Epidemiological transition in Indonesia : impact of helminths and urbanization on the development of Type 2 diabetes

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About the cover: Illustration of the two different study areas described in this thesis. The upper part depicts Nangapanda (Flores), a rural area, which is characterized by its beautiful tropical beach which has a stretch of green stones and coconut trees. The lower part depicts Jakarta, the capital city of Indonesia, an urban area, which is characterized by its skyscrapers and the National Monument (Monas). The border between the upper and lower part is represented by the presence of soil-transmitted helminths (the brown-coloured curved lines).

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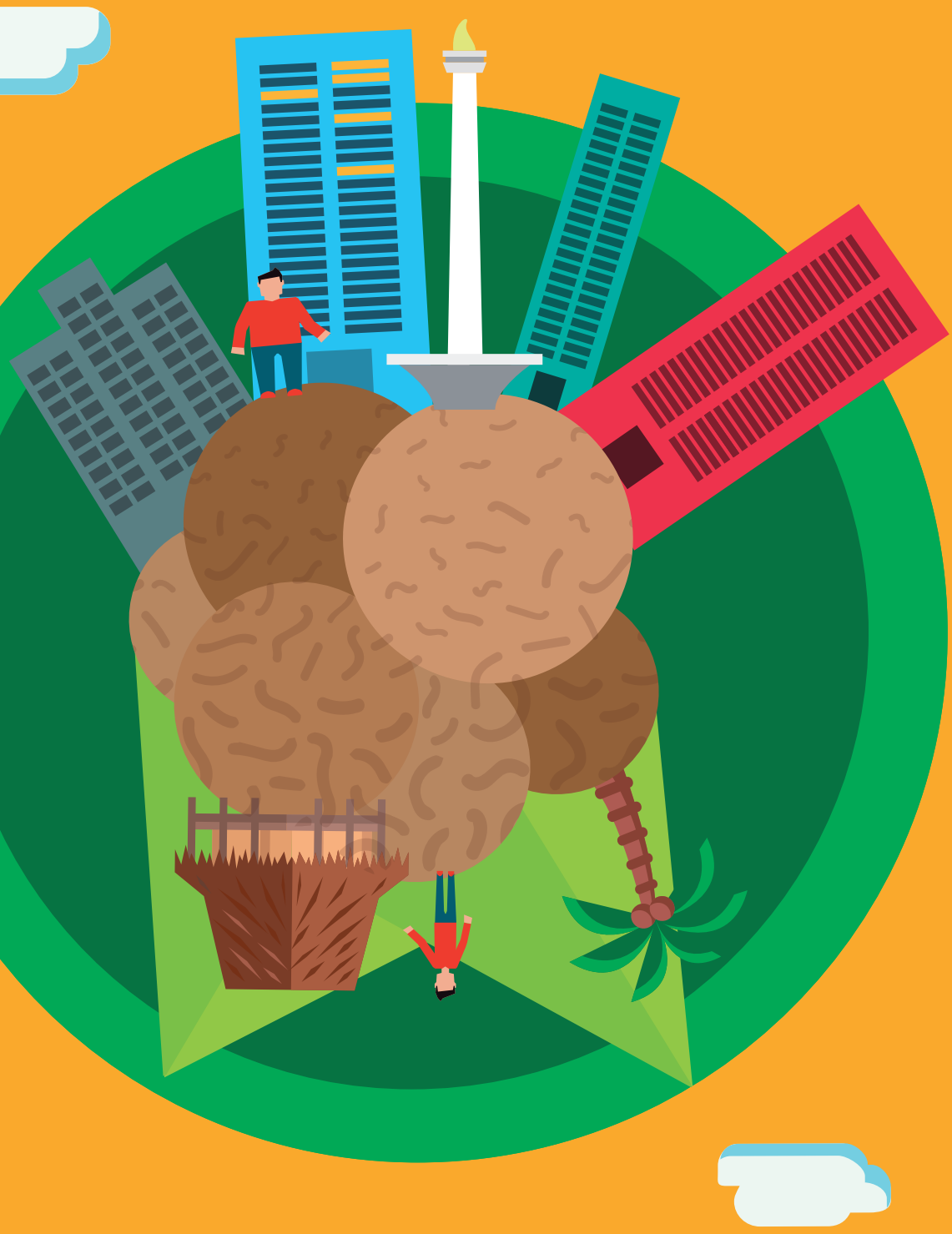
DEDICATION

This thesis is dedicated to the memory of my father, C.H.B. Benny Tahapary,
who inspired me;
to my mother, Endang Soeprapti,
who has provided unconditional love and patience;
to my wife, Maria Larasati Susyono,
who has supported me in all my endeavours;
and to my sons, Nicholas, Sebastian, and Olivier,
who make it all worthwhile.

He has made everything beautiful in its time.
Ecclesiastes 3:11

CONTENTS



Chapter 1

GENERAL INTRODUCTION

Adapted from:

A. Challenges in diabetes management in Indonesia: a literature review
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B. Helminths, hygiene hypothesis and type 2 diabetes
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EPIDEMIOLOGICAL TRANSITION: THE SITUATION IN INDONESIA

There has been an alarming increase in the worldwide burden of type 2 diabetes (T2D) [1], especially in low and middle-income countries (LMIC),[2] including Indonesia. As the fourth world's most populous country, Indonesia is currently undergoing an epidemiological transition. In 2014, 77% of total mortality was estimated to be attributed to non-communicable diseases (NCD), of which diabetes was the third highest after cardiovascular disease (CVD) and cancer.[3] This number considerably increased from the previous report in 2010, of which 63% of all deaths were attributed to NCDs. It is important to note, that whereas the percentage of mortality attributed to CVD and cancer were relatively constant, the diabetes-attributable death has doubled from 3% to 6%[3]. However, a marked geographical variation in disease patterns exists, which is exemplified by the high prevalence of infectious diseases in rural area, but of NCDs, including T2D, in urban areas.

In 2015, the International Diabetes Federation reported that, whereas, 6.5% of Indonesian adults have diabetes, 17.9% of them also have impaired glucose tolerance (IGT), which is associated with a higher risk to develop T2D.[1] These numbers have substantially increased from previous study in 2007 which reported that the prevalence of diabetes and IGT was 5.7% and 10.2%, respectively.[4] Epidemiological studies of diabetes have indicated that the prevalence in Jakarta, the capital city of Indonesia, rose from 1.7% in 1982 to 5.7% in 1993, and then more than doubled to 12.8% in 2001.[5] A study in Ujung Pandang, a metropolitan center on Sulawesi island, also showed similar results.[5] Despite the paucity of data from rural areas, a study in Ende, Flores island, a rural area of Indonesia, found a much lower prevalence of T2D of 1.56%.[6] The results of these epidemiological studies in urban and rural area of Indonesia, which are in line with studies elsewhere on the association between rural-to-urban migration and increased risk of T2D and other CVD risk factors,[7-11] indicates the importance of expanding studies to outside of urban centers. Collecting information from rural areas, not only would help prepare the health system for emerging diseases but it will also enable the identification of important risk factors for CVD and therefore guiding interventions. One potentially important aspect of rural areas, where 40% of the Indonesian population lives,[3] is the possible effect helminth infections might have on glucose metabolism.[12]

Helminth infections affect approximately 1.4 billion people worldwide, of which a significant proportion are in Southeast Asia,[13] including Indonesia [14] For

example soil-transmitted helminths (STH), which despite a decrease in overall national prevalence from 47.2% to 24.6%,[13] are still very prevalent in most rural area of Indonesia,[15-17] and in some villages more than 80% of the population are infected with these parasites[15, 17], which in part is due to the tropical and moist climate.[18] Poor sanitation infrastructure and hygiene practises, as well as lack of adequate clean water resources, might also contribute.[19]

INVERSE ASSOCIATION BETWEEN HELMINTH INFECTIONS AND T2D

Worldwide, there is little overlap between the prevalence of soil-transmitted helminths and T2D (**Figure 1**), which is supported by a number of epidemiological studies in different populations reporting an inverse association between helminths and metabolic diseases.[20-24]. Interestingly, these studies consistently reported an inverse association between previous [22, 23] or current [20, 21] helminth infections and metabolic diseases, namely metabolic syndrome prevalence [22, 23], T2D prevalence [21, 23] and Insulin Resistance (IR), as assessed by homeostatic model assessment (HOMA)-IR[20].

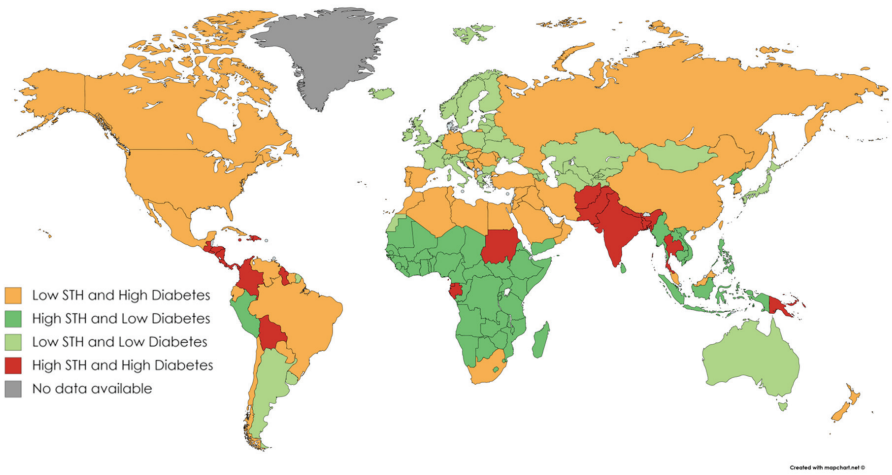

Figure 1. Worldwide prevalence of soil-transmitted helminths (STH) and diabetes. STH prevalence is defined as the estimated proportion of children (1-14 years of age) requiring preventive chemotherapy for STH per country,[14] of which in this figure, proportion of lower than 1/3 is considered low. The prevalence of diabetes in adults (20-79 years of age) per country,[1] of which in this figure prevalence of lower than 7% is considered as low).

The association between previous helminth infections and metabolic diseases has been reported in China. Chen *et al*. used self-reported disease and medication history, cross-referenced with local government registry data, as a method to diagnose previous schistosome infections (PSI)[23]. The prevalence of both T2D and metabolic syndrome was significantly lower in the group with PSI compared to the non-PSI group (14.9% vs 25.4%, P<0.0001; 14.0% vs 35.0%, P<0.0001, respectively). In addition, PSI was associated with lower levels of body mass index (BMI), HOMA-IR, plasma fasting blood glucose, postprandial blood glucose, and glycated haemoglobin A1c (HbA1c)[23]. A different method of PSI diagnosis was used by Shen *et al*., because schistosome-associated liver pathology can be present for years, therefore ultrasonography was performed to detect chronic schistosomal liver disease [22]. PSI was significantly associated with a lower prevalence of metabolic syndrome (18.28% in PSI group vs 34.01% in control group) and its components, including central obesity, hypertension, low high-density lipoprotein cholesterol (HDL-C), hypertriglyceridemia, and hyperglycaemia. [22]

The inverse association between current helminth infections and metabolic diseases was first reported in Chennai, India. Aravindhan *et al*. reported a significantly lower prevalence of lymphatic filariasis among diabetic subjects compared to pre-diabetic and non-diabetic subjects [24]. Furthermore, Hays *et al*. found that among 259 Australian Aboriginal adults, participants with a chronic *S. stercoralis* infection, as defined by serological testing, were 61% less likely to be diagnosed with T2D compared to those who were uninfected, after adjustment for age, blood pressure, triglycerides and BMI [21]. Furthermore, it was shown by a study in rural area of Indonesia that subjects with a current STH infection had a lower BMI and lower levels of HOMA-IR, indicating that infected subjects were more insulin sensitive compared to uninfected subjects [20]. A significant negative association was found between the number of helminth species a subject was infected with and HOMA-IR, even after adjustment for age, sex and BMI [20].

A recent meta-analysis showed that individuals with a previous or current helminth infection were 50% less likely to have an outcome of metabolic dysfunction (hyperglycaemia, T2D, metabolic syndrome or insulin resistance) compared to those uninfected (OR 0.50; 95% CI 0.38-0.66) [12]. However, cross-sectional studies provide no information on the causal relationship between helminths and metabolic diseases and therefore longitudinal studies are required.

MECHANISM BEHIND THE INVERSE ASSOCIATION BETWEEN HELMINTHS AND T2D

Adiposity and Adipose Tissue Inflammation

Insulin resistance, a decrease in insulin-stimulated glucose uptake, is a hallmark of T2D leading to hyperglycaemia. Whereas increased adiposity has been associated with an increased risk of developing IR and T2D[25], helminth infection has been commonly associated with a poor nutritional status and reduced adiposity,[20, 22, 23] suggesting that helminth-associated lower adiposity may contribute to the observed lower IR.[20] However, despite the abundance of evidence on the role of human adipose tissue on IR, which will be summarized below, no data are available on the effect of helminth infections on human adipose tissue.

Obesity-induced chronic low-grade inflammation seems to be a key feature in the development of IR, hence T2D [26-29]. Initiation of inflammation in obesity involves inflammation of visceral adipose tissue (VAT), and the release of free fatty acids (FFA) as well as liver inflammation (called non-alcoholic steatohepatitis (NASH)), which then promote systemic inflammation, reflected in increased levels of pro-inflammatory cytokines,[26] such as TNF-α,[30, 31] that lead to an impairment in insulin signalling.[26] This will thereby affect glucose levels by leading to a reduced glucose uptake in skeletal muscles, increased hepatic gluconeogenesis, and an increase in circulating free fatty acids.[26]

Adipose tissue inflammation plays a prominent role in the development of obesity-induced inflammation [27], which is characterized by the accumulation of inflammatory cells in obese adipose tissue creating a pro-inflammatory milieu [32]. A number of studies have shown that there is a phenotypic switch [33-35] from anti-inflammatory alternatively activated macrophages (AAM or M2) in adipose tissue, which are activated by IL-4 and IL3 and express anti-inflammatory IL-10 [32, 36], towards pro-inflammatory classically activated macrophages (CAM or M1), which secrete various pro-inflammatory mediators (TNF-α, IL-6, IL-1β, IL-12, IL-23) [32, 36]. This phenotypic switch is positively correlated with IR.[33-35]

In addition, an imbalance between pro- and anti-inflammatory adipokines may also contribute to the development of IR [37]. Leptin, a pro-inflammatory adipokine [37], can increase and suppress the production of circulating Th1 and Th2 type cytokines, respectively [38]. Other pro-inflammatory adipokines, such as resistin, TNF-α, IL-6, IL-18, retinol-binding protein (RBP) 4, lipocalin 2, angiopoietin-like

protein (ANGPTL) 2, CC-chemokine ligand (CCL) 2, and CXC-chemokine ligand (CXCL) 5 have been reported to be upregulated in an obese state [37]. Adiponectin, an anti-inflammatory adipokine [39, 40], can stimulate the production of IL-10 by macrophages [41]. Recently, another anti-inflammatory adipokine, Sfrp5, was reported to have beneficial metabolic effects [39].

