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Original Article

Title

An additional bolus of rapid-acting insulin to normalise postprandial cardiovascular risk factors following high-carbohydrate high-fat meal in patients with type 1 diabetes: A randomised controlled trial

Short title

Cardiovascular risk and high fat feeding in type 1 diabetes

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Abstract

Aim: To evaluate an additional rapid-acting insulin bolus on postprandial lipaemia, inflammation, and pro-coagulation following high-carbohydrate high-fat feeding in people with type 1 diabetes.

Methods: Ten males with type 1 diabetes (HbA_{1c} 52.5±5.9 mmol/mol [7.0±0.5%]) underwent three conditions: 1) a low-fat meal with normal bolus insulin (**LF**), 2), a high-fat meal with normal bolus insulin (**HF**), 3) a high-fat meal with normal bolus insulin with an additional 30% insulin bolus administered 3-hrs post-meal (**HFA**). Meals had identical carbohydrate and protein content and bolus insulin dose determined by carbohydrate-counting. Blood was sampled periodically for 6-hr post-meal and analysed for TG, NEFA, APO_{B48}, glucagon, TNF-α, fibrinogen, HTF activity, and PAI-1. Continuous glucose monitoring captured interstitial glucose responses.

Results: TG concentrations following **LF** remained similar to baseline, whereas TG levels following **HF** were significantly greater throughout the 6-hour observation period. The additional insulin bolus (**HFA**) normalised TG similarly to **LF** 3-6-hrs following the meal. **HF** was associated with late postprandial elevations in TNF-α, whereas **LF** and **HFA** was not. Fibrinogen, PAI-1, and TFP levels were similar between conditions.

Conclusions: Additional bolus insulin 3-hrs following a high-carbohydrate high-fat meal prevents late rises in postprandial TGs and TNF-α, thus improving cardiovascular risk profile.

Clinical trial registration: clinicaltrials.gov; Reg. no. NCT02595658

Keywords:

Type 1 diabetes, high-fat feeding, lipaemia, inflammation, cardiovascular risk

Introduction

Structured education provided to patients with type 1 diabetes for managing meal-time insulin dose focuses on the carbohdyrate-counting method [1, 2], whereby people calculate the dose of insulin administered at meal-times based on the total carbohdyrate content of that meal [3]. Whilst this has been demonstrated as an effective strategy for HbA_{1c} reduction [3], typical eating patterns consist of the consumption of mixed-macronutrient meals [4], and in reality many people with type 1 diabetes still struggle to maintain postprandial euglycaemia [5]. This is particularly the case for individuals treated with modern insulin analogue injections, as this method of insulin delivery is associated with less meal-time insulin dose flexibility compared to Continuous Subcutaneous Insulin Infusion therapy (CSII) [6].

The addition of fat to a carbohydrate-based meal has been shown to cause postprandial hyperglycaemia, and increase insulin requirements late into the postprandial period [7, 8]. In clinical practice, people with type 1 diabetes are often reluctant to administer an additional injection of bolus insulin either at mealtime or late into the postprandial period for fear of hypoglycaemia or because increasing injection frequency is considered to be burdensome [6, 9]. Recently, we have showed that when consuming a carbohydrate-based meal with a high-fat content, adopting the carbohydrate-counting method for insulin dose adjustments at meal-time followed by the administration of an additional insulin-bolus late into the postprandial period is important for the normalisation of glycaemia [7]. Specifically, compared to the carbohydrate-counting method alone, administration of additional bolus insulin 3 hours later resulted in a 23% reduction in blood glucose area under the curve (AUC) [7]. Importantly, this method did not cause hypoglycaemia, whereas simply increasing the amount of rapid-acting insulin dose administered at meal-time did [7].

Insulin has an important role not only in the control of postprandial glucose excursions, but also in the regulation of postprandial lipaemia [10]. Excessive increases in both glycaemia and lipaemia can create a pro- inflammatory and -coagulant milieu [11-15], and are collectively and independently associated with cardiovascular disease (CVD) and early mortality [16, 17]. Considering the substantial pre-existing risk of CVD-associated early mortality in this cohort [18, 19], and the potential for this to be heightened by exaggerated post-prandial lipaemia [20-24], optimising meal time insulin dosage is important for cardiovascular risk management, not just normalisation of glycaemia per se. However, the influence of administering additional insulin late into the postprandial period on metabolic or cardiovascular risk factors in patients with type 1 diabetes treated with basal-bolus injection therapy has never been assessed. In this study, we manipulated rapid-acting insulin injection dosage and timing in response to a high-carbohydrate high-fat meal feeding to test the hypothesis that an additional but delayed rapid-acting insulin bolus is required to normalise postprandial lipaemia and the associated metabolic, inflammatory, and pro-coagulant response.

