Establishing a Controlled Human Hookworm Infection Model at Malaghan Institute of Medical Research

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Introduction: Background information and scientific rationale

Background:

It is suspected that the disappearance of gastrointestinal parasites from humans may be responsible for the upsurge in inflammatory and atopic autoimmune diseases. This concept is embodied by the hygiene hypothesis, which in simple terms states that people from developed, industrialized countries experience less infectious diseases, especially parasitic diseases, but more autoimmune and allergic diseases.

It is well established that gastrointestinal parasites can modulate the immune system of their hosts to promote their own survival. They accomplish this by producing immune suppressive compounds that blunt the immune system sufficiently to allow the parasites to be tolerated within the body while leaving the immune system sufficiently competent that the host is protected against other infectious diseases. An exemplar of this host-parasite interaction is the human hookworm *Necator americanus (Na).*

Human hookworm biology

Na infection occurs when the infective L3 larvae (iL3) come in contact with human skin. The iL3 penetrate the skin and traverse the lungs where they undergo further maturation before migrating via the respiratory tract to the throat where they are swallowed, completing their journey to the small intestine. It is here where the larvae mature to adulthood, mate, and produce eggs which are then passed out into the faeces[1].

Controlled human hookworm infection

Controlled human hookworm infection has made a major contribution to our understanding of the immunological response that occurs with this parasite. Additionally, it is a central component to assess efficacy of candidate vaccines. Controlled infections tend to be dose-controlled and usually with low larval inoculum which consequently is well tolerated by human volunteers[2-4].

Since the pioneering study in the late 70s involving self -infection with 250iL3 *Na* (Ogilvie et al, 1978) several studies have been published showing a range of immunological responses and the safety of low-dose controlled *Na* infection [5]. More recently, the impact that controlled human hookworm infection has on the gut microbiota has been investigated[6].

Production of Na

There are difficulties in using animal experimental models for *Na* as it is highly adapted to the human body [5], consequently a reservoir of *Na*-infected individuals is necessary. Production of the parasite has been rudimentary: involving mixing eggs from stool samples of infected individuals with charcoal and incubating on culture plates for up to 15 days. Eggs hatch and grow to the infective L3 stage where they are then harvested from the plates and stored for future use [7]. Though a standard method, there are limitations to this method which can result in contamination, inconsistent quality and yield of infective L3 larvae.

Therapeutic potential of human hookworm

Over the last decade, human hookworm has been shown to have therapeutic potential for a number of inflammatory disorders of the intestinal tract of humans, such as coeliac disease [8, 9] and Crohn's disease [10]. *Na* has also shown to have promise in allergic asthma [3, 11], and for some autoimmune diseases such as multiple sclerosis [12].Furthermore, it has been demonstrated in preclinical studies that hookworm secreted proteins can be used to treat animal models of inflammatory bowel disease [13].

Rationale:

Clinical studies for evaluating the potential therapeutic benefit of hookworm infection has been hampered by the lack of a defined source and production of hookworm larvae. We have established well defined methods to isolate mouse hookworm eggs and develop these to infective larvae. We now wish to translate these methods to the human hookworm which will allow the production of a consistent supply of good quality, well-characterised *Na* iL3, for conducting future clinical trials on a range of human autoimmune and allergic diseases.

Further, assessing the immunological phenotype *Na* induces along with the gut microbiome and metabolic change in the human host will provide valuable insights on the cross-talk between the parasite and its host.

Potential Risks and Benefits

Potential Risks

This study will expose healthy volunteers to a low dose human hookworm, *Na* infection. In the acute phase of hookworm infection, a rash and mild abdominal pain are fairly predictable. The following procedures will be in place to manage the small risk to participants.

Dermal Reactions

When using low doses of human hookworm early pruritic skin reactions are common, while severe skin reactions are infrequently reported. Topical steroids and antihistamines may be used to reduce the severity of pruritic dermal lesions, both of which are available over-the-counter and on prescription.

Abdominal Symptoms

Gastrointestinal symptoms of hookworm infection can include pain, diarrhoea, flatulence, nausea and vomiting. While low doses of human hookworm are tolerated, development of severe symptoms is not completely predictable. For symptoms severe enough to warrant treatment, anthelmintic therapy comprising of an over-the-counter (OTC) mebendazole 200 mg orally twice daily for 3 days (Vermox®/Combantrin®) will be offered to terminate the infection.

