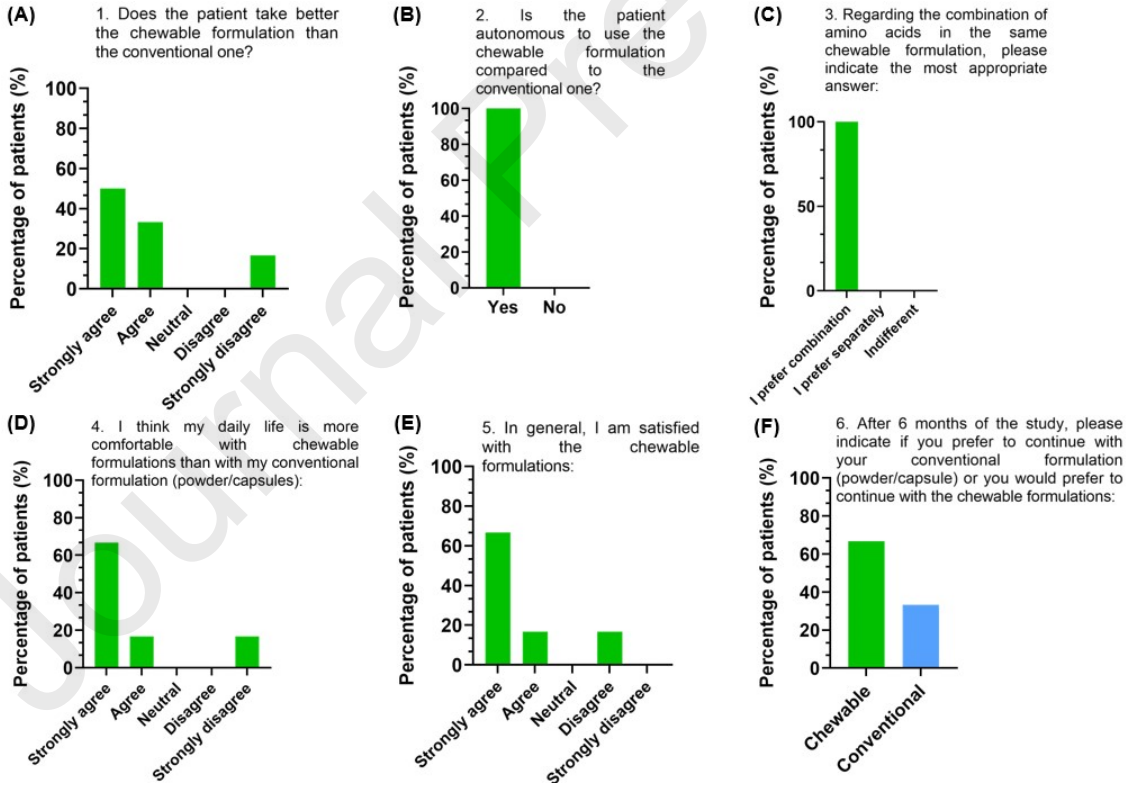

Figure 8. Graphical representation of the answers that were selected by patients in the questionnaire. The bars represent the percentage of patients who selected the option (total number of patients = 6).

Regarding question 1 (Figure 8A), half of the participants (patients 2, 3 and 4) strongly agreed, while patients 1 and 5 agreed, and patient 6 strongly disagreed. Remarkably, parents of patient 6 reported that they do not like her conventional medication either. Figure 8B shows that all patients felt autonomous in self-administering the chewable formulation. Patients 1 and 2, receiving combined isoleucine and valine, preferred this over separate amino acid intake (Figure 8C). For question 4 (Figure 8D), patients 2, 3, 4, and 5 strongly agreed, patient 1 agreed, and patient 6 strongly disagreed regarding comfort with chewable formulations. Regarding satisfaction (Figure 8E), patients 2, 3, 4, and 5 were satisfied, patient 1 strongly agreed, and only patient 6 disagreed. Four participants (patients 1, 2, 4, and 5) expressed a preference for chewable over conventional medication (Figure 8F), though patient 6 (six years old) favoured the conventional form, despite liking certain chewable flavours and showing good compliance. Notably, patient 3 expressed willingness to switch to chewable if the flavour was vanilla, the only one they accepted.

The questionnaire findings (Figure 8) indicate a general preference among children for chewable 3D printed medicine over conventional medication. However, one patient (patient 6) expressed strong disagreement with chewable medicines but also responded negatively toward conventional treatment, suggesting a general aversion towards medicines. As such, these findings suggest that the replacement of conventional amino acid supplementation with chewable 3D printed medicines improved patient experience and quality of life, likely improving adherence and therapeutic outcomes.

In 2019, isoleucine chewable 3D printed formulations manufactured with SSE technology were administered to four MSUD patients at Hospital Santiago de Compostela, with no significant difference between isoleucine blood levels for both conventional and chewable formulations [57]. The present study furthers the previous work through the combination of two amino acids in the same formulation (isoleucine and valine for patients 1 and 2), evaluating three amino acids for three different diseases, increasing patient numbers to six, and working with higher target doses (200 – 1900 mg vs 50 – 200 mg). The inclusion of new flavours (lemon, vanilla, and peach) to obtain more information on patient preferences and the implementation of a mobile app to obtain patient feedback in real-time also advances previous clinical studies.