Methods

Patients

The study population consisted of 10 male type 1 diabetes patients (mean ± SD; age 26 ± 4 years, BMI 25.4 ± 1.6 kg.m^2, duration of diabetes 17 ± 5 years, age at diagnosis 9 ± 4 years; HbA_{1c} 52.5 ± 5.9 mmol/mol [7.0 ± 0.5 %]). Patients were eligible for inclusion if they were aged between 18-50 years, with a duration of diabetes greater than 5 years on enrolment, treated on basal-bolus insulin regimen, and were familiar with carbohydrate counting and using a stable insulin-to-carbohydrate ratio. Patients were treated on a stable basal-bolus insulin analogue regimen consisting of either insulin glargine (n = 8) or detemir (n = 2) and fast-acting insulin analogue aspart (n = 10), for a minimum of 12 months. Patients were free of diabetes

related complications, and were receiving no additional medication other than insulin. All patients had received structured education in carbohydrate counting as part of their diabetes care. This study received approval by the local National Health Service Research Ethics Committee (R&D Ref: 7241). All patients who participated provided written informed consent. Eligible patients underwent randomization by computer program to determine the sequence of 3 crossover conditions.

Pre-Laboratory Phase

Patients arrived at the laboratory after an overnight fast (>10 hours) having replicated their diet in the previous 48 hours (assessed using weighed dietary recording sheets). Participants were fitted with a real-time continuous glucose monitor (CGM; Paradigm Veo, Medtronic Diabetes, Northridge, CA) as described previously [7, 11, 25-27] to aid in the maintenance of normal glycaemia during the pre-laboratory period. Additionally, patients were instructed to maintain their normal insulin regimen, with basal insulin dose standardized (dose, injection site, time of injection) across conditions. Patients were also given a pedometer (Omron Healthcare Europe B.V., Netherlands), which they were instructed to wear over the course of 24 hours prior to experimental visits. Patients were required to avoid strenuous activity in the previous 48 hours and maintain similar activity patterns between visits.

Main Experimental Visits

In a randomised and counter-balanced fashion participants attended three separate morning time (~07:00AM) laboratory-based visits, each interspersed by seven days. Upon arrival to the laboratory, patients assumed a seated and rested position whilst a 20-gauge cannula (Vasofix, B. Braun, Melsungen AG, Melsungen, Germany) was inserted into the antecubital vein of their

non-dominant arm; resting, fasted, venous blood samples were then collected prior to experimental testing.

Each experimental visit involved the consumption of meals matched for carbohydrate and protein content, but differing in 1) fat content and 2) rapid-acting insulin bolus dose and timing. The **LF** condition involved administration of rapid-acting insulin according to individual patient carbohydrate counting requirements (dose per 10 g: 1.1 ± 0.8IU) prior to the consumption of a low-fat meal (Table 1). The **HF** condition involved administration of rapid-acting insulin according to the individual patient carbohydrate counting requirements (as administered in **LF)**, however, the meal contained an additional 50 g of fat to constitute a high-fat meal (Table 1). The **HFA** condition involved the administration of rapid-acting insulin according to individual carbohydrate counting requirements (as administered in **LF** and **HF**) prior to the consumption of a high-fat meal, and an additional 30% of rapid-acting insulin administered at 180 minutes post-ingestion (Table 1). The aim of this was such that the additional units of insulin would enter the circulation to coincide with the occurrence of peak postprandial lipaemia [28].

To minimise the influence of injection location on insulin absorption kinetics, the site of bolus injection was standardised across visits using prominent anatomical landmarks (equidistant from the most medial portion of the iliac crest and navel).

Following meal consumption patients remained in a seated and rested position with blood samples drawn every 30 minutes for 6 hours following meal consumption. Following this, patients were discharged and returned home. Plasma Fibrinogen, Human Tissue Factor (HTF),

and Plasminogen Activator Inhibitor-1 (PAI-1) were sampled at baseline, 3 hours post-meal, and at 6 hours post-meal.