Although iron deficiency anaemia is well described in naturally acquired infection, anaemia has not been described as a consequence of experimental human infection.

It should be noted that hookworms do not reproduce asexually, therefore the maximum number of adult worms' resident in the gut will be the total number of infective larvae applied on the volunteers' skin. Moreover, patients will not become reinfected accidentally as in order for hookworm to propagate in the environment, infected subjects must defecate in the soil in humid environments and then frequent the soiled area weeks later with exposed skin coming in contact with the soiled ground. Given that the volunteers will exercise standard Western hygienic practises, there is no risk of infection to themselves or to others. As mentioned previously, the infection can be eliminated using over-the-counter anthelmintic treatment.

Potential Benefits

Current methods of hookworm production are still rudimentary, yielding inconsistent and unreliable quantities of *Na,* with contamination a major issue. This study will provide important methodology for producing a reliable and consistent supply of a defined source of *Na.*

Participating in this study will provide crucial information on the immune responses, gut microbiome and metabolic changes that occur upon infection of naïve individuals with low-dose hookworm

infection. Additionally, participants will be able to request information relating to their gut microbiome and metabolic profiles, immune function and dietary intake analysis.

Objectives

Study Objectives

The primary objective for this study is to establish a cohort of 15 healthy individuals infected with a low-dose of the human hookworm, *Necator americanus*, to ensure a reliable source of eggs and larvae for optimisation studies of production, storage and phenotyping for future clinical trials.

Additionally, we propose to use recently employed and published methods for controlled human hookworm infection, that have been approved by the Human Research Ethics Committee of James Cook University, Queensland Institute of Medical Research, Townsville Hospital and Princess Alexandra Hospital, Australia to measure the hookworm larvae functionality by Immuno-phenotyping the *Na* infected volunteers using a systems biology approach. This would be a world first in-depth study of controlled hookworm infection in naïve, healthy human volunteers to investigate immune profile, gut microbiota and the impact of metabolites.

Study Measures

Primary Outcome Measure

The detection of *Na* eggs in the stool of volunteers to confirm active *Na* infection via capsule endoscopy, PCR testing of blood and faecal sample for microscopic analysis. Once active *Na* infection is confirmed stool samples will be collected for the production and method optimisation of *Na* infective L3 larvae.

Secondary Outcome Measure

Antibody, immune cell, microbiome and metabolite profiling from blood and stool samples from volunteers pre *Na* infection and then with active *Na* infection . "Smart-pills" will also be used to measure gut motility.

Study Design

This study will be conducted within the Wellington region. The target sample size is 15 participants. The population studied will be healthy participants with no history of helminth infection (other than *E. vermicularis)*. Participants will be infected with 30 iL3 *Na*. Participants will be monitored for patent *Na* infection via egg release in their stool. Immunological cell and antibody phenotyping, metabolic changes and gut microbiota composition will be monitored pre-*Na* and post-*Na* infection at given time points over 52 weeks. Following confirmation of *Na* infection, participants will be asked to provide stool samples for the production and method optimisation of *Na* infective L3 larvae.

Study schedule

Schedule of assessments are shown in Appendix 1.

Screening/study visits

Study visits will be conducted by experience clinical researchers with training is clinical assessment and data collection. At screening participants will be interviewed regarding demographic data and medical history and a full physical examination will be completed.

Study Assessments and Procedures

Active Na Infection confirmation:

Participants develop a patent infection when adult hookworm establish in the gut and release eggs which can be detected in stool by microscopy and or PCR. This usually occurs between weeks 9 to 12 post percutaneous administration. Participants will be asked to provide a small stool sample pre-inoculation (Week 0) and every 2nd week from week 6 to 12, which will be processed at the Malaghan Institute of Medical Research, Wellington and the presence of eggs will be confirmed using a standard microscopic method. Confirmation of speciation along with infection intensity will be carried out using a PCR-based assay. Following positive PCR and microscopic confirmation, capsule endoscopy will be used at week 20 post inoculation, to visualise live *Na* worms in the gut. This will be performed by a gastroenterologist in Wellington. Capsule endoscopy has been shown to be semi quantitative in both animals and humans [10, 14].