Aside from the adipose tissue, inflammation in other tissues, such as liver, skeletal muscle and the pancreas, might also contribute to the development of obesity-induced inflammation and therefore the development of IR. In obesity, the activation state, but not the number of Kupffer cells (KC), in the liver changes[42, 43] promoting the expression of inflammatory genes,[42] as well as the production of inflammatory mediators which leads to an increased IR in the liver.[43] Liver inflammation might also be initiated by the abdominal adipose tissue-associated increased secretion of pro-inflammatory cytokines into the portal circulation. [27] In contrast, skeletal muscles may not be the site where inflammation is initiated, but the target of inflammation-induced IR.[27] An increased expression of inflammatory markers within skeletal muscle was only reported among obese people with T2D, but not among obese people without T2D.[44] In the pancreatic islets of diabetics, increased levels of the pro-inflammatory cytokine IL-1β have been found,[45] which is a master regulator of islet cell inflammation in T2D. [46] This pancreatic islet inflammation is a key step in the development of T2D, as the failure of beta cells to compensate IR will lead to the development of hyperglycaemia and T2D.[47]

Immunomodulatory Effects of helminths

Helminth has been shown to be a potent natural inducer of type 2 and regulatory responses.[48-51] As chronic low grade inflammation plays a key role in the development of IR and T2D,[29] helminth-associated immune responses may therefore dampen systemic inflammation, hence increasing insulin sensitivity. [52-54] Despite the possible main role of adiposity in mediating the helminths-associated protective effects in human, it has been reported that even after adjustment for adiposity, the association between helminth infections and lower IR is not completely attenuated [20], suggesting that other pathways, such as helminth-associated immunomodulatory effects, may be involved.

A number of experimental studies in mouse models have provided evidence for the beneficial effects of helminths and helminth-derived molecules on metabolic homeostasis,[55-60] and shed light on the immunomodulatory mechanisms that

could explain the link between helminths and T2D. Helminths influence metabolic homeostasis,[61-63] at least partly, by changing the immune cell composition in the adipose tissue. Whereas obesity-induced low-grade chronic inflammation is characterized by the accumulation of $CD8^+$ T cells, $CD4^+$ Th1 cells, CAMs, B cells and mast cells in the adipose tissue (AT), chronic helminth infections or helminth-derived molecules induce increased numbers of $CD4^+$ Th2 cells, eosinophils, AAMs, Tregs and ILC2s, dampening the inflammation and improving glucose tolerance.

Although there is very little human data available that could explain the mechanism by which helminths may protect against T2D, it can be speculated that helminths suppress chronic inflammation associated with T2D by modulating the immune response. Recent population-based study has shown that community deworming program alleviates helminth-associated immune hyporesponsiveness,[64] however, the question whether this would lead to the development of inflammatory disorders, including T2D, in the future, remains unanswered.

Taken together, there is evidence that suggests living a more traditional lifestyle in a rural environment might confer a protective effect against the development of NCDs, including T2D. With the rapid pace of socioeconomic development and increased rate of urbanization, these relatively protective lifestyle and environment will progressively subside. In urban areas or urbanized communities, changes toward a more sedentary lifestyle and increased consumption of energy-dense food, will lead to a positive energy balance and increased adiposity, contributing to the increased burden of T2D.[65] It is also hypothesized that improved hygiene and sanitation in parallel with the current deworming programs, leading to a decreased exposure to helminth infections, which have been shown to have a protective metabolic effect,[12, 20-23] might also contribute to the increasing prevalence of T2D.[52-54] The proposed association between epidemiological transition, urbanization, insulin resistance, and helminth infections are schematically summarized in **Figure 2**.

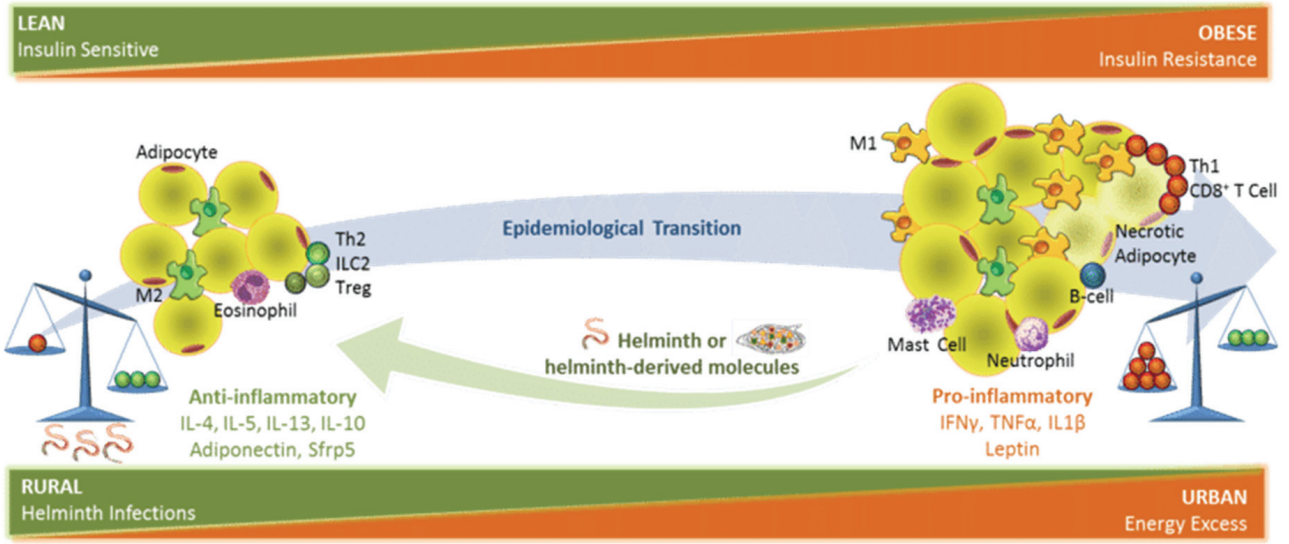

Figure 2. Epidemiological Transition, Insulin Resistance, and Helminth Infections. Along with epidemiological transition, the prevalence of obesity is higher and exposure to helminth infections is lower in urban areas compared to rural areas. With obesity, the immune cell composition in the adipose tissue shifts towards a pro-inflammatory profile associated with insulin resistance. Helminths or helminth-derived molecules are thought to prevent and/or reverse this shift by inducing an anti-inflammatory immune cell environment, which is associated with insulin sensitivity.

SCOPE AND AIMS OF THE THESIS

The main objective of this thesis is to improve understanding of the role of helminth infections in the development of IR, hence T2D, in the light of increasing urbanization in Indonesia. We aimed to unravel the causal effect of helminth infections on human metabolic homeostasis by assessing how anthelmintic treatment (deworming) could affect IR and other metabolic parameters. Our large scale cluster randomized controlled trial (RCT) was performed in a rural area of Indonesia, which is an area endemic for STH, and has been previously reported to have a low prevalence of IR and T2D. We also assessed the role of adiposity and adipokines in mediating the effect of helminths on IR. Next, we also aimed to assess the different metabolic profile between populations living in rural and urban area, and to study the relative protective effect of rural environment on the development of IR by performing a short-term high-fat diet intervention study.

The first part of this thesis addresses the following question:
What is the effect of anthelmintic treatment (deworming) on host metabolic homeostasis?
Chapter 2 describes the design of an RCT to answer the question whether anthelmintic treatment will affect host metabolic homeostasis by performing a household-clustered randomized double-blinded placebo-controlled anthelmintic trial in an area endemic for STH in Indonesia.
Chapter 3 describes the outcomes of this RCT on STH prevalence, Th2 responses, adiposity, and IR
Chapter 4 describes the outcome of this RCT on adipokines and how this mediates the changes in IR

The second part of this thesis we focus on the following question:
What are the differences in metabolic profiles between populations in rural and urban areas?
Chapter 5 describes the different metabolic profile of subjects with the same genetic background living in rural and urban areas. It also describes their responses to a short-term high fat diet intervention.

Chapter 6 summarizes our findings and provide directions for future research to understand the link between helminth infections, urbanization, adiposity, and insulin resistance.

Author Details

A. Challenges in diabetes management in Indonesia: a literature review

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B. Helminths, hygiene hypothesis and type 2 diabetes

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PART I

What is the effect of anthelmintic treatment (deworming) on host metabolic homeostasis?

Chapter 2

Study Protocol

HELMINTH INFECTIONS AND TYPE 2 DIABETES: A Cluster-randomized Placebo Controlled Sugarspin Trial in Nangapanda, Flores, Indonesia

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ABSTRACT

Background: Insulin resistance is a strong predictor of the development of type 2 diabetes mellitus. Chronic helminth infections might protect against insulin resistance via a caloric restriction state and indirectly via T-helper-2 polarization of the immune system. Therefore, the elimination of helminths might remove this beneficial effect on insulin resistance.

Methods/design: To determine whether soil-transmitted helminth infections are associated with a better whole-body insulin sensitivity and whether this protection is reversible by anthelmintic treatment, a household-based cluster-randomized, double blind, placebo-controlled trial was conducted in the area of Nangapanda on Flores Island, Indonesia, an area endemic for soil-transmitted helminth infections. The trial incorporates three monthly treatment with albendazole or matching placebo for one year, whereby each treatment round consists of three consecutive days of supervised drug intake. The presence of soil-transmitted helminths will be evaluated in faeces using microscopy and/or PCR. The primary outcome of the study will be changes in insulin resistance as assessed by HOMA-IR, while the secondary outcomes will be changes in body mass index, waist circumference, fasting plasma glucose, 2h-glucose levels after oral glucose tolerance test, HbA1c, serum lipid levels, immune system, and efficacy of anthelmintic treatment.

Discussion: The study will provide data on the effect of helminth infections on insulin resistance. It will assess the relationship between helminth infection status and immune responses as well as metabolic parameters, allowing the establishment of a link between inflammation and whole-body metabolic homeostasis. In addition, it will give information on anthelmintic treatment efficacy and effectiveness.

Trial registration: This study has been approved by the ethical committee of Faculty of Medicine Universitas Indonesia (ref: 549/H2.F1/ETIK/2013), and has been filed by the ethics committee of Leiden University Medical Center, clinical trial number: ISRCTN75636394. The study is reported in accordance with the CONSORT guidelines for cluster-randomised trials.

Keywords: Insulin resistance, Helminth, Type 2 diabetes, Parasite, Metabolism, Albendazole, Immunology

BACKGROUND

The number of people with diabetes mellitus is increasing worldwide [1-3]. At present, 8.3% of adults (382 million people) have diabetes mellitus [4] and Asia is a major site of this rapidly emerging epidemic [5]. In many Asian countries, including Indonesia, rapid socio-economic development has led to a shift in infrastructure, technology and introduction of Western style diets, which promotes overnutrition and sedentary lifestyles [5-8]. These changes have already led to an increasing prevalence of diabetes mellitus in Indonesia [9-12].

A strong predictor for the development of type 2 diabetes mellitus (DM2) is insulin resistance [13,14], which is caused by complex disturbances in multiple biological systems. There is now abundant evidence that inflammation [15] plays a role in the development of DM2, in addition to the more established relationship between an altered energy balance resulting from excess consumption of high-energy foods and/or decreased physical activity. In DM2 subjects, chronic low-grade inflammation is a common feature [15], which results, at least in part, from the activation of inflammatory pathways by fatty acids in multiple organs [16-18]. However, the fundamental molecular mechanisms are still incompletely understood [19].

In developing countries, infectious pressure might be one particular modifier of insulin resistance. Helminth infections, which are still endemic in many low to middle income countries, may therefore affect whole-body and tissue-specific insulin sensitivity owing to their immunomodulatory properties.[20] Previous studies have shown that helminth infections can adopt an immune evasion strategy by inducing regulatory T cells. [21-26] Hereby helminth infections may decrease systemic inflammation and subsequently the development of inflammatory diseases, including DM2 [27-30]. Studies examining the relationship between helminth infections and DM2 in both humans [31,32,32] and rodents [33,34] support this hypothesis. Recent landmark studies in animal studies have also established important links between metabolism and the immune response. At a molecular level, mTOR, a serine/threonine protein kinase located downstream of insulin signalling, plays an essential role in immune cell energy metabolism and function [35,36]. Furthermore, it has been shown that STAT6 signalling downstream of IL-4, as well as Th2 responses induced by helminths, improve glucose metabolism and insulin signalling [33,37]. Intriguingly, in humans, immune intervention with IL-1 receptor antagonist (Anakinra) has also been shown to influence glucose metabolism [38].

Helminths are also known to reduce energy intake and thereby change the energy balance [39], which may be beneficial in terms of insulin resistance [39,40]. Helminths may therefore both directly improve insulin sensitivity via a caloric restriction state and indirectly via Th2 activation. It appears, the immune system, which has evolved with helminths [41] and under conditions of low energy intake, seems to be out of balance in situations of nutritional overload and decreasing exposure to parasites [23,42]. In line with the proposed beneficial effects of helminth infections on glucose metabolism, our previous unpublished cross sectional study in Flores Island, Indonesia, has shown that subjects infected with intestinal helminths have a significantly lower insulin resistance as expressed by HOMA-IR.

Although aforementioned studies strongly suggest that there is an association between helminth infections, systemic inflammation and glucose metabolism, the causality in these relationships has not been demonstrated as yet. Therefore, we have initiated a large scale cluster randomized controlled trial (RCT) with the aim to assess the effect of anthelmintic treatment on insulin resistance, the hypothesis being that removing helminth infections will lead to a higher degree of insulin resistance. While study outcomes will be analysed at the individual participant level, a household cluster randomization was chosen to minimise contamination between treatment groups and therefore reinfection of treated individuals.

STUDY DESIGN

Study area

The study area is located in Nangapanda, a sub-district of the Ende District of Flores Island, Indonesia [43,44]. Nangapanda is a semi-urban coastal area with a population of approximately 22.000 people being divided over 29 villages. Our study area includes three of these villages (Ndeturea, Ndorurea 1, Ndorurea, with a total population of 3698 people, from which most of the adult population are farmers. Previous studies have shown that this area is endemic for soil-transmitted helminth (STH) infections [45]. A detailed map of the study area has been published [43].

Trial design

The study is designed as a household-based cluster-randomized, double-blind trial with two arms. In one arm treatment is given with albendazole (single dose of 400 mg) on 3 consecutive days, while the other arm consists of matching placebo treatment (both albendazole and placebo are manufactured by PT Indofarma

Pharmaceutical, Bandung, Indonesia). The treatment is provided every three months for a period of 1 year (total 4 rounds) to all household members except children below 2 years of age, while subjects aged 16 or above will undergo clinical and laboratory examination. Subjects with active treatment for diabetes mellitus, serious concomitant disease and pregnancy will be excluded.

The population was randomised by JWAS and JJH using computer aided block randomization at household level, utilizing Random Allocation Software to assign treatment groups. Both study investigators and patients are blinded for treatment codes. The treatment code will be unblinded when all data needed for analysis are cleaned and entered into the database. An additional randomization was performed in a subgroup of individuals, who will undergo an oral glucose tolerance test and immunological studies in order to study glucose metabolism and immune mechanisms in more detail. For this subgroup, we aimed to select one subject per household and stratified by age group (16-36 years of age, 36-56 years of age, and >56 years of age) to ensure that sufficient numbers of all age groups are participating. Randomization was based on households.

Well trained community workers were recruited and trained to distribute the drugs. These workers were also trained to assist during clinical examination and sample collection and were involved in health promotion within the population. Community workers and research team members will directly supervise the study participants while taking the study medication, and will collect empty drug canisters at each visit to confirm compliance. Furthermore, assessment of side effects will take place during these visits and migration and death will be noted. Adverse events spontaneously reported by the patient or observed by the investigators, will be monitored throughout the study. After completion of the study, the whole study population will be treated with a single dose of albendazole (400 mg) for 3 consecutive days.

Outcomes

As this study aims to assess the effect of anthelmintic treatment on whole-body insulin sensitivity, our primary outcome is a change in insulin resistance as assessed by HOMA-IR between both treatment arms after one year of treatment. Secondary outcomes are changes in body mass index and waist circumference, fasting plasma glucose, 2h-glucose levels after oral glucose tolerance test, HbA1c, serum lipid levels, immunological parameters, and efficacy of anthelmintic treatment.

Sample size

Sample size is calculated according to intention to treat analysis in which we will need 1580 subjects in total. Based on our previous study [45] we assume that the average household size is 4 and that around 20% will be lost to follow up after one year. We use a significance level of 5% and a power of 80%. Correlations within households are taken into account by using the correction factor 1+(m-1) ICC, with m being the household size and ICC the intra-class correlation. The sample size is computed for a difference in mean between the two treatment groups of 0.18 and an ICC of 0.1.