Meal Composition

The macronutrient contribution to each meal is presented in Table 1; meal carbohydrate and protein content were matched across conditions. The low-fat and high-fat meals were based upon the composition of a curried dish consisting of basmati rice (Basmati Rice Basics, Tesco, UK), tikka masala sauce (Mild Spice Tikka Masala, Weight Watchers, UK), and chicken (Everyday Sliced Chicken, Tesco, UK). The amount of each food item was identical in each condition and calculated such that carbohydrate content was individualised, equalling 1 g of carbohydrate per Kg of body mass. In addition to the above, the high-fat meals included the addition of an absolute amount of 50 g of clarified butter (Butter Ghee, East End Foods, UK) which constitutes 99.9% fat.

Blood Sampling

At each time point 10 ml of venous whole blood was taken and dispensed into serum separation and lithium-heparin (Vacuette, Greiner Bio-One GmbH, Kremsmünster, Austria) tubes before being centrifuged for 15 minutes at 2,000 g at 4˚C and stored at -80˚C for retrospective analysis of Triglycerides (TG; Serum Triglyceride Determination Kit; Sigma-Aldrich, St. Louis, MO, USA). Apolipoprotein B48 (APO_{B48}; Apolipoprotein B48, Antibodies-online, USA), Non-Esterified-Fatty Acids (NEFA; RANBUT, Randox Laboratories, London, UK), plasma Glucagon (Glucagon EIA, Sigma-Aldrich, St. Louis, MO, USA), Tumour Necrosis Factor alpha (TNF-α; Human TNF-α Quantikine ELISA, R&D Systems, Roche Diagnostics, West Sussex, UK) were measured hourly. Plasma Fibrinogen (ab108842, Fibrinogen Human ELISA Kit, Abcam, Japan), HTF (Human Tissue Factor activity ab108906, abcam, UK), and PAI-1

(Human PAI-1/serpin ELISA Kit DSE100, R7D systems, UK) were measured at Rest, 3 hours and 6 hours post-meal. The intra-assay coefficient of variation was < 10% for all assays. Due to increased assay cross-reactivity with insulin detemir, only participants treated with insulin glargine were included in serum insulin analysis (n = 8).

Data Analysis

Sample size analyses was performed using data from Cohen and Berger [29], whereby increasing insulinaemia via the co-ingestion of glucose lowered postprandial (3 hours) TG concentrations from ~1.36 ± 0.24 mmol.l^{-1} to 0.85 ± 0.24 mmol.l^{-1}. Based on these data, 10 participants should provide >90% chance of statistically detecting a similar effect size with an α-level of 0.05. Statistical analysis was performed using PASW Statistics 18 software (IBM, Armonk, NY) with significance set at $p \leq 0.05$. Data were examined using repeated measures ANOVA (condition*time). Where significant p-values were identified for interaction effects (condition*time), Bonferroni corrected post-hoc analysis was performed. Significant main effects of time were investigated using pairwise comparisons. Where relevant, one-way ANOVA with Bonferroni adjusted pairwise comparisons was used to compare between conditional differences. Data are presented as mean±SD unless stated otherwise.

***** INSERT TABLE 1 *****

Results

Pre-Laboratory Phase

Patients displayed similar glycaemic control during the 24 hours before arriving to the laboratory, with similar mean (**LF** 7.5±1.6, **HF** 7.0±1.1, **HFA** 8.2±1.5 mmol.l^{-1}; p = 0.519) and total AUC (**LF** 11123±2224, **HF** 10,080±1543, **HFA** 11762±2159 mmol.l^{-1}.min^{-1}; p = 0.328)