Faecal samples for production of Na infective L3 larvae:

N. americanus is highly adapted to the human body. *Na* infections have been attempted in several species but have proven unsuccessful. Mice have also been attempted and while larvae traverse the lung, infective larvae do not mature to blood-feeding adult hookworms [15]. Hamsters have been used successfully [16] but are not available in New Zealand. Consequently, human hosts are essential for the long-term maintenance and propagation of this parasite in order to maintain a reliable source of *Na*. Several labs around the world, including our collaborators in Australia have successfully maintained *Na* in human volunteers for this purpose, with no adverse events.

Once patent infection has been established in participants, they will be scheduled to donate stool samples on request; for up to 1 year. Eggs will either be isolated from stool samples and developed to iL3 and stored for up to 3 weeks for future use as described above, or developed to juvenile larvae and cryopreserved for future iL3 development. Participants will be given the option of either collecting a stool sample at home or at the Malaghan Institute of Medical Research in Wellington. All participants will be provided with clear instructions on stool collection and necessary collection aides. For participants who prefer home collections a sterile plastic container to store the stool sample for transportation will also be provided along with clear instructions on the storage of the sample. As samples are required to be processed as quickly as possible following collection, an urgent courier service will be employed to deliver samples to the Malaghan Institute of Medical Research, Wellington.

Skin:

Previous studies have shown that application of low dose *Na* iL3 to healthy adults is well tolerated though early skin reactions at the application site are common[4]. Participants will be asked to take photographs (using their mobile-phones) of the inoculation site every week for up to week 8 post infection.

Immune profile:

A small number of controlled hookworm infections in naïve human volunteers have been conducted looking at limited systemic responses[4, 17, 18] .

We will take blood samples as per the schedule and using a flow cytometry-based technique, which utilizes a spectral analyzer, we will look at a panel of immune cell populations and their expression of functional markers, cytokines and transcription factors. We will perform standard complete blood counts as well as antibody responses to hookworm as per schedule.

An in-depth immune profiling of participants pre- and post- *Na* infection will be highly informative and may help elucidate key mechanisms of how the parasite interacts with the immune system.

Blood samples will be taken by a trained individual as per schedule and stored at the Malaghan Institute of Medical Research until analysis for the immune profiling. Complete blood counts will be performed at Malaghan Institute of Medical Research and iron testing will be carried out by a commercial pathology lab.

Microbial profile:

Human intestines harbour a plethora of commensal microbes which include bacteria, viruses, fungi and other unicellular and multicellular microorganisms; collectively termed the microbiota. Previous human studies and a raft of rodent studies have established that helminths can modify the gut microbial profile, such as inducing a more diverse microbiota profile [19, 20]. Further to this, results from a number of trials using controlled helminth infections in a range of inflammatory disorders look promising and it is thought that this is partly due to cross-talk between the parasite and the commensal microbes that reside within the gut [19].

In order to assist in the development of effective therapies, an understanding of the effect that controlled helminth infections have on healthy individuals and their microbes is necessary.
This knowledge may provide insights on how the parasite is interacting with the gut microbiota and provide a better understanding of the therapeutic potential of *Na*; assisting in the design of future clinical trials.

We aim to assess how the commensal make up of healthy volunteers is altered upon infection with *Na*. Shotgun metagenome sequencing on stool samples will enable an in-depth analysis of the gut microbial ecosystem.

Stool samples will be collected at home by participants as per the schedule and according to the instructions provided. Briefly, fresh stool will be added immediately to 4 sterile 25mL containers. An instant ice pack will be used to lower the temperature of the stool and keep it cold during transit to the Malaghan Institute of Medical Research. Samples will then be urgently couriered to the Malaghan Institute of Medical Research where they will be stored at -80°C until analysis.

Metabolic profile:

It has been reported that *Na* infections can modify the metabolic profiles of its host [21]. *Na* has been shown to decrease acetate in the serum of infected hamsters [19]. Further, rodent studies have established that helminths can promote short chain fatty acid production [20], and an increased abundance of selected amino acids in faecal samples [22].