For the subgroup of individuals undergoing an oral glucose tolerance test, a sample size of 335 subjects in total is calculated assuming that around 20% will be lost to follow up after one year and using a significance level of 5% and a power of 80%. The sample size is computed for a difference in mean of 10.3 mg/dL and a standard deviation of glucose level of 30 mg/dL.

METHODS

Sample collection

At baseline all eligible subjects, aged 16 and above will be invited to visit the examination centre after an overnight fasting and to provide stool, blood and first morning urine samples. During this visit, participants education level and profession will be registered. After 1 year of treatment, follow-up sample collection will take place as shown in **Table 1**.

Clinical anthropometry assessment

Anthropometric measurements of body weight, height, waist and hip circumference are obtained using the National Heart, Lung, and Blood Institute (NHLBI) practical guidelines. To measure body weight a flat scale for mobile use (SECA Model 876, Seca Gmbh Co, Hamburg, Germany) is used, while a portable stadiometer (SECA Model 213, Seca Gmbh Co, Hamburg, Germany) is used to measure height. Waist and hip circumference are measured using ergonomic circumference measuring tape (SECA Model 201, Seca Gmbh Co, Hamburg, Germany). In addition, body fat composition is measured using a Tanita body composition analyser (TBF-300A, Tanita Corp, Tokyo, Japan). Three blood pressure measurements (left arm, sitting upright position, after resting 5 minutes) are taken from each subject, using a digital sphygmomanometer (HEM-7200, Omron Healthcare Co, Ltd, Kyoto, Japan), and calibrated using a Riester nova-presameterH-Desk model

mercury sphygmomanometer (Gerhard Glufke Rudolf Riester GmbH & Co, Jungingen, Germany) and a 3MTM LittmannH Classic II S.E. Stethoscope (3M, St. Paul, Minnesota, USA). The average of three systolic/diastolic blood pressure measurements will be used for analysis.

Table 1. Study schedule of the Sugarspin project

		3 monthly treatment				1 year
Outcome	**Baseline**	**1st**	**2nd**	**3rd**	**4th**	**follow up**
Clinical Anthropometry	X					X
Parasitological examination	X					X
Metabolic parameters	X					X
Immunological parameters	X					X
Assessment of side effects		X	X	X	X	X

Parasitological examination

To assess intestinal helminth infection, stool containers are distributed and collected by health workers. Stool samples are examined by the Kato Katz method [46] for identification and quantification of STH eggs using 2 slides for each sample. An aliquot of the fresh stool samples is frozen at -20°C in the field and subsequently at -80°C in laboratories of the Departments of Parasitology at Leiden University Medical Center, Leiden, The Netherlands and Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia for DNA extraction [43]. Part of the stool sample will be saved for potential future analysis of the microbiome.

DNA isolation and helminth real-time PCR

DNA isolation from stool will be performed as described elsewhere [43], with some minor modifications. Real-time PCR will be performed to detect the presence of *A. duodenale*, *N. americanus* (hookworm), *A. lumbricoides* and *T. trichiura* using a method described previously [43] with some modifications.

Blood collection

Peripheral blood is collected into EDTA and SST Vacutainers (BD, Franklin Lakes, NJ, USA). Giemsa-stained peripheral blood smear is prepared to evaluate neutrophil and eosinophil count. In a subset of the study population, additional blood is collected in PAXgene Blood RNA Tubes (PreAnalytiX GmbH, Hombrechtikon,

Switzerland) and Sodium Heparin Vacutainers (BD). Blood collected in PAXgene Blood RNA Tubes will be used to study RNA expression profiles, while blood collected in Sodium Heparin Vacutainers will be used for detailed immunological measurements as described below (section Immunological methods). All samples deriving from EDTA and SST Vacutainers (serum, plasma, cell pellet and whole blood) and all PAXgene Blood RNA Tubes are kept at -20°C at the Field Clinical Research Centre (FCRC) and will be sent on dry ice to University's laboratory for storage at -80°C.

Metabolic parameters

Fasting blood glucose is measured in capillary blood using Breeze®2 glucose meters (Bayer Health Care LLC, Basel, Switzerland). An oral glucose tolerance test performed in a subset of the study population uses WHO protocol. [47,48] Glucose levels are measured in capillary blood using Breeze®2 glucose meters after overnight fasting and 2 hours after ingesting 75g of anhydrous glucose dissolved in 200 cc of water. Insulin, HbA1c and lipid profiles will be measured in an internationally accredited laboratory. HOMA-IR, a well-validated measure of insulin resistance will be calculated to estimate insulin resistance.[49]

Immunological methods

The immunological parameters that will be studied are 1) Total IgE levels as one of the markers of Th2 response and its relation to metabolic parameters, 2) Circulating pro and anti-inflammatory cytokines in order to study their relationship to metabolic parameters, 3) Antigen specific IgE and IgG to *Ascaris lumbricoides* to monitor antibody responses to one of the helminths studied as a marker of changing immune responses as a result of anthelmintic treatment, 4) Granulocyte (neutrophil and eosinophil) frequencies and their activation to assess whether granulocytes, in particular eosinophils which are associated with Th2 response, are linked to helminth infections and metabolic parameters, 5) Peripheral blood mononuclear cells (PBMC) subset analysis and polarisation by flow cytometry in order to assess the relationship between immune cell frequencies (T cell subsets, B cell subsets, monocyte subsets, NK cells and myeloid suppressor cells) in situ as well as after activation and metabolic parameters.

Total IgE

Total IgE will be measured using ELISA with rabbit anti-human IgE antibodies (Dako, Glostrup, Denmark) and goat anti-human IgE biotinylated antibodies

(Vector Laboratories, Burlingame, CA, USA) as capture and detection antibodies, as described previously [43]. The World Health Organization standard of human serum IgE was used as a reference (National Institute for Biological Standards and Control). The results will be expressed in International Units (IU).

Circulating cytokines

Pro and anti-inflammatory cytokines (TNFα, IFNγ, IL-1, IL-6, IL-10, TGFβ) will be measured in serum samples using cytokines kit with high sensitivity.

Ascaris-specific IgE

Ascaris antigen will be prepared from *Ascaris lumbricoides* worms as described previously.[50] Maxisorp plates (Thermo Fisher Scientific, Roskilde, Denmark) will be coated overnight with 5 µg/ml *Ascaris* antigen in 0.1M carbonate buffer (pH 9.6). Plates will be blocked for 1 hour with PBS containing 2% bovine serum albumin. Samples will be diluted 1/60 in 0.1 M Tris-HCl containing 0.05% Tween-20 and incubated overnight together with a pool of positive standard plasma containing $1X10^6$ arbitrary units (AU) parasite specific IgE. After a washing step, goat anti-human IgE biotinylated antibodies (Vector) will be incubated followed by streptavidin-HRP (Sanquin, Amsterdam, the Netherlands). The color is developed by adding 3,3',5,5' tetramethylbenzidine (TMB) (KPL, Gaithersburg, MD, USA). The reaction will be stopped by adding 1.8 M H_2SO_4 and absorbance will be read at 450 nm in an automated plate reader.

Ascaris-specific IgG isotypes

Maxisorp plates will be coated with *Ascaris* antigen as described for *Ascaris* specific IgE above. Blocking will be done using PBS containing 5% bovine serum albumin. Samples will be diluted 1/1000, 1/10, 1/5 or 1/25 for IgG1, IgG2, IgG3 and IgG4 respectively, and a pool of positive standard plasma containing $1X10^6$ arbitrary units (AU) parasite specific IgG isotypes will be included in each plate. After overnight incubation, HRP-labelled anti human IgG isotypes (Sanquin) in PBS 0.05% Tween-20 will be added for 4 hours incubation at 37°C using the following dilutions: 1/3000 for anti IgG1 (HP6188) and anti IgG4 (HP6196); 1/1000 for anti IgG2 (HP6014) and anti IgG3 (HP6095). TMB substrate will be used to develop the color and the reaction will be stopped as described above.

Whole blood stimulation and fixed granulocyte cryopreservation

To study the expression of activation markers on granulocytes, 600 µl of heparinised venous blood is divided over 3 polystyrene tubes (200 µl/tube). After a pre-incubation of 5 minutes in a 37°C waterbath, a 5 minutes stimulation

at 37°C is performed with medium/control, N-Formyl-Met-Leu-Phe (FMLP, 10^{-5} M; Sigma, Saint Louis, MO, USA) or eotaxin (10^{-7} M; R&D systems, Abingdon, UK). Subsequently, 4 ml of FACS lysing solution (BD) is added and after an incubation period of 15 minutes at room temperature the red blood cells is lysed while white blood cells, including granulocytes, become fixed. Cells are washed with RPMI 1640 containing 10% heat-inactivated FCS and then resuspended in RPMI 1640 containing 10% of heat-inactivated foetal calf serum (FCS) and 10% dimethyl sulfoxyde (DMSO). Cryovials containing the cell suspension are placed at -80°C for minimum of 4 hours, followed by storage in liquid nitrogen until further analysis.

PBMC cryopreservation

Peripheral blood mononuclear cells (PBMCs) are isolated from heparinised venous blood using Ficoll density gradient centrifugation within 12 hours after blood collection. After isolation, cells are cryopreserved in RPMI 1640 containing 20% of heat-inactivated foetal calf serum (FCS) and 10% dimethyl sulfoxyde (DMSO). Cryovials containing the cell suspension are transferred to a freezing unit which is placed in a -80°C freezer for minimum of 4 hours. Subsequently, vials are stored in liquid nitrogen until analysis.

Metabolomics for metabolic profiling

Urine samples and blood samples from heparinized blood are kept at -20°C at the FCRC and subsequently stored at -80°C at the University's laboratory for possible future metabolomics measurements. The exploratory metabolomics analysis will be performed by 1H-NMR and LC-MS metabolomics, a combination of NMR and LC-MS proposed for this study provides a comprehensive coverage of metabolome and as such increase the probability finding physiologically meaningful associations within the data.

Data management and statistical analyses

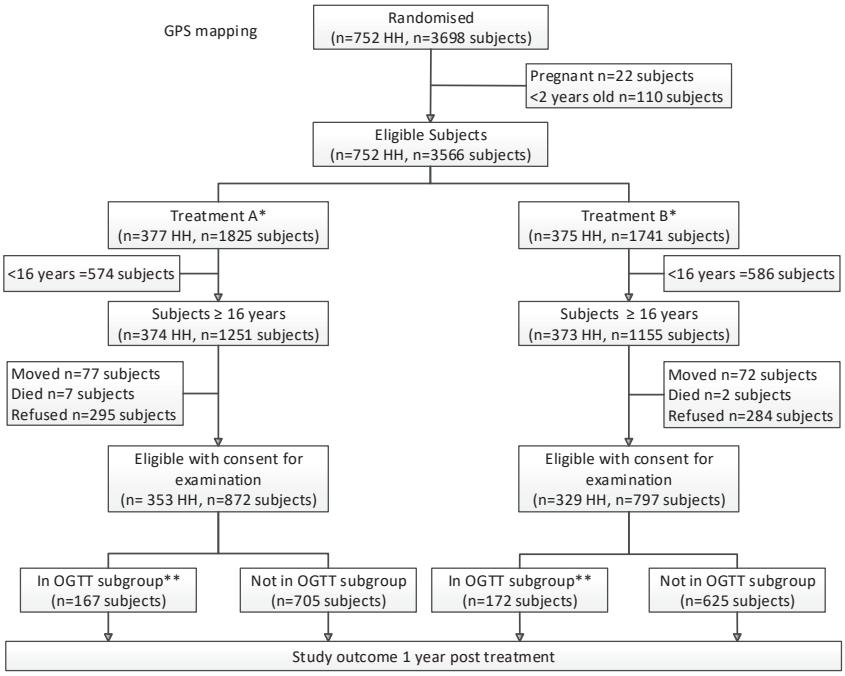
A centrally accessible database designed in Microsoft Access is established and the data is entered by well-trained data entry personnel. Descriptive data will be summarized for continuous variables as mean +/- SD for normally distributed data and median (range) for non-normally distributed data. Categorical data will be expressed as proportions.

The effect of anthelmintic treatment on change in insulin resistance (HOMA-IR) as our primary outcome will be assessed using an intention to treat approach

after 1 year of treatment using mixed models to account for the correlation within households, in which relevant confounders (including age, gender, BMI, village) are entered. The characterization of immune responses to helminth infections and systemic inflammation will be assessed by measuring cytokine profiles. Moreover, for these analyses multilevel modelling will be used and the use of longitudinal data will take repeated measurement into account.[51] The mediation of helminth's effects on insulin resistance via immune responses will also be assessed.

Ethical approval, trial registration and consent

This study has been approved by The Health Research Ethical Committee, Faculty of Medicine, Universitas Indonesia Cipto Mangunkusumo Hospital, Jakarta, Indonesia (reference number:549/H2.F1/ETIK/2013). It has also been filed by the ethics committee of Leiden University Medical Center and is registered as a clinical trial ref: ISRCTN75636394 (http://www.controlled-trials.com/isrctn/pf/75636394). The local health authorities have been informed about this study and have given



*Households were assigned to three monthly treatment with albendazole or placebo for 1 year
**For the oral glucose tolerance test (OGTT) and immunological studies, a random selection was made and 339 individuals were invited to participate.

Figure 1. Flow diagram of the Sugarspin project.

their approval and support. The study, its benefits and risks are explained to the population and consent forms are distributed to be signed by the subjects who are willing to participate in this study. They are informed that they can withdraw from the study at any time, for any reasons and without any consequences.

Description of the population recruited

So far, the study has provided the following data (**Figure 1**). At baseline, a total of 3698 individuals were registered in 752 households. Of the 2428 subjects aged 16 years or older, 1669 subjects were eligible with consent for examination. For the oral glucose tolerance test and immunological studies 339 subjects were randomly selected and gave approval.

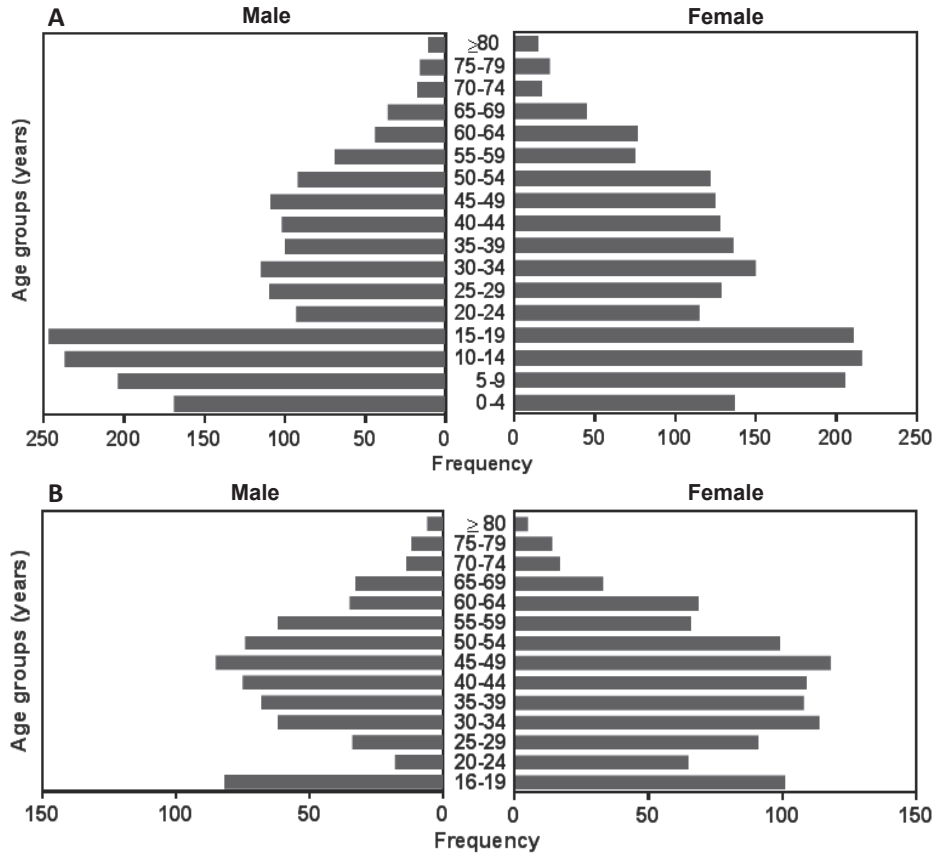


Figure 2. Age pyramid. Age pyramid of all individuals living in the study area in Nangapanda, Flores Island, Indonesia (n=3698 subjects, 52% female) (A), and of study participants (n=1669 subjects, 60% female) (B).