198 interstitial glucose across visits. Throughout this time, patients demonstrated similar dietary
199 patterns, rapid-acting insulin administrations, and activity levels across conditions ($p > 0.05$;
200 Supplement 1).
201
202 **Laboratory Phase**
203 TG concentrations following **LF** remained similar to baseline (Figure 1A; $p > 0.05$), whereas
204 TGs under **HF** were significantly greater throughout the 360 minute observation period (Figure
205 1A, B, C; $p < 0.05$). **HFA** elicited an increase in TGs similar to **HF** concentrations during the
206 first 180 minutes, but beyond 180 minutes concentrations returned to baseline and were
207 comparable to **LF** (Figure 1A, B, C; $p > 0.05$). **HF** was also associated with elevated TNF-α
208 late into the postprandial period, whereas **LF** and **HFA** was not (Figure 2A, B; $p < 0.05$). The
209 CGM interstitial glucose responses are presented in Figure 2C. There was a significant
210 time*condition interaction ($p = 0.02$, partial-$\eta 2 = 0.199$), and a significant time ($p < 0.01$,
211 partial-$\eta 2 = 0.753$) and condition ($p = 0.29$, partial-$\eta 2 = 0.324$) effect in CGM interstitial glucose
212 responses to the conditions (Figure 2C), whereby **HF** resulted in higher interstitial glucose
213 concentrations in the late postprandial period compared to both **LF** and **HFA** (Figure 2C; $p <$
214 0.05). Interstitial glucose was comparable between conditions during the first 180 minutes with
215 similar total $AUC_{0\text{-}180}$ (**LF** 4104±831, **HF** 5401±545, **HFA** 4959±525 $mmol.l^{-1}.min^{-1}$; $p =$
216 0.418) and absolute interstitial glucose levels at 180 minutes (**LF** 6.0±1.3, **HF** 7.0±0.7, **HFA**
217 6.3±0.7 $mmol.l^{-1}$; $p > 0.05$; Figure 2C). Beyond 180 minutes, interstitial glucose levels were
218 greater under **HFA** ($AUC_{180\text{-}360}$: **LF** 8518±1876, **HF** 14,591±1957 vs. **HFA** 11,011±1509
219 $mmol.l^{-1}.min^{-1}$; $p < 0.05$). The APO_{B48}, NEFA, Glucagon, fibrinogen, HTF activity, and PAI-
220 1 responses are presented in Table 2.
221
222

***** INSER FIGURE 1 *****

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Discussion

This is the first study to show that in adult type 1 diabetes patients treated with modern insulin analogue injections, an additional rapid-acting insulin dose, provided 3 hours after ingestion of a high-carbohydrate high-fat meal, reduces the late rise in lipaemia seen with when the carbohydrate counting method for insulin administration at meal time is used alone. Moreover, such a strategy provides a similar postprandial glycaemic and inflammatory response to a meal with negligible fat content and does not augment the pro-coagulant response of fibrinogen, HFP or PAI-1. In comparison, when following the carbohydrate counting method at meal time alone patients are likely to experience raised lipaemia, hyperglycaemia, and elevated TNF-α concentrations late into the post-prandial period. These findings further highlight the importance of an additional but delayed insulin bolus not just for glucose control per se, but for normalisation of a milieu potentially promoting vascular damage.

Our data show that the addition of dietary fat increases rapid-acting insulin dose requirements, similar to that shown previously in patients using (CSII) [8, 30-33]. For example, Wolpert et al. [8] showed that under closed-loop glucose control, the insulin requirement for a high-fat evening meal was increased by ~42%, in comparison to a carbohydrate-matched, low-fat meal. The present study furthers these previous findings by examining how adjusting the dose and timing of rapid-acting insulin administration influences the metabolic milieu and cardiovascular risk factors associated with consuming mixed macronutrient meals; to date has not been examined within the literature. Our data demonstrate that when administering rapid-acting insulin to

cover only the carbohydrate content of the meal (as in the **HF** condition) patients are exposed to raised triglycerides and TNF-α at 4-6 hours post-meal (Figure 1A-C, Figure 1A). In addition, we observed a trend towards an increase in fibrinogen late into the postprandial period; our sample size was likely too small for yield statistical significance in this individual marker, however our findings indicate an increased inflammatory and thrombotic response following high-carbohydrate, high-fat meal feeding in people with type 1 diabetes that can be prevented with an additional delayed bolus of insulin. These data call for a larger scale observation of the thrombotic responses to high-carbohydrate high-fat meal feeding, and it is recommended that subsequent interventions to reduce post-prandial lipaemia consider this as a potentially important outcome.

Prior research has shown that high-fat meals (> 70 grams of fat) can increase pro-coagulation markers [34, 35], however in the measures we chose, we saw no influence of meal type or dosing strategy. The fat content of the meals within this study was chosen such that they replicated meals that may habitually be consumed by patients (~50 grams of fat), and may simply not have been large enough for subtle changes in insulin dose (+30% equalling ~2.6 IU) and timing to cause a demonstrable effect at the respective sample points. Additionally, the postprandial glucose excursions were only moderately hyperglycaemic under both high-fat conditions.