It is possible that any changes in the gut microbiota profiles associated with *Na* colonisation may exert downstream effects on both intestinal and systemic metabolism. Inducing changes in individual metabolites may in turn have potential implications for the overall health of an infected individual. We aim to assess both serum and faecal metabolite profiles of participants pre- and post-*Na* infection. Part of this analysis will include: a lipid profile blood test conducted by a commercial pathology lab alongside the iron testing; and screening for the protein Growth Differentiation Factor 15 (GDF15) in the serum or plasma which is related to tissue tolerance to infections via triglyceride activity.
Additionally, we have access to the only New Zealand-available metabolic flux analyser, which will allow us to gain insights into how circulating metabolites influence immune cell function pre- and post-*Na* infection.

Stool samples will be collected at home by participants as per the schedule and according to the instructions provided as described above. Blood samples will be taken as per schedule and stored at the Malaghan Institute of Medical Research until analysis.

Gut Motility:

Helminths are known to induce changes in the gut physiology such as increasing smooth-muscle contractility and mucus production, and enhancing fluids in the gut lumen[23].

Smart pills are ingestible capsules that pass through the entire gastrointestinal tract. They allow for the continuous measurement of gastrointestinal pH, pressure, temperature and transit time; providing information on gut motility and physiology. These devices will be included in the study for use in capturing characteristic differences in gut motility of participants pre- and post-*Na* infection.

Faecal calprotectin assessment:

Calprotectin is a protein released by inflammatory immune cells, called neutrophils, upon activation. The amount of this protein is commonly measured in conditions such as ulcerative colitis and Crohn's disease as a measure of inflammation. We intend to measure the faecal calprotectin of participants in order to determine changes induced by *Na* infection. Results may help inform directions of future *Na* studies for inflammatory gut conditions. This will be carried out by a commercial pathology laboratory and requires a small sample of stool. Acquiring this sample does not require additional visits by participants.

Tolerance of hookworm infection:

The likelihood of adverse events in people inoculated with low dose *Na* is low. No serious adverse events relating to hookworm infection occurred in our collaborator's studies[4, 24] or other studies [2, 11]. Participants infected with hookworms sometimes developed a mild skin irritation shortly after application of larvae and the inoculation site remained itchy in some subjects until week 4. Some subjects experienced gastrointestinal pain during the initial colonisation of the intestine, but this resolved completely by week 16 (P. Giacomin, personal communication, February, 2018).

Tolerance of the hookworm infection will be monitored by symptom responses using a symptom questionnaire including a modified version of the Talley gut symptom questionnaire[25]. However, participants will be encouraged to report immediately unexpected or adverse events to study staff. The symptom questionnaire will be completed by the patient in electronic form from week 1-12 .

Dietary intake assessment:

Dietary intake will be assessed using a three-day diet record (3DDR) in week 0 (baseline), week 4, week 8, week 12, week 24, week 36 and week 48 to coincide with gut microbiome stool collection. The dietary intake information will be used to determine whether significant changes in dietary intake occur during the study. Dietary analysis will be conducting using FoodWorks version 9 (Xyris software). Dietary intake misreporting will be assessed [26].

Investigational product

Hookworm dose: 30 L3 infective larvae; administered percutaneously.

Low dose *Na* infective larvae (iL3) that will be used in our study has been proven to be safe in reservoir donors and in previous and recent trial experiences when used with coeliac disease patients [9].
The initial source of *Na* iL3 will be obtained from James Cook University, Australia with future iL3 produced at the Malaghan Institute of Medical Research, Wellington, NZ using a standardized protocol developed in-house. Larvae are developed from eggs isolated from stool samples provided by human donors infected for this purpose. The larvae are repeatedly washed in an iodine solution

before testing for morphological integrity and viability/motility by an experienced technician using dissecting microscopy. Aliquots of 15 L3 are stored in deionized water contained in small glass vials. The L3 survive for 6 weeks when kept at approximately 25°C and protected from light. Inocula will comprise 30 L3 to be used within 3 weeks of harvesting.

The L3 will be presented as a 200 µL aqueous solution contained in a small glass vial. Administration will be supervised by a suitable trained researcher. The solution will be extracted using a glass pipette (single use only) and placed on the gauze of a 5-7 cm commercial dressing. The carrier tube will be rinsed with 200 µL of deionized water with the rinse waste to be collected and applied to the gauze. Two dressings, each with 15 L3 larvae, will then be applied. One dressing to the ventral surface of each forearm to be left in place until the end of the day. The Participants will not be required to keep the dressing dry.