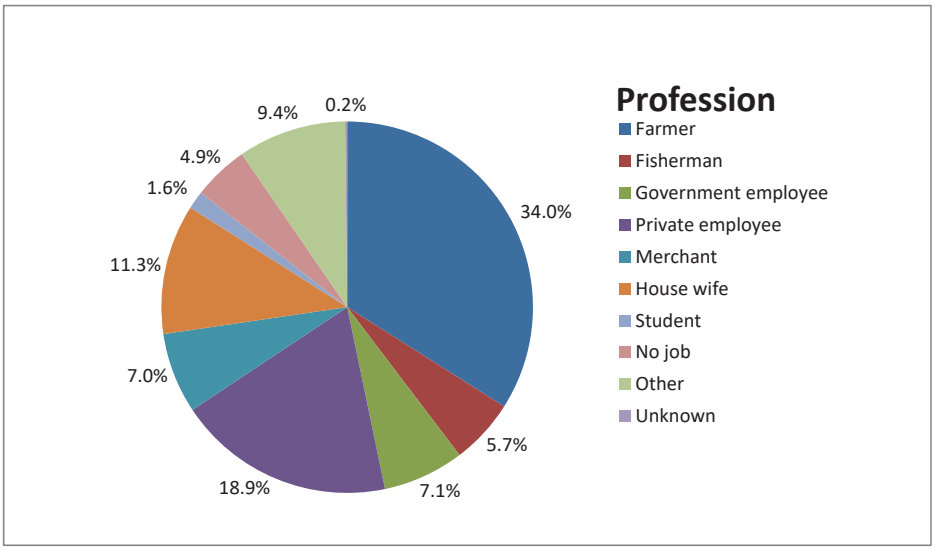


Figure 3. Job distribution. At baseline, profession was assessed for study participants (n=1669 subjects).

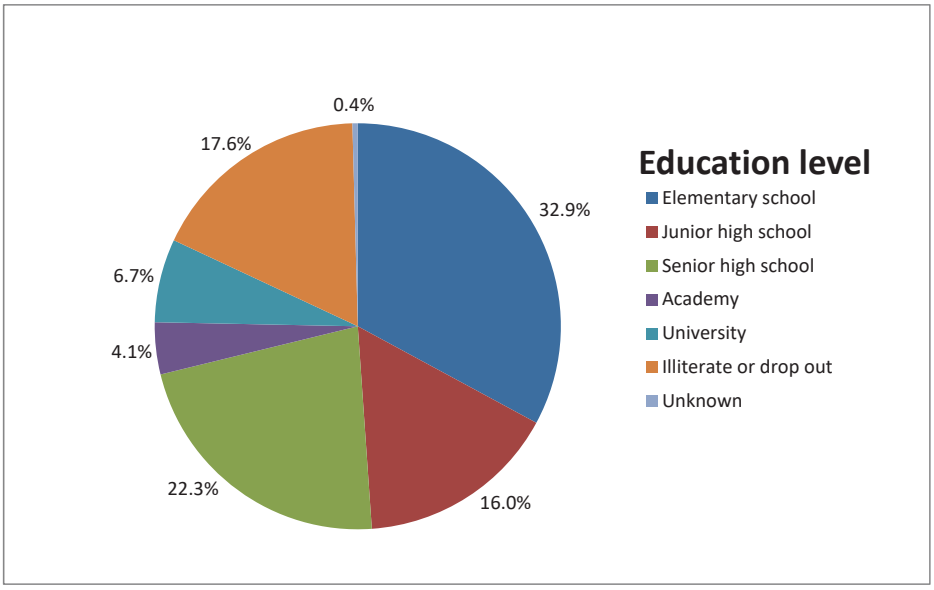


Figure 4. Education level. At baseline, education level was assessed for study participants (n=1669 subjects).

Figure 2 shows the age pyramid of both the whole population in the study area and the study population. In this area farming and fishing are the traditional source of income while some individuals engage in jobs at government offices or in the private sector (**Figure 3)**, a similar distribution is seen in the total population.

The education level of the majority of subjects in the study population is elementary school (33%), followed by senior high school (22%), and junior high school (16%), while 11% has college or university degrees. Moreover, 18% of the subjects is illiterate, either not educated at all or dropped out from elementary school (**Figure 4**). A similar distribution of education levels is seen in the total population.

DISCUSSION

The SUGARSPIN trial is the first and currently the only longitudinal study investigating the effects of anthelmintic treatment on whole-body insulin sensitivity. This placebo-controlled trial enables us for the first time to investigate the causal relationship between helminth infections, systemic inflammation and glucose metabolism in humans. In addition, this study will provide data on anthelmintic treatment efficacy and effectiveness in a large population in a developing country like Indonesia.

Abbreviations

DM2: Type 2 diabetes mellitus; FCRC: Field Clinical Research Center; OGTT: Oral Glucose Tolerance Test; STH: Soil-transmitted helminth; Th2: T-helper-2

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

DLT is a medical doctor in charge of the field study, involved in setting up the study, supervising gathering of data, treatment, clinical care, follow up of the study population and wrote the paper. KR is a medical biologist in charge of the field study, involved in setting up the laboratory in the study area, performing the immunological analysis, supervising the data cleaning, follow up of the study population and wrote the paper. IM is a mathematician who is developing methods to analyse the complex data generated during the lifetime of the project and is involved in the randomization. LvL is a parasitologist who is involved in the performance and analysis of diagnostic assays for the detection of helminths in stool samples. BG is a biologist who advises on the metabolic aspects of the study. PS is an endocrinologist who advises on the metabolic aspects of the study. YD is a medical doctor who is involved in coordinating the study and advises on the immunological and parasitological aspects of the study. AEW is a medical doctor who is involved in setting up and supervising the database and providing the treatment. OM is a biochemist who advises on the metabolomics studies in urine and serum. JJH is a biostatistician who developed the study, and is involved in supervising sample size calculation, randomization and statistical analysis. HT is a mathematician who is in charge for census, GPS-mapping and baseline preliminary database

update. ES is an immunoparasitologist who is involved in coordinating the study and advising on parasitological and immunological aspects of the study and supervised the writing of the manuscript. MY is an immunologist who developed the study, supervised the writing of the manuscript and and is the scientific coordinator of the SUGARSPIN program. JWAS is an endocrinologist who developed the study, supervised the writing of the manuscript, and is the Dutch coordinator of the SUGARSPIN program. TS is a parasitologist who developed the study and is the Indonesian coordinator of the SUGARSPIN program. All authors read and approved the final manuscript.

Author's information

Dicky L. Tahapary and Karin de Ruiter are equal contributors in writing the manuscript, while Johannes W.A. Smit and Taniawati Supali are equal contributors in the term of coordinators of the project from The Netherlands and Indonesian side.

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Chapter 3

EFFECT OF ANTHELMINTIC TREATMENT ON INSULIN RESISTANCE: A Cluster-randomized Placebo-controlled Trial in Indonesia

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ABSTRACT

Background. Emerging evidence suggests that helminth infections are associated with lower insulin resistance (IR). Current deworming programs might remove this helminth-associated protective effect. Therefore, we evaluated the anthelmintic treatment effect on changes in IR.

Methods. We conducted a double-blind, household-cluster-randomized, placebo-controlled clinical trial on Flores island, Indonesia, an area endemic for soil-transmitted helminths (STHs). All subjects received four rounds of albendazole or matching placebo with 3-month intervals, for 3 consecutive days. The primary outcome was the change in homeostatic model assessment of IR (HOMA-IR) in those aged ≥16 years. An intention-to-treat analysis was performed involving all subjects and ad hoc in the helminth-infected subjects.

Results. We examined 797 (in 329 households) and 872 (in 353 households) subjects, who were assigned randomly into the albendazole and placebo arm, respectively. Albendazole was associated with a significant reduction in STH prevalence, total Immunoglobulin E (IgE) and eosinophil count. Whereas albendazole had no effect on IR [estimated treatment effect, 0.006 (95% confidence interval, -0.010 – 0.021), p=0.48] at the community level, it was associated with a significant increase in IR [0.031 (0.004 – 0.059), p=0.04, p-value for interaction=0.01] among helminth-infected subjects as detected by microscopy. Pathway analysis suggested that this might in part be due to an increased body mass index or a reduced eosinophil count.

Conclusions. Anthelmintic treatment reduces STH prevalence, total IgE and eosinophil count but has no effect on IR at the community level. In helminth-infected subjects, treatment significantly increases IR, highlighting the need for metabolic health monitoring with ongoing deworming programs.

Clinical Trials Registration. ISRCTN 75636394.

Keywords. anthelmintic, insulin resistance, deworming, diabetes, helminths

INTRODUCTION

The increasing prevalence of type 2 diabetes mellitus (DM2) is a major health concern worldwide, in particular in low- and middle-income countries (LMIC).[1] The rapid socio-economic development in these countries has led to a shift in dietary habits and infrastructure which promotes overnutrition and decreased physical activity,[2] ultimately increasing the risk for DM2. DM2 is characterized by increased insulin resistance (IR). Although the pathophysiology of DM2 is complex and involves several defects,[3] there is evidence that in addition to an altered energy balance, chronic low-grade systemic inflammation plays a key role, linking the immune system and the impairment in metabolic homeostasis.[4]

Helminth infections, which are still endemic in many LMIC,[5] are associated with skewed immune responses towards type-2 and regulatory immune responses.[6] This may lead to a decreased systemic inflammation and consequently increased whole-body and tissue-specific insulin sensitivity.[7] In addition, helminths are associated with a lower body mass index (BMI),[8] which may be beneficial in terms of IR. Helminths may therefore improve insulin sensitivity via immunological and non-immunological pathways.[6, 9, 10]

Interleukin (IL)-4[11] and IL-10,[12] key cytokines in helminth infections, have been shown to regulate peripheral nutrient metabolism and insulin sensitivity.[11] Recent studies in animal models of diet-induced obesity [13-17] have also shown that helminth infections [13, 15-17] and helminth-derived molecules [14, 16-18] can increase insulin sensitivity through direct and indirect control of metabolic pathways.[18] Furthermore, several population based studies have reported a lower DM2 risk in subjects with previous [19, 20] or current [21, 22] chronic helminth infections. In a previous study on Flores island in Indonesia, we reported that chronic soil-transmitted helminth (STH) infections were associated with lower whole-body IR, independent of BMI.[8]

However, all human studies performed so far have been cross-sectional, preventing any insight on a causal relation between helminth infections and IR. Therefore, we performed a cluster-randomized controlled trial (RCT) of anthelmintic treatment in an area endemic for STHs, studying the hypothesis that a reduction of helminth infections will lead to a higher degree of IR.

METHODS

Study Overview

We conducted a household-based cluster-randomized double-blind trial in 3 villages in Nangapanda, Ende, Flores island, Indonesia. The trial was approved by the ethics committee of Faculty of Medicine, Universitas Indonesia (FKUI), filed by the ethics committee of Leiden University Medical Center (LUMC), and registered as a clinical trial (http://www.isrctn.com/ISRCTN75636394). The protocol was published previously.[23]

Participants

All subjects in the study area, except children <2 years of age and pregnant women, were included in the trial to avoid cross-contamination between household members. Subjects aged ≥16 years underwent clinical and laboratory examination, excluding subjects with active treatment for diabetes mellitus and serious concomitant diseases.

Study Design and Treatments

After obtaining written informed consent, the population was randomised by household blocks using Random Allocation Software for assignment to treatment. Both study investigators and participants were blinded for the treatment code. After randomisation, all study subjects received a tablet of albendazole (400 mg) or matching placebo (both manufactured by PT Indofarma Pharmaceutical, Bandung, Indonesia) for three consecutive days with direct supervision. This treatment regimen was given 4 times with 3-month intervals (week 9-10, 21-22, 33-34, and 45-46). Clinical measurements, as well as blood and stool sample collection, were performed during the first 8 weeks before the start of the drug administration(baseline or t=0) and 6 weeks after the last drug administration (follow-up or t=52 weeks) (**Supplementary Figure 1**). After completion of the study, the whole study population was treated with a tablet of albendazole (400 mg) for 3 consecutive days.

Study Procedures and Outcomes

All clinical measurements and blood sample collections were performed after an overnight fast. Detailed information on study procedures are available at the **Supplementary Appendix**. In brief, body weight, height, waist circumference, hip circumference were measured, from which BMI and Waist-to-hip-ratio were calculated. Fasting blood glucose was determined in capillary blood. All sera, plasma, whole blood, and stool samples were frozen at −20°C and subsequently

stored at −80°C. Insulin, haemoglobin A1c (HbA1c), total cholesterol, high-density lipoprotein cholesterol (HDL-C), triglycerides and high-sensitivity C-reactive protein (hs-CRP) were measured pairwise (baseline and follow-up) in the same analytical runs at the LUMC. A Giemsa-stained peripheral thin blood smear was read at FKUI to assess the differential white blood cell count, resulting in a relative percentage of basophils, eosinophils, neutrophils, lymphocytes and monocytes. Total IgE was measured at LUMC as described previously.[24]

Fresh stool samples were examined by microscopy using the Kato-Katz method to detect STHs (hookworm, *Ascaris lumbricoides,* and *Trichuris trichiura*). Multiplex real-time polymerase chain reaction (PCR) was performed to simultaneously detect the presence of hookworm (*Ancylostoma duodenale, Necator americanus*), *Ascaris lumbricoides, Trichuris trichiura,* and *Strongyloides stercoralis*. Stool samples were considered positive by PCR when cycle threshold (Ct) values were <50, and further grouped into 3 categories: Ct <30, 30 to <35, and ≥35 representing a high, moderate and low DNA load, respectively.[25]

Primary outcome was IR, assessed using the homeostatic model assessment of IR (HOMA-IR), a well-validated measure of whole body IR in humans (HOMA-IR= fasting serum insulin x fasting glucose / 22.5).[26] Secondary outcomes included BMI, waist circumference, fasting blood glucose, HbA1c, lipid levels, total IgE, eosinophil count, hs-CRP and prevalence of STHs as assessed by microscopy and stool PCR. Adverse events reported by subjects or observed by the investigators were monitored during the trial.

Statistical Analysis

The sample size was calculated according to intention-to-treat analysis. Based on our previous study,[27] we assumed that the average household size is 4 and that around 20% of participants would be lost to follow-up after one year. We used a significance level of 5% and a power of 80%. Correlations within households were taken into account by using the correction factor 1 + (m-1) ICC, with *m* being the household size and ICC the intra-class correlation. The sample size was calculated to aim at a difference in mean HOMA-IR between the 2 treatment groups of 0.18 and an ICC of 0.1 indicating 1580 subjects in total.

For continuous variables, normally distributed data were summarized as mean and standard deviation [mean (SD)], while non-normally distributed data (HOMA-IR, insulin, hs-CRP, total IgE and eosinophil count) were summarized as geometric

mean and 95% confidence interval (CI), and log-transformed for analyses. HOMA-IR and hsCRP were log transformed as $\log_{10}(1+[value])$. Categorical data were expressed as proportions.