It is noteworthy that foods with different fatty acid profiles may elicit different postprandial lipaemic [28] and inflammatory [36] responses, potentially mediated via modulation of insulin sensitivity [37], gastric emptying [38], gut hormones responses [38], circulating adhesion molecules [39], and oxidative stress generation [39, 40]. Fats predominantly saturated and of long-chain in composition cause a delayed postprandial lipaemic response [28, 34]. The fatty acid profile of the clarified butter added to the meals in the present study was ~62% saturated and 29%

monounsaturated fat, which, as highlighted in this study is likely to result in a delayed and exaggerated lipaemic response occurring beyond the action time profiles of modern rapid-acting insulin analogues if administered as a single bolus at the time of meal ingestion [41]. As such, the differential responses between **HF** and **HFA** in late lipaemia can be attributed to our insulin administration strategy, considering i) glycaemia was similar between conditions up to 180 minutes post-meal, and ii) the triglyceride response under **HF** beyond 180 minutes is comparable to previous observations profiling time-course lipaemic responses in individuals without type 1 diabetes following high-fat feeding [42].

Prior research examining the interactions of protein in isolation [43, 44] and in combination with carbohydrate and fat [32], shows that protein can raise postprandial glucose late after feeding, with additive effects when combined with fat [32]. Meal protein content was kept under 30 g, such that no bolus insulin dose adjustment for the protein content would be needed [45]; indeed, under the **LF** condition, patients demonstrated no late postprandial hyperglycaemia, with all patients remaining within euglycaemic ranges when the carbohydrate counting method was employed.

Conclusions

In conclusion, these are the first data to demonstrate that when eating a meal with a high-carbohydrate and high-fat content, an additional insulin dose provided 3 hours into the postprandial period reduces plasma triglyceride concentrations and inflammatory markers in type 1 diabetes patients. Thus people with type 1 diabetes treated with basal-bolus insulin injections should be encouraged to carbohydrate count at meal time and administer additional insulin units 3 hours into the postprandial period when consuming a high-carbohydrate, high-fat meal. Not accounting for the fat component of the meal is associated with raised blood

lipids, delayed glucose excursions, and increased inflammation. Based on our findings, patients should be advised of the importance of the late bolus not just for glucose control, but for also normalising other markers that may negatively influence vascular health.

List of abbreviations

APO_{B48} = Apolipoprotein B48; AUC = Area Under the Curve; BMI = Body Mass Index; CGM = Continuous Glucose Monitoring; CSII Continuous Subcutaneous Insulin Infusion; CVD = Cardiovascular Disease; HF = High-Fat; HFP = Human Tissue Factor; HFA = High-Fat Split; LF= Low-Fat; NEFA = Non-Esterified Fatty Acids; TG = Triglycerides; TNF-α = Tumor Necrosis Factor Alpha

Figure legends

Figure 1 A-C. A Time course changes in plasma triglycerides; **B** Total plasma triglyceride $AUC_{0\text{-}180}$; **C** Total plasma triglyceride $AUC_{180\text{-}360}$. Red trace/bar = **HF**; Blue trace / bar = **HFA**; Black trace/bar = **LF**. Data presented as mean±SD. * indicates significantly different to **LF**, ** indicates significantly different to **LF** and **HFA**. Dashed line break on panel B indicates additional insulin bolus administration.

Figure 2 A-C. A Time course changes in TNF-α; **B** Total plasma TNF-α $AUC_{180\text{-}360}$; **C** Time course changes in CGM interstitial glucose. Red bar/trace = **HF**; Blue bar/trace = **HFA**; Black bar/trace = **LF**. Data presented as mean±SD. CGM data presented as mean±SEM for reader clarity. * indicates a significantly different to **LF**, ** indicates significantly different to **LF** and **HFA**. Dashed line break on panel B indicates additional insulin bolus administration.

Tables

Table 1. Experimental meal composition and accompanying insulin administration

		LF	HF	HFA
Energy	**MJ**	4±0	4±0	4±0
Carbohydrate	**%E**	34	34	34
	g	68±3	68±3	68±3
Fat	**%E**	10	55	55
	g	5±0	58±2	58±2
Protein	**%E**	11	11	11
	g	26±1	26±1	26±1
Total Insulin Administration (IU)	**IU**	9±2	9±2	9±2 + 3±1

Note: Data are presented as mean ± SD; n = 10. All meals composed of 1 g carbohydrate Kg body mass. All meals were composed equally of basmati rice (Tesco, UK), chicken breast (Tesco, UK), and a low fat curry sauce (Tikka Masala Sauce, Weight watchers, UK). **HF** and **HFA** contained an additional 50 g of fat in the form of clarified butter (Ghee, East End Foods, UK). **%E** = percentage of energy intake.