As hookworm infection will diminish over time, subjects will be offered a once yearly top-up inoculations with 10-15 L3 only if egg counts are within the "low intensity" range for hookworm infection as (<1,000 eggs per gram of faeces).

Study Enrolment and Withdrawal

Participant Inclusion Criteria

- Participant has provided written informed consent and is willing to comply with all Protocol scheduled visits, laboratory tests, and other trial procedures and in the opinion of the Investigator has a good understanding of the Protocol, the length of the study and the demands of the study.
- Participants will be male and non-pregnant, non-lactating females aged between 18 to 65 years.
- Participants must weigh more than 50kg with a BMI within the 18 – 35kg/m^2 range
- Participants must understand the procedures involved and agree to participate in the study by giving fully informed, written consent prior to any study assessment.
- Participants must be contactable and available for the duration of the clinical trial.
- If female, has met either of criterion "a or "b" below:
  (a) If of non-childbearing potential, has met 1 of the following – Amenorrhoeic for at least 2 years, or has had a hysterectomy and/or bilateral oophorectomy at least 8 weeks prior to screening, or has had a tubal ligation at least 8 weeks prior to screening.
  (b) If of childbearing potential, must be willing to use the acceptable methods of contraception
- In the opinion of the investigator is in good general health

Participant Exclusion Criteria

- Current or history of helminth infection (other than *E. vermicularis*).
- Have any finding at screening that in the opinion of the Investigator or medical monitor would compromise the safety of the Participant or affect their ability to adhere to protocol scheduled visits, treatment plan, laboratory tests, and other trial procedures.
- Have participated in any other clinical trial and/or have received an investigational drug or device within 30 days of screening.
- History or current evidence of any of the following: compromised respiratory function (chronic obstructive pulmonary disease, respiratory depression, signs or symptoms of hypoxia at screening); thyroid pathology (unless stabilized and euthyroid for >3 months at the time of screening); hepatitis B, hepatitis C, or human immunodeficiency virus (HIV) infection; evidence of clinically significant chronic cardiac, hepatic or renal disease; psychiatric illness (poorly controlled); seizure disorder or any other chronic health issues that in the opinion of the Investigator would exclude the Participant from the trial.

- Have one of the following laboratory abnormalities: ferritin <20 µg/L, transferrin <2.04 g/L or Hb <120 g/L for females or 130 g/L for males.
- History of severe asthma or other health conditions that may require future steroid use;
- History of substance abuse or current substance abuse that in the opinion of the Investigator would exclude the Participant from the trial.
- History of intolerance, allergy or hypersensitivity to the proposed anthelmintic – mebendazole.
- History of intolerance, allergy or hypersensitivity to the Betadine (iodine) solution used in preparation of *N. americanus* that in the opinion of the Investigator would exclude the participant from the trial.
- History of malignancy of any organ system (other than localized basal cell carcinoma of the skin), treated or untreated, within the past 5 years;
- For female subjects: positive urine pregnancy test at screening.
- Current or past scars, tattoos, or other disruptions of skin integrity at the intended site of larval application.
- Poor venous access making the Participant unable to comply with the safety laboratory testing requirements.
- Probiotic or prebiotic supplementation within 1 month of screening or commencement during the study
- Laxative or gastric motility medication use within 1 month of screening or commencement during the study
- Significant dietary change or weight loss (>5%) within 6 months of screening or during the study
- Smokers or high alcohol consumers

Recruitment

For this study, healthy male and non-lactating and non-pregnant female adult participants between 18-65 years of age will be enrolled. Participants will be recruited by word of mouth in the Malaghan Institute of Medical Research or an approved advertisement via social media, print, or radio. The expected population may include all New Zealand races and ethnic groups.

Subject Withdrawal

Participants are free to withdraw from the study at any time.
Participants may be withdrawn by the investigators should there be an occurrence of serious adverse effects, any medical condition or situation occurs such that continuation in the study will not be in the best interest of the participant. Investigators may also withdraw participants should the subject meet an exclusion criterion, either newly developed or not previously recognised, that precludes further study participation. Additionally, failure by the participant to comply with the requirements of the protocol will lead to withdrawal from the study. The reasons for the withdrawal will be clearly recorded in the participant's clinic file.