The effect of anthelmintic treatment on HOMA-IR was assessed at community level using an intention-to-treat approach, using mixed models to account for the correlation within households. As an ad hoc analysis, we stratified by infection status by including helminth infection status (no infection, any infection) at baseline and its interaction with treatment into the model. We also stratified by the number of helminth species a subject was infected with, by including the number of helminth species (no infection, single infection, multiple infection) at baseline and its interaction with treatment into the model. Two random effects were used: to model clustering within households a random household specific intercept was used and to model correlation within subjects random subject-specific intercept was used. Parameter estimates for treatment effect and 95% CIs were reported. The reported p-values were obtained using a likelihood ratio test comparing the model with and without the treatment effect. We used the same model for secondary outcomes. For the binary outcome (helminth infection status), a logistic model was used with random household effects and random subject effects. All models were fitted using the lme4 package (R software).

RESULTS

Between 1 April 2014 and 3 June 2014, we initially included 752 households with 3566 subjects in the trial. Randomization resulted in 1825 subjects assigned to placebo and 1741 subjects to albendazole (377 and 375 households, respectively). The overall trial profile is shown in **Figure 1**, with a total of 1669 subjects aged 16 and above who were examined at baseline [872 subjects (353 households) and 797 subjects (329 households) in the placebo and the albendazole group respectively]. Baseline (t=0) characteristics were similar between both treatment arms (**Table 1**).

The overall loss to follow-up, from baseline to 52 weeks, was 18.9%. The main reason for loss to follow-up was permanent or temporary movement out of the village for employment or study. Those who moved out and refused to come for follow-up, were younger in comparison to the whole population. There were no significant differences between both treatment arms in terms of loss to follow-up (**Supplementary Table S1**). With respect to compliance, 87.9% (1189/1353) of the subjects took the maximum of 12 tablets [87.0% (574/660) vs 88.7% (615/693), in

the albendazole and the placebo group, respectively]. We collected stool samples from 92.0% (1535/1669) of the subjects at baseline and 89.9% (1217/1353) of the subjects at follow-up. Data to calculate HOMA-IR were available for 1604 subjects at baseline, and for 1272 subjects at follow-up. Sixteen subjects who were receiving active treatment for DM2 were excluded from analysis.

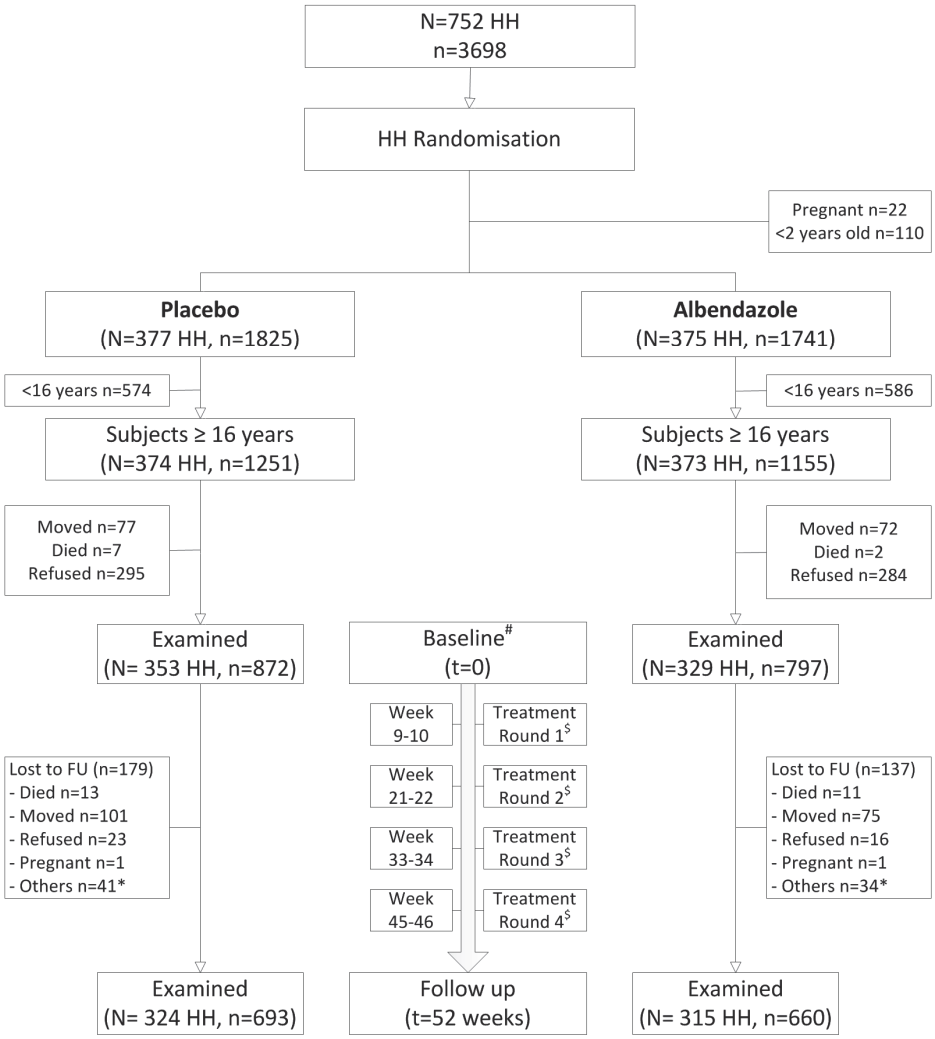

Figure 1. Trial profile. #Baseline data (t=0) were collected during the first 8 weeks before the start of the drug administration. $Single dose of albendazole or matching placebo was given for 3 consecutive days to all household members, except children <2 years of age and pregnant women. *Other reasons of lost to follow-up were harvesting crops, working on funeral ceremonies, severely ill, hospitalized, and nursing mother. Abbreviations: HH, households; FU, follow-up.

Table 1. Baseline Characteristics of Study Population

Characteristic	No.	Placebo	No.	Albendazole
Age, y, mean (SD)	872	42.5 (15.7)	797	42.5 (15.7)
Sex, female, No. (%)	872	534 (61.2)	797	478 (60.0)
Body mass index, kg/m2, mean (SD)	860	22.4 (4.2)	790	22.4 (4.0)
Waist circumference, cm, mean (SD)				
Female	532	76.5 (12.6)	476	77.0 (12.6)
Male	333	76.5 (11.6)	317	76.6 (11.3)
Waist-to-hip-ratio, mean (SD)				
Female	507	0.88 (0.07)	473	0.89 (0.08)
Male	317	0.94 (0.07)	315	0.94 (0.07)
Systolic BP, mmHg, mean (SD)	871	129.3 (23.4)	765	129.5 (23.8)
Diastolic BP, mmHg, mean (SD)	871	76.4 (12.1)	765	76.5 (12.1)
Total cholesterol, mmol/L, mean, (SD)	836	4.9 (1.1)	764	4. 9 (1.1)
HDL cholesterol, mmol/L, mean, (SD)				
Female	517	1.3 (0.4)	457	1.3 (0.4)
Male	319	1.1 (0.3)	307	1.1 (0.3)
LDL cholesterol, mmol/L, mean, (SD)	836	3.0 (0.9)	763	3.0 (0.9)
Triglycerides, mmol/L, mean, (SD)	836	1.4 (0.7)	764	1.5 (0.7)
HbA1c[a], mmol/mol, mean (SD)	715	32.5 (9.0)	683	32.3 (8.5)
Fasting blood glucose, mmol, mean, (SD)	836	5.5 (1.6)	768	5. 5 (1.6)
Fasting Insulin, mU/L, GM (95%CI)	836	3.5 (3.2 – 3.7)	768	3.5 (3.3 – 3.8)
HOMA-IR, GM (95%CI)	836	1.09 (1.02 – 1.15)	768	1.08 (1.01 – 1.14)
hs-CRP, mg/L, GM (95%CI)	836	1.26 (1.16 – 1.36)	764	1.26 (1.16 – 1.37)
Total IgE, IU/mL, GM (95%CI)	835	557.2 (498.1 – 623.3)	766	601.6 (534.8 – 676.7)
Eosinophil count, %, GM (95%CI)	829	5.9 (5.6 – 6.1)	763	6.1 (5.8 – 6.4)
Helminth infection by microscopy, No. (%)	655	283 (43.2)	602	251 (41.7)
Single		185 (28.2)		160 (26.6)
Multiple		98 (15.0)		91 (15.1)
Helminth infection by PCR, No. (%)	783	425 (54.3)	710	393 (55.4)
Single		256 (32.7)		252 (35.5)
Multiple		169 (21.6)		141 (19.9)

Abbreviations: BP, blood pressure; CI, confidence interval; GM, geometric mean; HDL, high-density lipoprotein; HbA1c, hemoglobin A1c; HOMA-IR, homeostasis model assessment for insulin resistance; hs-CRP, high-sensitivity C-reactive protein; IgE, immunoglobulin E; LDL, low-density lipoprotein; PCR, polymerase chain reaction; SD, standard deviation. [a]After excluding subjects with unidentified hemoglobinopathy on the Tosoh G8 high-performance liquid chromatography analyzer (13.9% [116/831] in the placebo group and 10.5% [80/763] in the albendazole group).

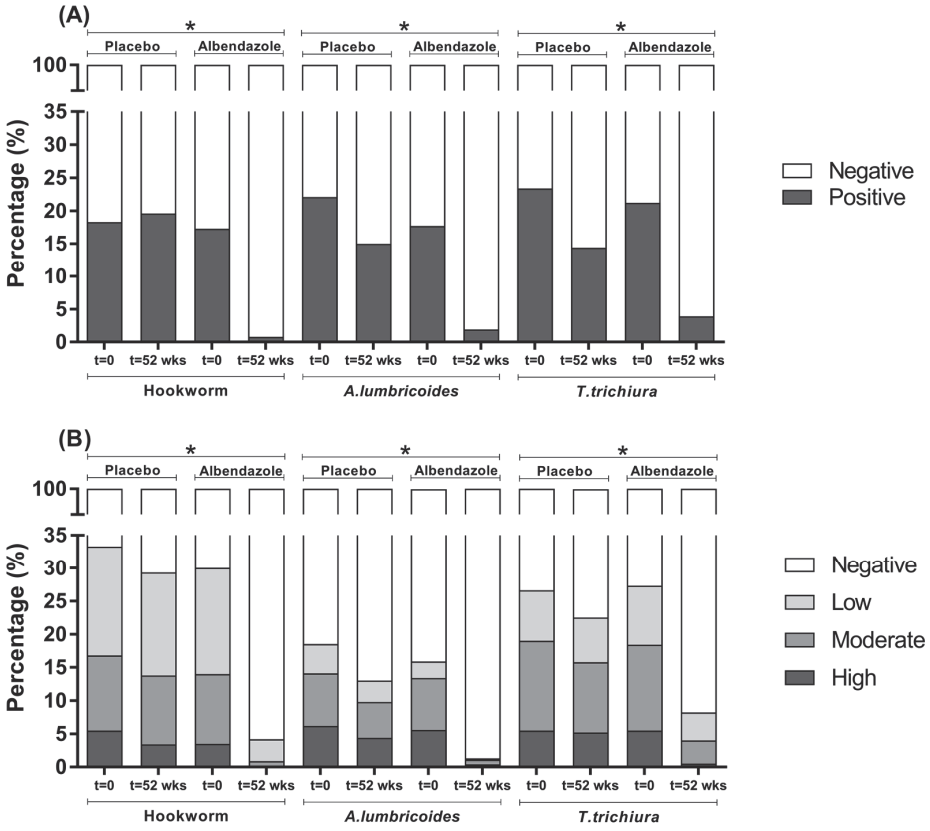

Figure 2. The effect of albendazole treatment on the prevalence and intensity of soil-transmitted helminths. Percentage of hookworm-, *Ascaris lumbricoides*-, and *Trichuris trichiura*-infected subjects at baseline (t=0) and following treatment (t=52 weeks), in placebo and albendazole treatment arms, as detected by microscopy (n=1011; A) and polymerase chain reaction (n=1144; B). Albendazole treatment was associated with a significant reduction of hookworm, *A. lumbricoides* and *T. trichiura*. p-values were calculated using a logistic model with random household effects and random subject effects. *corresponds to p-value <0.0001.

Effect of Treatment at the Community Level

Albendazole treatment reduced the percentage of subjects with any helminth infection as assessed by either microscopy [41.7% (251/602) to 5.6% (27/486) in the albendazole arm vs 43.2% (283/655) to 34.4% (181/526) in the placebo arm, p<0.0001] or PCR [55.4% (393/710) to 11.3% (62/550) in the albendazole arm vs 54.3% (425/783) to 46.8% (278/594) in the placebo arm, p<0.0001]. The highest reduction was seen for hookworm, followed by *A. lumbricoides* and *T. trichiura* infection **(Figure 2).** When assessing the infection intensity in categories based on PCR, albendazole treatment resulted in a reduction in intensity across these

three helminths species with the least effect on *T. trichiura* infection **(Figure 2B)**. S. stercoralis prevalence, which was already low, was eliminated in the albendazole group (**Supplementary Table S2**).

At the community level, neither HOMA-IR nor BMI, waist circumference, fasting blood glucose, HbA1c, total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides and hsCRP were found to be affected by albendazole treatment **(Table 2)**. However, the significant reduction of infection prevalence and intensity by albendazole treatment, was accompanied by a significant decrease in total IgE level [Estimated treatment effect (95% CI), p value, -0.066 (-0.094 – -0.037), p<0.0001] and eosinophil count [-0.057 (-0.086 – -0.028), p=0.0001] **(Table 2)**.

Effect of Treatment in STH-infected Subjects

Next, the effect of treatment was assessed only in those who were infected with helminths at baseline, as detected by microscopy. Albendazole treatment resulted in a significant increase in HOMA-IR [0.031 (0.004 – 0.059), p=0.04] **(Figure 3.A)**. This effect was greater in comparison to subjects without helminth infections at baseline (p=0.01 for the interaction between helminth infection status at baseline and treatment). Moreover, with an increasing number of helminth species infecting a subject at baseline, there was a gradual increase in HOMA-IR after treatment. Thus, whereas we saw no significant effect of treatment among those with no infection [-0.013 (-0.035 – 0.010), p=0.28] or those infected with single species [0.017 (-0.017 – 0.050), p=0.34], treatment in those infected with multiple helminth species resulted in a significantly higher HOMA-IR [0.061 (0.015 – 0.106), p=0.02, p value for interaction=0.005] **(Figure 3.A)**. These effects were also reflected for BMI **(Figure 3.B)**, eosinophil count **(Figure 3.C)**, and total IgE level (**Figure3.D**), but not for HbA1c, total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides and hsCRP levels. Pathway analysis showed that adjustment for BMI, eosinophil count but not total IgE level, attenuated the treatment effect on HOMA-IR among helminth-infected subjects (**Supplementary Table S3**).

When using PCR to detect STH infection, a significant increase of HOMA-IR was only observed among subjects who were infected with multiple helminth species at baseline **(Supplementary Figure S2.A)**. A significant increase of BMI was also observed in these subjects (**Supplementary Figure S2.B**).The group of subjects infected with multiple helminth species had a significantly higher infection intensity in comparison to the group of subjects infected with single helminth species (**Supplementary Table S4**).