Another study explored personalised medicine preparation using 3DP in a hospital environment, focusing on low-dose sildenafil citrate for pulmonary arterial hypertension in young children [58]. The 3D printed tablets were also prepared using SSE technology, and bioequivalence with the marketed product was demonstrated in healthy adults, though not yet in patients. Additionally, SSE technology was assessed as an alternative method to avoid the subdivision of levothyroxine sodium tablets in 91 infants with transient hypothyroxinaemia, displaying better disorder control compared to manual subdivision [59]. While this study employed specific healthcare software (M3DIMAKER Studio™) for dose calculations, the main limitation was the small sample size due to the rarity of metabolic diseases, hindering significant differences between formulations.

It is vital to continue gathering evidence and data through such studies to support the findings in this and the previous MSUD study, specifically on the improvements in clinical efficacy and patient acceptability afforded by 3D printed medicines. Despite lacking an established regulatory framework, medicines regulatory agencies like the U.S Food Drug Administration (FDA) and Medicines and Healthcare products Regulatory Agency (MHRA) are adapting regulations to support point-of-care manufacturing [60]. This study supports the benefits of 3D printed medicines in real clinical practice, such as reducing pill burden, improving palatability, and maintaining drug levels closer to target concentrations.

5. Conclusions

This study showed the feasibility of 3DP technology in preparing tailored, safe, and effective treatments for a heterogeneous group of patients who require precise doses of amino acids that need to be altered in a hospital setting. Innovative treatments have been offered to fill the therapeutic gap in the field of rare diseases. Amino acid plasma levels achieved with the new form of administration (chewable formulation) were similar to the levels obtained with the conventional formulation. Notably, the fluctuations in citrulline levels were significantly lower with the chewable formulation although the differences were not significant, most likely due to the small sample size. The levels reached did not show significant differences; therefore, the efficacy of both formulations was comparable.

The shape and texture of the chewable formulations were more accepted than the conventional form, although there were no major statistical differences due to the sample size. The acceptability results in terms of taste varied and were dependant on the age and personal preferences of the patient. According to the responses reported in the questionnaire, the chewable formulations were well accepted since four patients would prefer to continue with the new form of administration (there would be five patients if patient 3 could choose her most accepted flavour). Moreover, there was an improvement in the daily life of children/caregivers due to the ease of administration (self-administration) and the possibility of amino acid combinations in the same formulation, affecting the adherence to the treatment. Therefore, it was possible to improve both acceptability and adherence with the chewable 3D printed medicines. The results obtained in this study are not only applicable to rare diseases, but also to other pathologies in which adherence to treatment is low because the medication is not well adjusted to the needs of the paediatric population. With further development such as in non-destructive analytical technologies for quality control, pharmaceutical 3DP may eventually be deployed in clinics to improve patient experience and clinical outcomes.

List of Abbreviations

3D: Three-dimensional

3DP: Three-dimensional printing

SSE: semi-solid extrusion

ECHS1: Short-chain Enoyl-CoA Hydratase

FDA: Food and Drug Administration

MHRA: Medicines and Healthcare products Regulatory Agency

MSUD: Maple Syrup Urine Disease

OTC: Ornithine Transcarbamylase

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Conflict of interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. Alvaro Goyanes and Abdul Basit are co-founders and directors at FabRx.

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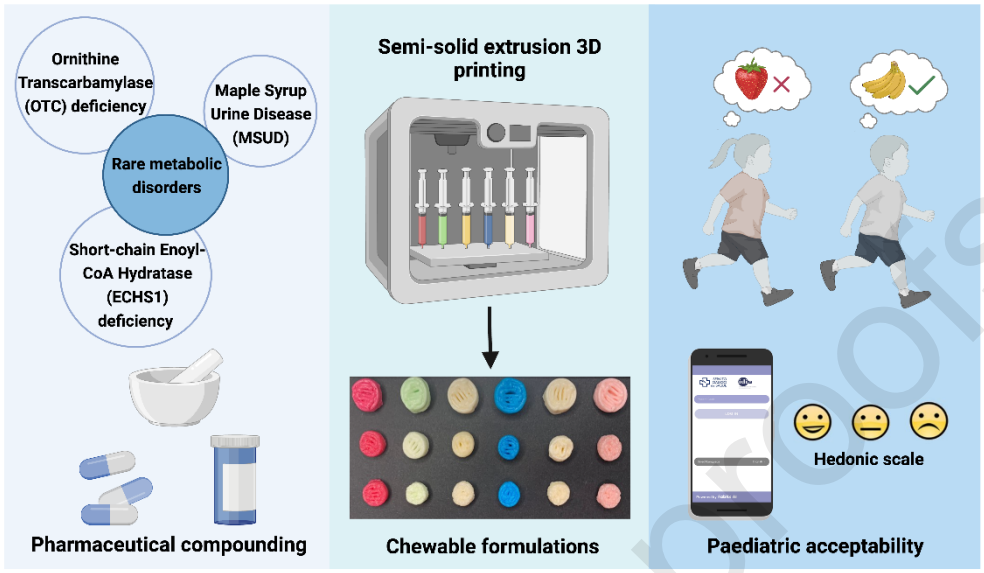

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863 **Declaration of interests**

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865 ☐ The authors declare that they have no known competing financial interests or personal
866 relationships that could have appeared to influence the work reported in this paper.

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868 ☒ The authors declare the following financial interests/personal relationships which may be
869 considered as potential competing interests:

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Abdul W. Basit reports a relationship with FABRX Ltd. that includes: employment and equity or stocks. Alvaro Goyanes reports a relationship with FABRX Ltd. that includes: employment and equity or stocks. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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