Continuation Phase

At conclusion of study (week 52) participants with be able to either take Mebendazole (Vermox®/Combantrin®) to remove *Na* larvae, or keep their *Na* larvae. Participants who keep their *Na* larvae will be offered 6 monthly egg checks to monitor their infection and can take mebendazole at any time. Participants who take Mebendazole (either at 52 weeks or sometime after) will be asked to provide a stool sample two weeks after completion of treatment to confirm lack of *Na* eggs and end of infection.

Premature Termination or Suspension of Study

We do not anticipate it will be necessary to suspend or terminate the study prematurely.
However, this study may be suspended or prematurely terminated if there is sufficient reasonable cause. Written notification, documenting the reason for suspension or termination of this study, will be provided by the suspending or terminating party.

Statistical Consideration

The main aim of this study is the establishment of the safety and efficacy of controlled *Na* infection in healthy volunteers. The presence and burden of infection will be estimated by simple worm count at capsule endoscopy and by egg count in stool. A convenience sample of 10 participants will be sufficient to achieve that aim.

The secondary outcome measures examine the changes in gut motility, physiology and gut microbiota induced by controlled *Na* infection. Gastrointestinal transit time before and after inoculation using Smartpill transit studies will be compared using paired student T-tests. Stool microbiota and metabolites; and blood immune, metabolite and antibody profiling before inoculation and at intervals following inoculation will be compared using multiple n of 1 trials. Variations within an individual will be compared followed by any common signatures between individuals associated with *Na* infection.

Ethics

Informed Consent

Participants will be fully informed of the nature of the study, the properties and side effects of the investigational products, and all relevant aspects of study procedures in the 'Participant Information Sheet'. Participants will receive a copy of the 'Participant Information Sheet'. The nature of the study, the study agents and their side effects will also be discussed with the participants by the investigator during recruitment. The participants may ask questions of the investigator or the clinic staff at any time.
The 'Informed Consent' form will be signed and dated by the participants in the presence of an investigator. Participants will also be given a copy of their signed 'Informed Consent'.

Anticipated ethical issues

Participants may feel uncomfortable providing a stool sample. Participants will be provided with a 'stool collection kit' with clear instructions on how to collect the stool sample in the comfort of their own home. Stool sample will then be urgently couriered to the Malaghan Institute of Medical Research for processing. Participant reluctance to provide stool samples will jeopardise the completion of this study. Therefore, it is vital for the participants to be comfortable with providing stool samples as required.

Issues affecting Māori population

Cultural issues for Māori are related to whakama and collection of human tissue. In Māori tradition there is a preference for face-to-face communication, and it is important to communicate information about the study in this manner. Support from participant's whanau will be encouraged. Separation of body parts or tissue from the body is at variance with Māori belief in waiora and has potential to cause stress. It is important that Māori participating in this study are aware that any blood or stool samples taken cannot be returned due to laboratory safety regulations.

Participant Confidentiality

All data will be stored using linked anonymised labelling, protecting the identification of individual subjects. Identifiable data will be held on a secure drive and accessible only by the principal investigator, co-investigators and the research assistants, and destroyed after the period of time required for the ethics approval.

Data Management

Data management and source document management will be supervised primarily by study team members. Information will be stored on an ID-protected online accessible database.

Appendix 1 Schedule of Assessments

Assessment	Week																						
	Pre	0	1	2	3	4	5	6	7	8	9	10	11	12	16	20	24	28	32	36	40	44	48	52
Evaluation Visit	X																							
Study Visit	X	X				X		X		X				X		X	X			X			X	
Informed consent	X																							
Review eligibility criteria	X																							
Demographic data	X																							
Medical history	X																							
Full-physical examination	X																							
Clinical safety laboratory tests	X																							
30iL3 Inoculation		X																						
Stool sample (for eggs count)		X						X		X		X		X										
Capsule endoscopy																X								
"Smart pill" – gut motility		X						X						X									X	
Haematology (CBC)		X				X				X				X			X			X			X	
Skin imaging		X	X	X	X	X	X	X	X	X														
Stool sample for microbiome and metabolites		X				X				X				X			X			X			X	
Bloods for immune, metabolite, antibody profiling		X				X				X				X			X			X			X	
Stool collection for culturing iL3														X	X	X	X	X	X	X	X	X	X	X
Symptom questionnaire			X	X	X	X	X	X	X	X	X	X	X	X					X					X
Three-day diet record		X				X				X				X			X			X			X	

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