Table 2. Effect of Albendazole Treatment on Primary and Secondary Outcomes at the Community Level

		Placebo		Albendazole		Treatment Effect (95%CI)
Outcome	**Parameter**	**Baseline**	**Follow-up**	**Baseline**	**Follow-up**	**p-value**
Insulin resistance	HOMA-IR	1.09 (1.02 – 1.15)	1.16 (1.09 – 1.24)	1.08 (1.01 – 1.14)	1.17 (1.13 – 1.25)	0.006 (-0.010 – 0.021)
		n=836	n=659	n=768	n=635	p=0.48
Glucose-related	Fasting blood glucose	5.49 (1.61)	5.47 (1.09)	5.45 (1.58)	5.52 (1.49)	0.018 (-0.105 – 0.142)
	(mmol)	n=836	n=650	n=768	n=634	p=0.77
	Fasting insulin	3.5 (3.2 – 3.7)	3.8 (3.5 – 4.1)	3.5 (3.3 – 3.8)	3.9 (3.6 -4.2)	0.006 (-0.032 – 0.043)
	(mU/L)	n=836	n=646	n=768	n=628	p=0.77
	HbA1c	32.5 (9.0)	32.7 (7.4)	32.3 (8.5)	32.7 (8.4)	0.051 (-0.350 – 0.452)
	(mmol/mol)	n=715	n=564	n=683	n=556	p=0.80
Adiposity-related	Body mass index	22.4 (4.2)	22.8 (4.2)	22.4 (4.0)	22.9 (4.1)	0.104 (-0.011 – 0.220)
	(kg/m^2)	n=860	n=690	n=790	n=659	p=0.08
	Waist circumference	76.5 (12.2)	77.2 (11.5)	76.8 (12.1)	77.4 (11.1)	-0.229 (-0.855 – 0.397)
	(cm)	n=865	n=692	n=793	n=657	p=0.47
Lipid-related	Total cholesterol	4.9 (1.0)	5.0 (1.1)	4.9 (1.1)	5.0 (1.1)	-0.031 (-0.098 – 0.035)
	(mmol/L)	n=836	n=659	n=764	n=632	p=0.35
	HDL –C	1.2 (0.3)	1.3 (0.3)	1.2 (0.3)	1.3 (0.4)	-0.008 (-0.031 – 0.016)
	(mmol/L)	n=836	n=659	n=764	n=632	p=0.52
	LDL-C	3.0 (0.9)	3.1 (0.9)	3.0 (0.9)	3.1 (1.0)	-0.032 (-0.089 – 0.024)
	(mmol/L)	n=836	n=658	n=763	n=631	p=0.26
	Triglycerides	1.4 (0.7)	1.5 (0.7)	1.5 (0.7)	1.5 (0.7)	-0.003 (-0.023 – 0.090)
	(mmol/L)	n=836	n=659	n=764	n=632	p=0.25
Immune-related	Total IgE	557.2 (498.1 – 623.3)	441.8 (386.7 – 504.7)	601.6 (534.8 – 676.7)	399.0 (347.9 – 457.7)	-0.066 (-0.094 – -0.037)
	(IU/mL)	n=835	n=651	n=766	n=628	p<0.0001
	Eosinophil count	5.9 (5.6 – 6.1)	5.9 (5.6 – 6.1)	6.1 (5.8 – 6.4)	5.2 (5.0 -5.5)	-0.057 (-0.086 – -0.028)
	(%)	n=829	n=641	n=763	n=619	p=0.0001
	hs-CRP	1.26 (1.16 – 1.36)	1.30 (1.19 – 1.42)	1.26 (1.16 – 1.37)	1.34 (1.23 – 1.46)	0.010 (-0.017 – -0.038)
	(mg/L)	n=836	n=659	n=764	n=632	p=0.46

The estimated treatment effect after 12-months of follow-up for HOMA-IR and other glucose-related parameters, adiposity, lipid, and immunological parameters at community level is displayed with corresponding 95% CI. The estimated treatment effects were obtained by mixed models and p-values are indicated. HOMA-IR, fasting insulin, total IgE, eosinophil count and hs-CRP were log transformed.

Abbreviations: CI, confidence interval; HbA1c, hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment for insulin resistance; hs-CRP, high sensitivity C-Reactive Protein. IgE, Immunoglobulin E; LDL-C, low-density lipoprotein cholesterol

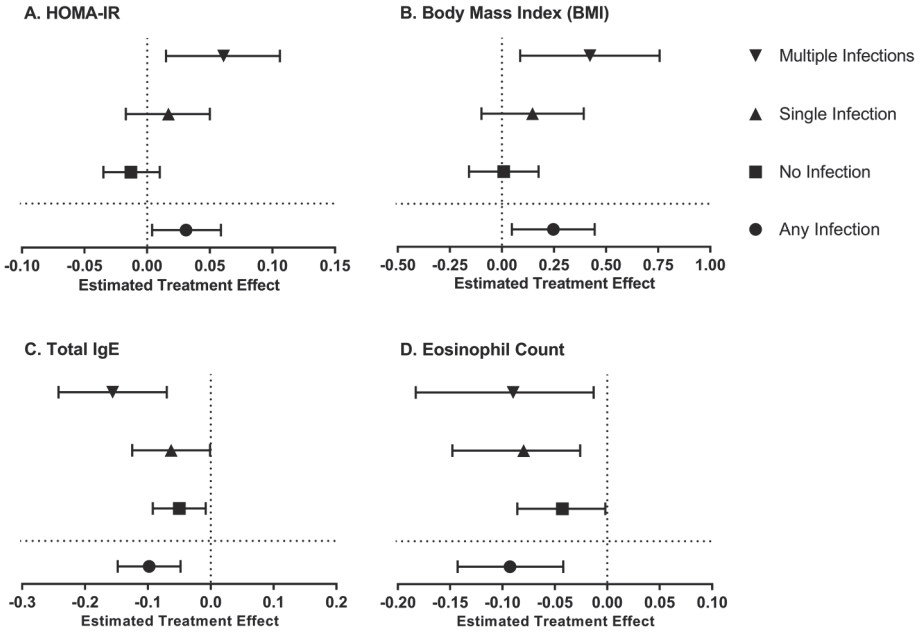

Figure 3. Effect of albendazole treatment on homeostatic model assessment for insulin resistance (HOMA-IR), body mass index (BMI), total immunoglobulin E (IgE), and eosinophil count, stratified by the number of helminth species carried by infected subjects at baseline as detected by microscopy. Effect of albendazole treatment on HOMA-IR (n = 1211; **A**), BMI (n = 1233; **B**), total IgE (n = 1209; **C**), and eosinophil count (n = 1200; **D**), stratified by the number of helminth species infecting subjects at baseline, as detected by microscopy. The estimated treatment effects are displayed with the corresponding 95% confidence interval. Circle, infected with at least 1 helminth species; square, no infection; triangle, infected with 1 helminth species; inverse triangle, infected with >1 helminth species.

Adverse Events

Adverse events were reported in 3.9% (31/797) and 2.6% (23/872) of subjects in the albendazole and the placebo group respectively. Abdominal pain was the most commonly reported complaint [35% (11/31) vs 13% (3/23) in the albendazole and the placebo group respectively]. Other commonly reported complaints were diarrhea and nausea, which were similar in both treatment arms.

DISCUSSION

Here, we report the first cluster-randomized trial in humans investigating the causal relationship between helminth infections and whole-body IR in an area endemic for STHs. We found that after 12 months of follow-up, 4 rounds of anthelmintic treatment with 3-month intervals did not lead to an increase in IR or other parameters such as

BMI, waist circumference, fasting blood glucose, HbA1c, serum lipid levels and hs-CRP at the community level, when all participants irrespective of their helminth status were included in the analysis. This despite the fact that the prevalence and infection intensity of STHs, as well as its associated type 2 immune responses, measured by total IgE and eosinophil count, were significantly reduced in albendazole-treated subjects.

When considering helminth-infected subjects, we observed that albendazole treatment resulted in a significant increase of IR among helminth-infected subjects when infection was detected by microscopy. Moreover, the effect of treatment on IR was stronger in those infected with multiple STH species at baseline compared to those with a single STH infection. We observed a similar pattern of the treatment effect on BMI. Even though significant, it is important to note that the magnitude of the effect of 1 year deworming on IR was modest. The effect of deworming in increasing IR seemed to be partly mediated through an increase in BMI, as adjustment for BMI, a strong predictor of IR,[28] attenuated the treatment effect on IR. Similar to BMI, eosinophil count and total IgE were significantly decreased in helminth infected subjects and this was stronger in those with multiple helminth infections. The possible importance of eosinophils in IR, shown in animal models [13, 14, 16, 17] and in 1 epidemiological study,[29] is also seen in our study as correction for eosinophil count reduced the treatment effect on IR.

When infection was assessed by PCR, which in comparison to the Kato Katz method, has a better ability to detect low intensity infections that may be clinically less relevant,[25] we only observed a significant increase in IR in the group of subjects who were infected with multiple helminth species at baseline. The infection intensity (DNA load) in this group of subjects was significantly higher than in those infected with a single STH species. Albendazole treatment led to a strong reduction of infection intensity in those infected with multiple STH species, which might explain the significant increase in IR following albendazole treatment.

The observed modest increase of IR after treatment among helminth-infected subjects, as detected by microscopy, could also contribute to the lack of a significant effect of albendazole treatment on IR at the community level. Two recent meta-analysis on deworming in children support this notion as they show that whereas a mass deworming approach, thus irrespective of helminth infection status, resulted in no change in weight gain, targeted anthelmintic treatment of infected children resulted in a significant weight gain.[30, 31]

However, several other explanations for the absence of a treatment effect on IR at the community level need to be considered. Although our study design was successful in lowering STH infection prevalence and its associated Th2 responses, it is possible that longer treatment and follow up would show stronger effects. It is also possible that both immune and non-immune-related effects of helminths on IR are not only associated with current helminth infections [8] but also with exposure to helminth infections in the past and therefore sustained. [13, 19] The causal relationship between helminth infection and IR, as found in the subgroup of infected subjects, might have a relatively small contribution to the multifactorial pathogenesis of IR.[28] Therefore, longer follow-up studies involving assessment of other more established factors, such as diet and physical activity,[28] will be needed to investigate this.

The use of PCR in our study, in addition to microscopy for detection of helminths, has helped us realize that the burden of infections, in terms of the number of helminth species as well as the infection intensity (DNA load), might influence the effect of anthelmintic treatment on IR. Deworming in subjects with increasing burden of infections resulted in an increasing change in IR. In addition, treatment of uninfected subjects, as assessed by either microscopy or PCR, did not influence IR, which suggests that an undetectable or a low level of helminth infection might be irrelevant for IR. The question whether a high burden of helminth infection causes different modulating effects on the immune system or energy balance, remains to be answered.

In conclusion, intensive anthelmintic treatment in an STH endemic area significantly reduces both the STH infection prevalence and intensity, as well as its related type 2 responses. This treatment does not lead to an increase of whole-body IR at the community level, but it does increase IR among those with a microscopy-detected STH infection. Studies are needed to determine the long term metabolic consequences of anthelmintic treatment in communities where STH are highly prevalent. However, in terms of policy, countries implementing helminth control programmes need to be aware that this may exacerbate or accelerate the deterioration in metabolic health, and that education and prevention strategies for non-communicable diseases such as DM2 need to go hand in hand with infectious disease control measures.

Supplementary Data

Supplementary materials are available at Clinical Infectious Diseases online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. DLT is a medical doctor in charge of the field study, involved in setting up the study, supervising gathering of data, treatment, clinical care, follow up of the study population, analyze the data and wrote the manuscript. KR is a medical biologist in charge of the field study, involved in setting up the laboratory in the study area, performing the immunological analysis, supervising the data cleaning, follow up of the study population and involved in the writing of the manuscript. IM is a mathematician who is developing methods to analyze the complex data generated during the lifetime of the project and involved in the randomization and data analysis. LvL is a parasitologist who is involved in the performance and analysis of diagnostic assays for the detection of helminths in stool samples. EATB is a technician who develop, optimized and performed multiplex real time PCR for detection of helminth infection. CMC is a clinical chemist who advised on the type, quality and metrological traceability of medical tests and who was responsible for the measurements of the metabolic parameters at the Department of Clinical Chemistry and Laboratory Medicine at LUMC. PS is an endocrinologist who advised on the metabolic aspects of the study. YD is a medical doctor who is involved in coordinating the study and advises on the immunological and parasitological aspects of the study. AEW is a medical doctor who is involved in clinical care and setting up the database. JJH is a biostatistician who developed the study, and is involved in supervising sample size calculation, randomization and statistical analysis. ES is an immunoparasitologist who is involved in coordinating the study and advising on parasitological and immunological aspects of the study and supervised the writing of the manuscript. JWAS is an endocrinologist who developed the study, supervised the writing of the manuscript, and is the Dutch coordinator of the SUGARSPIN program. MY is an immunologist who developed the study, supervised the writing of the manuscript and is the scientific coordinator of the SUGARSPIN program. TS is a parasitologist who developed the study and is the Indonesian coordinator of the SUGARSPIN program. All authors read and approved the final manuscript. The senior authors TS, MY and JWAS had final responsibility for the decision to submit for publication.

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SUPPLEMENTARY APPENDIX

Supplementary Methods

Study Procedures

All clinical measurements and blood sample collections were performed after an overnight fast. Anthropometric measurements of body weight (SECA Model 876, Seca Gmbh Co, Hamburg, Germany), height (SECA Model 213, Seca Gmbh Co, Hamburg, Germany), waist and hip circumference (SECA Model 201, Seca Gmbh Co, Hamburg, Germany) were obtained using the National Heart, Lung, and Blood Institute (NHLBI) practical guidelines by a team of trained researchers. BMI was calculated as weight in kg divided by square of height in meter, while Waist Hip Ratio (WHR) was calculated as waist circumference divided by hip circumference. Three blood pressure measurements (left arm, sitting upright position, after resting 5 minutes) were taken from each subject, using a digital sphygmomanometer (HEM-7200, Omron Healthcare Co, Ltd, Kyoto, Japan). The average of all three measurements was used for analysis.

Fasting blood glucose was measured in capillary blood using Breeze®2 glucose meters (Bayer Health Care LLC, Basel, Switzerland). All sera, plasma and whole blood samples were frozen at −20°C in the field study centre and subsequently stored at −80°C at the Department of Parasitology of FKUI and LUMC. Insulin, HbA1c, total cholesterol, high-density lipoprotein (HDL)-cholesterol, triglycerides and high-sensitive C-reactive protein (hsCRP) were measured pairwise (baseline and follow-up) in the same analytical runs at the Department of Clinical Chemistry and Laboratory Medicine at LUMC, which is ISO 15189:2012 accredited. Accuracy of test results is periodically and independently verified in EQA-schemes organized by the Dutch EQA-organization, the SKML.

Serum insulin concentrations were determined by a solid-phase, enzyme-labeled chemiluminescent immunometric assay (Siemens IMMULITE 2000XPi). The measuring range of the insulin assay was 2–300 mU/L (CV_a <7% at all levels). IR was assessed by HOMA-IR, a well-validated measure of whole body IR in humans (HOMA-IR = fasting serum insulin x fasting glucose / 22·5).[26] HbA1c was measured using a cation-exchange chromatography (IC)-based high performance liquid chromatogtaphy (HPLC) assay (Tosoh G8 HPLC Analyzer, from Tosoh Corporation, Tokyo, Japan and distributed through Sysmex in the Netherlands), with a measuring range of 20–125 mmol/mol Hb (CV_a<5% at all levels). Test results are in accordance to the IFCC Reference Measurement System for HbA1c. The

non-porous ion exchange HPLC-column and four-steps buffer gradient enables a clear separation of HbA1c from other fractions and haemoglobin variants such as HbC, HbD and HbS. As these variants can be detected, the software automatically corrects the HbA1c results for these variants. However, other variants which may typically be present in non-Caucasian populations, are not detected and lead to inaccurate HbA1c results. Therefore, all chromatograms were visually inspected by experienced technicians in order to detect the unidentified Hb-variants. HbA1c results from unidentified Hb-variants were excluded as these test results were inaccurate.

Total cholesterol, HDL-cholesterol and triglycerides assays were based on enzymatic colorimetric methods (Modular P analyzers, Roche Diagnostics, Mannheim, Germany). The measuring range of total cholesterol, HDL-cholesterol and triglycerides were 0·08–20·7 mmol/L (CV_a<2%), 0·08–3·10 mmol/L (CV_a<2%) and 0·05–11·4 mmol/L (CV_a<5%), respectively. Lipid test results are standardized to internationally recognized CDC Reference Measurement Systems. Low-density lipoprotein (LDL)-cholesterol (in mmol/L) was calculated using The Friedewald formula, [LDL-chol, mmol/L] = [Total chol] - [HDL-chol] - (0·456 X [TG]). A latex-enhanced immunoturbidimetric method was used to measure hsCRP on Roche Modular P-instrumentation, the measuring range being 0·1–20·0 mg/L. HsCRP test results are ERM-DA470k/IFCC standardized.