Table 2. Responses of metabolic, hormonal, inflammatory, chylomicron, and coagulation markers following high-fat meals / insulin administration

									ANOVA p	
		Rest	**60**	**120**	**180**	**240**	**300**	**360**	**T**	**T*C**
APO_{B48}	**LF**	6.65±5.98	7.72±5.52	8.16±4.98	8.75±5.68	10.90±9.61	10.98±8.43	11.27±13.53	=0.410	=0.267
(mg.ml^{-1})	**HF**	4.93±2.94	7.25±6.83	6.88±7.27	9.56±8.82	14.52±14.92	9.69±13.91	12.28±10.52		
	HFA	6.06±5.96	9.85±7.14	7.50±5.36	9.91±10.34	10.93±10.16	9.59±10.01	11.59±17.21		
NEFA	**LF**	0.39±0.21	0.21±0.06†	0.14±0.10†*	0.17±0.11†*	0.24±0.14†‡*	0.36±0.13†‡*	0.41±0.15	<0.001	<0.001
(mmol.l^{-1})	**HF**	0.47±0.33	0.20±0.10†	0.26±0.07†	0.36±0.10†	0.41±0.18†*	0.41±0.19†*	0.38±0.18†		
	HFA	0.52±0.20	0.22±0.10†	0.30±0.11†	0.40±0.14†	0.43±0.21†	0.28±0.13†‡*	0.36±0.13†		
Glucagon	**LF**	482±128	502±150	493±148	498±102	475±97	432±47	465±64	=0.195	=0.700
(pg.ml^{-1})	**HF**	471±160	500±167	524±164	498±164	458±156	449±156	440±156		
	HFA	467±135	480±150	498±152	483±127	453±121	428±85	438±102		
Fibrinogen	**LF**	2326±1131	---	---	2360±2184	---	---	2300±2268	=0.056	=0.398
(ug.ml^{-1})	**HF**	1988±1385	---	---	3314±3191	---	---	4436±5388		
	HFA	2286±1094	---	---	3660±5750	---	---	3346±3075		
HTF Activity	**LF**	131.74±61.53	---	---	183.71±81.73	---	---	119.02±44.79	=0.087	=0.328
(pmol.ml^{-1})	**HF**	124.18±68.89	---	---	192.69±76.55	---	---	129.42±35.94		
	HFA	134.00±62.65	---	---	191.02±110.96	---	---	218.30±64.84		
PAI-1	**LF**	1.34±0.90	---	---	1.41±0.72	---	---	1.33±0.62	=0.311	=0.100
(ng.ml^{-1})	**HF**	0.92±0.60	---	---	1.01±0.40	---	---	1.88±1.46		
	HFA	1.00±0.62	---	---	1.25±1.15	---	---	2.63±4.67		

Note: Data presented as mean±SD (n = 10). * indicates significantly different from **HF**, ** indicates significantly different from **HF** and **LF**, † indicates significantly different from rest, ‡ indicates significantly different from 180 minutes. T = time effect, T*C = time X condition interaction effect.

Declarations

Ethics approval and consent to participate

This study received approval by the local National Health Service Research Ethics Committee (R&D Ref: 7241). All patients who participated provided written informed consent.

Consent for publication

Not applicable – no presentation of individual data

Availability of data and material

All data generated or analysed during this study are included in the published article [and its supplementary information films]

Competing interests

The authors declare that they have competing interests

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This study was funded by Newcastle University. Only the named research team were involved in the design of the study, collection, analysis, and interpretation of data, and in writing the manuscript

Authors' contributions

MDC designed the study, collected, analysed and interpreted data, and wrote the manuscript. MW assisted in data collection and prepared the manuscript. RAA contributed to the interpretation of data and preparation of the manuscript. KMB contributed to the interpretation of data and preparation of the manuscript. JTG designed the study, collected, and interpreted

data, and wrote the manuscript. DJW designed the study, analysed and interpreted data, and wrote the manuscript.

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