A Giemsa-stained peripheral thin blood smear was read at the Department of Clinical Pathology, FKUI to assess differential white blood cell count, resulting in a relative percentage of basophils, eosinophils, neutrophils, lymphocytes and monocytes. Total IgE was measured at the Department of Parasitology, LUMC using ELISA with rabbit anti-human IgE antibody (Ab) (Dako, Glostrup, Denmark) as capture Ab and goat anti-human IgE biotinylated Ab (Vector Laboratories, Burlingname, CA, USA) as detection Ab.[24]

Fresh stool samples were examined by the Kato Katz method to identify and quantify STH (hookworm, *Ascaris lumbricoides* and *Trichuris trichiura*) eggs using 2 slides for each sample. Aliquots of fresh stool samples were frozen at –20°C in the field study centre and subsequently at –80°C at the Department of Parasitology of FKUI and LUMC for DNA extraction. Stool DNA isolation and real-time PCR were performed pairwise (baseline and follow-up). DNA isolation from stool was performed as described elsewhere,[24] with an additional step of bead beating (1800 rpm for 3 minutes) inside the Fastprep-96™ system to optimize DNA

extraction. Multiplex real-time PCR was performed to simultaneously detect the presence of hookworm (*Ancylostoma duodenale, Necator americanus*), *Ascaris lumbricoides, Trichuris trichiura* and *Strongyloides stercoralis* using a method described previously[24] with some modifications, adding the *T. trichiura* primers and probe, and combining the fluorescence of *A. duodenale* and *N. americanus* as one reading of hookworm. Stool samples were considered positive by PCR when cycle threshold (Ct) values were below 50. Positive Ct values were further grouped into 3 categories: $Ct<30$, $30\leq Ct<35$ and $Ct\geq 35$ representing a high, moderate and low DNA load, respectively.[25] An additional grouping was made based on the number of different STH species that subjects were infected with: no infection, single infection (one of the four STHs: hookworm, *A.lumbricoides*, *T.trichiura, S.stercoralis*) and multiple infections (more than one of these STHs).

Supplementary Figures

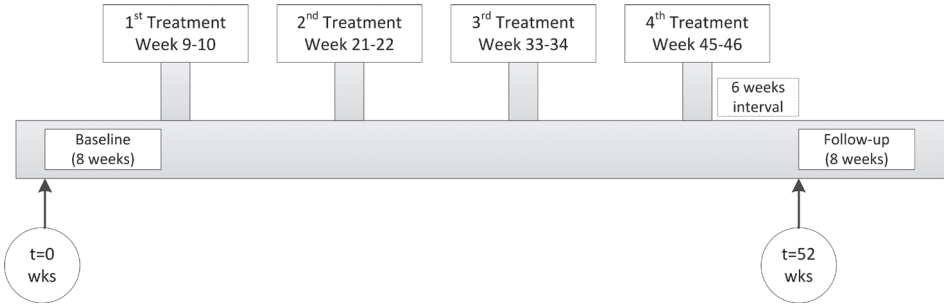

Figure S1. Trial time frame. Baseline data (t=0) were collected during the first 8 weeks before the start of the drug administration. Treatment regimen was given 4 times with three months time intervals (week 9-10, 21-22, 33-34, and 45-46). Follow-up data (t=52 weeks) were collected 6 weeks after the last treatment round.

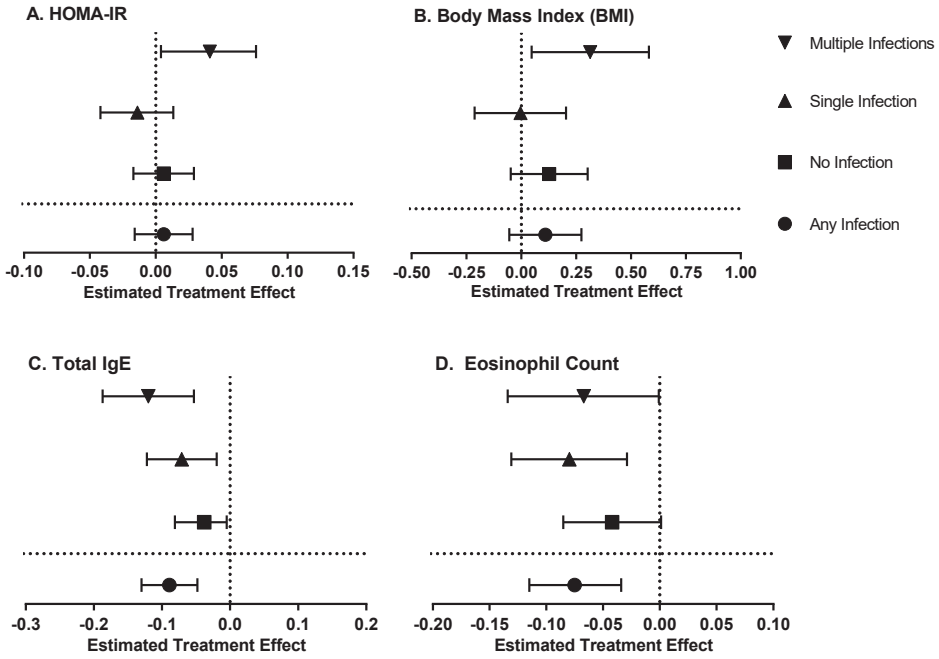

Figure S2. Effect of albendazole treatment on HOMA-IR, BMI, Total IgE and Eosinophil count stratified by the number of helminth species carried by infected subjects at baseline as detected by PCR. Effect of albendazole treatment on (A) HOMA-IR (n= 1433), (B) BMI (n= 1464), (C) Total IgE (n=1430), and Eosinophil count (n=1422) stratified by the number of helminth species infecting subjects at baseline (t=0), as detected by PCR. The estimated treatment effects are displayed with corresponding 95% confidence interval. Circle= infected with at least one helminth species, square= no infection, triangle= infected with one helminth species, inverse triangle= infected with more than one helminth species. Abbreviations: HOMA-IR=Homeostatic Model Assessment for Insulin Resistance, BMI=Body Mass Index, IgE= Immunoglobulin E.

Supplementary Tables

Table S1. Summary of subjects lost to follow-up

	Died		Moved		Refused		Other Reasons		Total Lost to Follow Up	
	Pla	Alb	Pla	Alb	Pla	Alb	Pla	Alb	Pla	Alb
Subjects (n, %)*	13 (1.5)	11 (1.4)	101 (11.5)	75 (9.4)	23 (2.6)	16 (2.0)	42 (4.8)	35 (4.4)	179 (20.5)	137 (17.2)
Age (mean, SD)	56.8 (10.1)	63.1 (10.7)	29.7 (14.1)	29.5 (14.0)	36.6 (13.9)	39.9 (13.8)	42.4 (17.2)	39.7 (41.1)	35.5 (16.5)	36.0 (17.1)
Sex (male) (n/N, %)	10/13 (77)	6/11 (55)	40/101 (40)	34/75 (45)	14/23 (61)	11/16 (69)	22/42 (52)	19/35 (54)	86/179 (48.0)	70/137 (51.1)

*% was calculated from all subjects in each treatment arm (placebo n=872, albendazole n=797
Abbreviations: Pla, Placebo; Alb, Albendazole

Table S2. Effect of albendazole treatment on infection intensity detected by PCR

		Baseline (t=0)		Follow-Up (t=52 weeks)	
Type of STH	Infection Intensity*	Placebo n=594	Albendazole n=550	Placebo n=594	Albendazole n=550
Hookworm n (%)	Negative	396 (66.7)	385 (70.0)	420 (70.7)	527 (95.8)
	Low	98 (16.5)	88 (16)	92 (15.5)	18 (3.3)
	Moderate	67 (11.3)	58 (10.5)	62 (10.4)	5 (0.9)
	High	33 (5.5)	19 (3.5)	20 (3.4)	0 (0.0)
A. lumbricoides n (%)	Negative	484 (81.5)	462 (84.0)	517 (87.0)	543 (98.7)
	Low	26 (4.4)	14 (2.5)	19 (3.2)	1 (0.2)
	Moderate	47 (7.9)	43 (7.8)	32 (5.4)	4 (0.7)
	High	37 (6.2)	31 (5.6)	26 (4.4)	2 (0.4)
T. trichiura n (%)	Negative	436 (73.4)	400 (72.7)	460 (77.4)	505 (91.8)
	Low	45 (7.6)	49 (8.9)	40 (6.7)	23 (4.2)
	Moderate	80 (13.5)	71 (12.9)	63 (10.6)	19 (3.5)
	High	33 (5.5)	30 (5.5)	31 (5.2)	3 (0.5)
S. stercoralis n (%)	Negative	588 (99.0)	547 (99.5)	589 (99.1)	550 (100.0)
	Low	3 (0.5)	1 (0.2)	3 (0.5)	0 (0.0)
	Moderate	2 (0.3)	1 (0.2)	1 (0.2)	0 (0.0)
	High	1 (0.2)	1 (0.2)	1 (0.2)	0 (0.0)

*Ct value of 50 indicates no infection (negative). Ct values <50 were further grouped into 3 categories: Ct<30, 30≤Ct<35 and Ct≥35 representing a high, moderate and low DNA load, respectively. Abbreviations: Ct=Cycle threshold

Table S3. Pathway analysis of the treatment effect on insulin resistance in helminth-infected subjects as detected by microscopy

Model	Estimated Treatment Effect (95%CI), p value
Unadjusted	0.031 (0.004 – 0.059), p=0.04
Adjusted for BMI changes	0.025 (-0.001 – 0.051), p=0.10
Adjusted for total IgE changes	0.030 (0.002 – 0.057), p=0.06
Adjusted for eosinophil count changes	0.026 (-0.002 – 0.053), p=0.13
Adjusted for BMI and eosinophil changes	0.020 (-0.007 – 0.046), p=0.23

Abbreviations: BMI=Body Mass Index, IgE= Immunoglobulin E.

Table S4.A. Comparison of baseline infection intensity in subjects infected with single and multiple helminth species as detected by PCR

Helminth species	Single (n=491) [Ct value, mean (SD)]	Multiple (n=296) [Ct value, mean (SD)]	Mean differences (95%CI), p value*
Hookworm	35.9 (4.0), n=259	34.3 (4.4), n=226	-1.6 (-2.3 – -0.8), p<0.0001
A.lumbricoides	32.9 (3.2), n=75	31.0 (3.7), n=179	-1.9 (-2.9 – -0.9), p<0.0001
T.trichiura	33.7 (2.9), n=156	32.3 (3.3) n=247	-1.4 (-2.0 – -0.8), p<0.0001

This analysis was conducted in subjects analyzed in figure S1. Infection intensity was assessed using Ct values of PCR, which represent the amount of DNA in the stool sample. The lower the Ct value, the higher the amount of DNA. Number of subjects with *S.stercoralis* was very low (one within the single infection group and nine within the multiple infection group). *Independent t-test.

Table S4.B. Comparison of baseline infection intensity in subjects infected with single and multiple helminth species as detected by microscopy

Helminth species	Single (n=334) [epg, median (IQR), n]	Multiple (n=180) [epg, median (IQR), n]	p value*
Hookworm	96 (48 – 267), n=108	138 (72 – 474), n=108	0.087
A·lumbricoides	360 (72 – 1596), n=103	1980 (432 – 6264), n=143	<0.001
T·trichiura	36 (12 – 120), n=123	84 (36 – 261), n=152	<0.001

This analysis was conducted in subjects analyzed in figure 3. Infection intensity was assessed using the number of egg per gram of stool sample (epg) from Kato Katz method. *Independent-samples Mann Whitney U Test.

Chapter 4

EFFECT OF ANTHELMINTIC TREATMENT ON LEPTIN, ADIPONECTIN, AND LEPTIN TO ADIPONECTIN RATIO: A Randomized Controlled Trial

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(Manuscript in submission)

ABSTRACT

Aims/hypothesis: We aimed to assess the role of adipokines in mediating the effect of helminths on insulin resistance. We hypothesized that the increase in IR after anthelmintic treatment in helminth-infected subjects is mediated by a shift in leptin to adiponectin ratio driving a more pro-inflammatory state.

Methods: Serum samples were obtained from a randomized-controlled trial of anthelmintic treatment in an area endemic for soil-transmitted helminths (STH), Flores Island, Indonesia. All subjects in the study area received albendazole or matching placebo for three consecutive days. This three-monthly treatment regimen was given for four rounds. We measured leptin, adiponectin, and resistin changes in those >16 years old. STH infections were assessed by microscopy and PCR.

Results: In STH-infected subjects, anthelmintic treatment significantly increased the ratio of leptin to adiponectin [treatment effect factor (95% CI), p-value for interaction: 1.20 (1.06 – 1.35), p=0.010], which largely stemmed from a significant reduction in adiponectin [0.91 (0.85 – 0.98), p=0.020] and a trend for an increase in leptin level [1.10 (1.00 – 1.21)], p=0.119]. No significant effect on resistin level was observed. This increase in leptin to adiponectin ratio seemed to contribute to the observed effect of deworming on increased IR as adjustment for leptin to adiponectin ratio attenuated the effect on IR from 1.07 (1.01 – 1.14, p=0.023) to 1.05 (0.99 – 1.11, p=0.075).

Conclusions: Anthelmintic treatment in STH-infected subjects increases leptin to adiponectin ratio which may in small part contribute to the modest increase in IR. Further studies will be needed to assess the effect of the changes in adipokine levels on the host immune response and metabolism.

Keywords: Helminths. Adipokines. Leptin. Adiponectin. Leptin to adiponectin ratio. Insulin resistance

Abbreviations: AT: adipose tissue, L/A: leptin to adiponectin, STH: soil-transmitted helminth, T2D: type 2 diabetes.

INTRODUCTION

Emerging evidence suggests that helminths might confer protection against the development of type 2 diabetes (T2D),[1-5] presumably by modulating the host immune responses.[6-8] Thus, in addition to the more established risk factors, such as sedentary lifestyle and high-energy foods, current deworming programs in parallel with rapid socioeconomic development might potentially contribute to the development of T2D in many low and middle-income countries.[6] In line with this, we have recently reported that removal of helminth infections increases insulin resistance (IR),[9] which is mainly mediated by the increase in adiposity,[9] suggesting a central role of adipose tissue (AT).[10-13]

Human AT secretes various adipokines, most notably leptin and adiponectin, affecting metabolic homeostasis and immune regulation.[14] Leptin and adiponectin have been consistently shown to be positively and negatively associated with IR, respectively.[14] Whereas leptin promotes pro-inflammatory immune responses and inhibits the proliferation of regulatory T-cells, adiponectin induces the secretion of anti-inflammatory cytokines.[15] The imbalance between those two adipokines, leptin to adiponectin (L/A) ratio, has been reported to be associated with pro-inflammatory conditions and IR.[16, 17]

Assessment of adipokines might provide a valuable insight into the role of human AT in mediating the helminths effect on metabolic homeostasis. To our knowledge, no studies have been published so far on the association between helminth infections and adipokines, except for resistin.[18] Therefore, we measured leptin, adiponectin, and resistin in serum samples obtained from a randomized-controlled trial of anthelmintic treatment in an area endemic for soil-transmitted helminth (STH).[19] We hypothesized that the increase in IR after anthelmintic treatment in helminth-infected subjects might be mediated by a shift in L/A ratio towards a more pro-inflammatory state.

METHODS

This present study is part of a household-based cluster-randomized double-blind placebo-controlled anthelmintic trial (The Sugarspin study), conducted in Nangapanda, Flores, an endemic area for soil-transmitted helminth (STH).[19] The primary outcome of the Sugarspin study is changes in insulin resistance (IR), as assessed using the homeostatic model assessment of IR (HOMA-IR), after anthelmintic treatment, which has been published recently.[9] Written informed

consent was obtained from all participants. The study was approved by the ethics committee of Faculty of Medicine, Universitas Indonesia (FKUI) (ref: 549/H2·F1/ETIK/2013), and filed by the ethics committee of Leiden University Medical Center (LUMC). The trial is registered as a clinical trial (http://www.isrctn.com/ISRCTN75636394).

The population was randomised by blocks at household level. After randomisation, all subjects in the study area, except children <2 years old and pregnant women, received a single tablet of albendazole (400mg) or matching placebo for three consecutive days with direct supervision. This three-monthly treatment regimen was given for four rounds. All measurements and sample collections were performed at baseline and 6 weeks after the end of the fourth treatment round (follow-up).[19] All subjects without sufficient sera samples, and/or incomplete data on body mass index, and soil-transmitted helminth (STH) infection status at baseline were excluded from the present study. Subjects receiving active treatment for diabetes were also excluded from analysis.

All subjects ≥16 years old were invited to undergo clinical measurements and blood drawing after an overnight fast.[19] Body weight and height were measured, and body mass index (BMI) was calculated as weight (kg) divided by square of height (m). Adipokines (leptin, adiponectin and resistin) were measured by ELISA using commercial reagents (DuoSet ELISA R&D System Europe Ltd, Abingdon, UK), according to the manufacturer's protocol. Leptin to adiponectin (L/A) ratio was calculated by L/A= leptin level (ng/ml) / adiponectin level (ug/ml).[17] Soil-transmitted helminth infection status was assessed using both microscopy (Kato Katz) and PCR, which was further stratified by the number of species a subject was infected with at baseline (no infection, single infection, multiple infection).[9]

Statistical Analysis

Leptin, adiponectin, L/A ratio, and resistin were log-transformed (log10) for analysis and summarized as geometric mean [95% confidence interval (CI)]. The effect of anthelmintic treatment on adipokine was assessed using mixed models to account for the correlation within households, as described previously.[9] The treatment effect estimates were the regression coefficient obtained from mixed models (β) indicating changes in log10 (leptin, adiponectin, L/A ratio, resistin) of subjects using albendazole compared to placebo. The treatment effect factors (10^{β}) are multiplicative instead of additive. Thus treatment effect factors indicate the proportional change for each

variable (leptin, adiponectin, resistin, L/A ratio), in comparison to the placebo. All models were fitted using the lme4 package (R software).

RESULTS

At baseline, the prevalence of STH infection was 42.0% (503/1195) and 54.1% (760/1405), as assessed by microscopy and PCR respectively. Serum leptin, adiponectin, L/A ratio, and resistin levels were similar in both treatment arms (**Table 1**). The consort diagram of the present study is shown in **figure S1**.

Similar to the main study, anthelmintic treatment significantly reduced the prevalence of STH infections, as assessed by microscopy or PCR (**Table S1**). In comparison to placebo, albendazole treatment had no effect on adipokine levels in subjects without STH infections (**Figure 1A**). In STH-infected subjects, as assessed by microscopy, albendazole treatment increased L/A ratio [treatment effect factor (95% CI), p-value for interaction: 1.20 (1.06 – 1.35), p=0.010], which was mostly derived from a significant reduction in adiponectin level [0.91 (0.85 – 0.98), p=0.020] and a trend for an increase in leptin level [1.10 (1.00 – 1.21)], p=0.119] (**Figure 1B**). No significant treatment effect on resistin level was observed [1.00 (0.94 – 1.05), p=0.363] (**Figure 1B**).

Table 1. Study Population

	Placebo N=807	Albendazole N=750
Age (in years, mean, SD)	41.9 (15.4)	42.6 (15.5)
Sex (female %, n/N)	62.0 (500/807)	59.9 (449/750)
Body Mass Index (kg/m^2, mean, SD)	22.5 (4.0)	22.5 (4.0)
Leptin to adiponectin ratio [geomean (95% CI)]	1.38 (1.25 – 1.53)	1.35 (1.21 – 1.51)
Leptin (ng/ml) [geomean (95% CI)]	7.1 (6.5 – 7.7)	6.7 (6.1 – 7.4)
Adiponectin (ug/ml) [geomean (95% CI)]	5.1 (4.9 – 5.4)	5.0 (4.7 – 5.3)
Resistin (ng/ml) [geomean (95% CI)]	15.6 (15.0 – 16.2)	15.7 (15.1 – 16.4)
Helminth-infected by microscopy (%, n/N)	43.5 (270/620)	40.5 (233/575)
- Single species	28.2 (175/620)	26.4 (152/575)
- Multiple species	15.3 (95/620)	14.1 (81/575)
Helminth-infected by PCR (%, n/N)	53.8 (392/729)	54.4 (368/676)
- Single species	31.7 (231/729)	35.2 (238/676)
- Multiple species	22.1 (161/729)	19.2 (130/676)

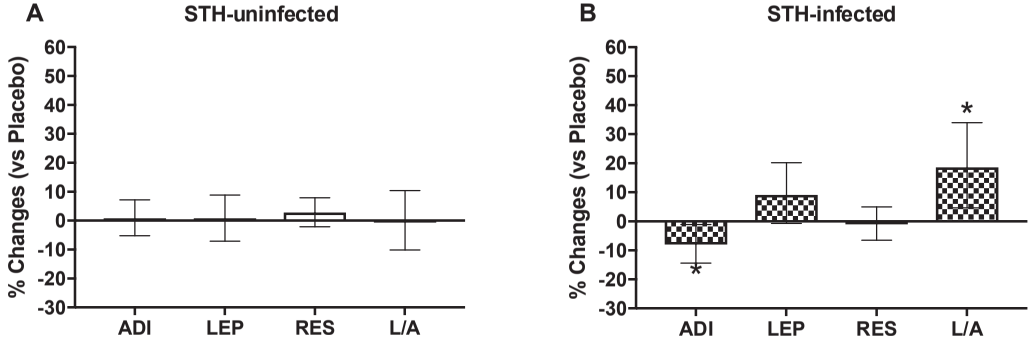

Figure 1. Effect of anthelmintic treatment on adiponectin, leptin, resistin, and leptin to adiponectin ratio in soil-transmitted helminth (STH)-infected and uninfected subjects. The effect of anthelmintic treatment on adiponectin (ADI), leptin (LEP), resistin (RES), and leptin to adiponectin ratio (L/A) in (**A**) STH-uninfected and (**B**) STH-infected subjects, as assessed by microscopy, are presented as proportion of changes (95% CI) between pre and post treatment in the albendazole group compared to the placebo group which is set to zero. Adiponectin, leptin, resistin, and L/A ratio were log-transformed for analysis. Analysis was performed on 1183 subjects, after excluding 12 subjects with diabetes. Treatment effect estimates were the regression coefficient (β) obtained from mixed models indicating changes in log (ADI or LEP or RES or L/A); the treatment effect factors (10β) are proportional instead of additive. Thus, treatment effect factors indicate the proportional change in each variable in comparison to the placebo group. *p<0.05

Pathway analysis showed that adjustment for changes in BMI partly attenuated the treatment effect on adiponectin level [to 0.92 (0.86 – 0.99), p=0.030] and L/A ratio [to 1.13 (1.02 – 1.26), p=0.040]. Analysis of the Sugarspin trial primary outcome revealed that the increased IR after anthelmintic treatment in infected subjects might be due to an increased BMI and a reduced eosinophil counts.[9] Therefore we also assessed whether the increase in L/A ratio contributes to the increased IR after treatment in helminth-infected subjects. This analysis showed that adjustment for changes in L/A ratio, attenuated the treatment effect on IR from 1.07 (1.01 – 1.14, p=0.023) to 1.05 (0.99 – 1.11, p=0.075), even more than adjustment for changes in BMI [to 1.06 (1.00 – 1.12), p=0.048].

When light infections were also considered by using PCR, albendazole treatment did not significantly increase L/A ratio [1.10 (1.00 – 1.22), p=0.31], despite a significantly reduced adiponectin level [0.94 (0.88 – 0.99), p=0.048]. No significant treatment effect was observed on the level of leptin, nor resistin (**Figure S2**). Next, we further stratified STH-infected subjects based on the number of STH species a subject was infected with at baseline. In subjects with multiple STH infections, albendazole significantly increased L/A ratio [1.25 (1.06 – 1.48), p=0.041] which derived from a significant reduction in adiponectin level [0.88 (0.80 – 0.97), p=0.013] and a non-significant increase in leptin level [1.10 (0.97 – 1.25), p=0.47]. (**Figure S3**) Using microscopy, a more pronounced reduction in adiponectin [0.88 (0.78 – 0.99), p=0.049] was observed in subjects infected with multiple STH species. The treatment effect on L/A ratio [1.18 (0.96 – 1.45), p=0.157] and leptin level [1.04 (0.89 - 1.22), p=0.71] in subjects infected with multiple species did not reach statistical significance (**Figure S4**).

DISCUSSION

Our study is the first to report the effect of anthelmintic treatment on serum adipokine levels. In STH-infected subjects, treatment significantly increased L/A ratio, which has been reported to be associated with low-grade inflammation [16] and IR.[16, 17] The increased L/A ratio was derived by the significant reduction in adiponectin level, and to a lesser extent, a trend of increase in leptin level. As adiponectin induces the secretion of anti-inflammatory cytokines,[15] while leptin increases Th1, suppresses Th2, and can act as a negative signal for the proliferation of human T regulatory cells [20], these changes may reverse the helminth-associated type 2 and regulatory immune responses, and presumably contribute to the development of IR. Indeed, adjustment for the increase in L/A ratio attenuated

the treatment-associated increase in IR, observed in the main trial,[9] even more than adjustment for increase in BMI. This suggests that adipokines play a relatively more important role than the adiposity in the mediation of helminth-associated beneficial effect on IR.

Using PCR, a more sensitive method, able to detect non-clinically relevant STH infections, the treatment effects were less in magnitude, as it significantly reduced adiponectin level only, but to a lesser extent. In line with this, in subjects with multiple STH infections, associated with a higher infection intensity,[9] treatment resulted in more pronounced effects, namely a significant reduction in adiponectin level, a trend for increase in leptin level, as well as a significant increase in L/A ratio. Except for the effect on adiponectin, these pronounced treatment effects were not observed when infection was assessed by microscopy, which might be due to the lower number of subjects who were found to be infected with multiple species, when using microscopy.

Despite having an ideal study design to study the causal relationship between helminth infections and adipokine levels, and to assess the contribution of adipokine levels to the increased IR after anthelmintic treatment, our study would have been more complete if we would have assessed food intake, appetite, and physical activity. In addition, measurements of other hormones that influence metabolism, such as ghrelin and cortisol, as well as analysis of AT biopsies and gut microbiome, could provide a more complete overview on how helminths may modulate human metabolism.

In conclusion, anthelmintic treatment in STH-infected subjects increases L/A ratio which may in small part contribute to the increased IR. Further studies will be needed to assess the effect of these changes in adipokine levels on the host metabolism and modulation of the host immune responses.

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Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Duality of interests

All authors declare no competing interests. The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The senior authors TS, JWAS, MY had final responsibility for the decision to submit for publication.

Contribution statement

D.L.T. is a medical doctor in charge of the field study, involved in setting up the study, supervising gathering of data, treatment, clinical care, follow up of the study population, analyzed the data and wrote the manuscript. K.R. is a medical biologist in charge of the field study, involved in setting up the laboratory in the study area, performing the immunological analysis, supervising the data cleaning, and the follow up of the study population. I.M. is a mathematician who is developing methods to analyze the complex data generated during the lifetime of the project and was involved in the randomization and data analysis. L.v.L. is a parasitologist who was involved in the performance and analysis of diagnostic assays for the detection of helminths in stool samples. E.A.T.B. is a technician who develop, optimized and performed multiplex real time PCR for detection of helminth infections. P.S. is an endocrinologist who advised on the metabolic aspects of the study. Y.D. is a medical doctor who was involved in coordinating the field study and advised on the immunological and parasitological aspects of the study. C.C.D. is a nurse who was involved in the field study, especially gathering of data, supervising anthelmintic treatment, and follow up the study population. J.J.H. is a biostatistician who developed the study, and was involved in supervising sample size calculation, randomization and statistical analysis. E.S. is an immunoparasitologist who was involved in coordinating the study and advising on parasitological and immunological aspects of the study and supervised the writing of the manuscript. T.S. is a parasitologist who developed the study and is the Indonesian coordinator of the SUGARSPIN program. J.W.A.S. is an endocrinologist who developed the study, supervised the writing of the manuscript, and is the Dutch coordinator of the SUGARSPIN program. M.Y. is an immunologist who developed the study, supervised the writing of the manuscript and is the scientific coordinator of the SUGARSPIN program. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIALS

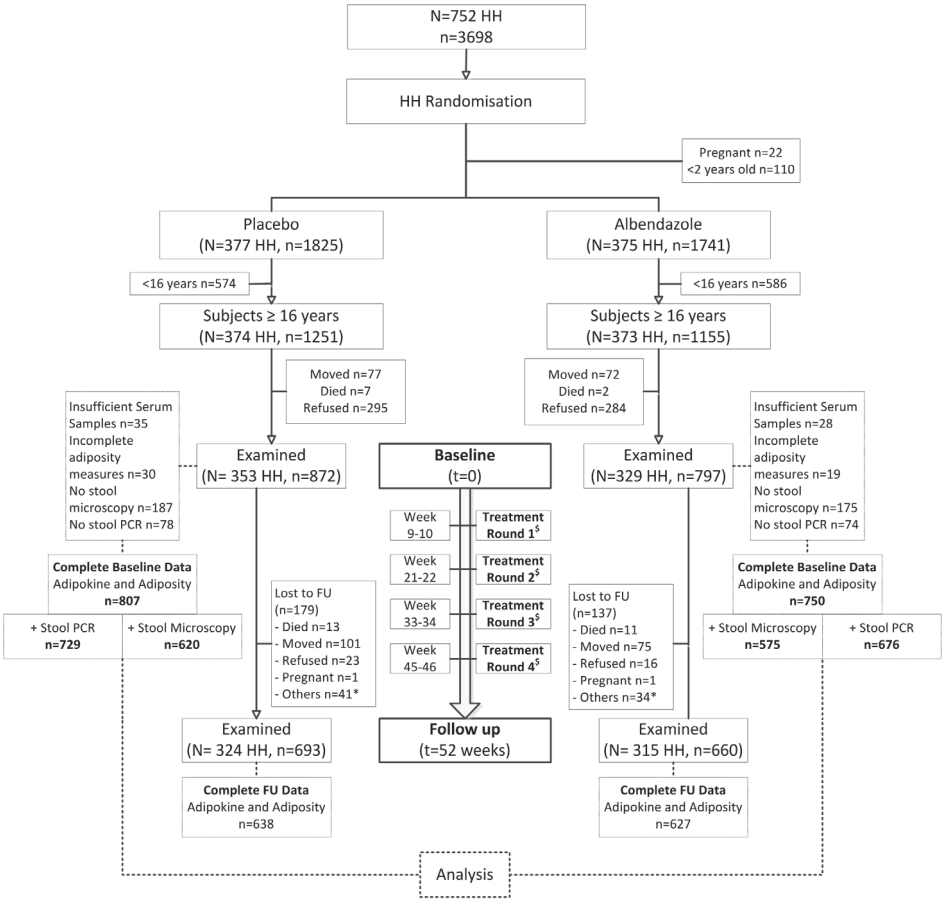


Figure S1. Consort Diagram. Baseline data (t=0) were collected during the first 8 weeks before the start of the drug administration. $Single dose of albendazole or matching placebo was given for three consecutive days to all household members, except children below 2 years of age and pregnant women. *Other reasons of lost to follow-up were harvesting crops, working on funeral ceremonies, severely ill, hospitalized, nursing mother. HH: Household, FU: Follow Up.

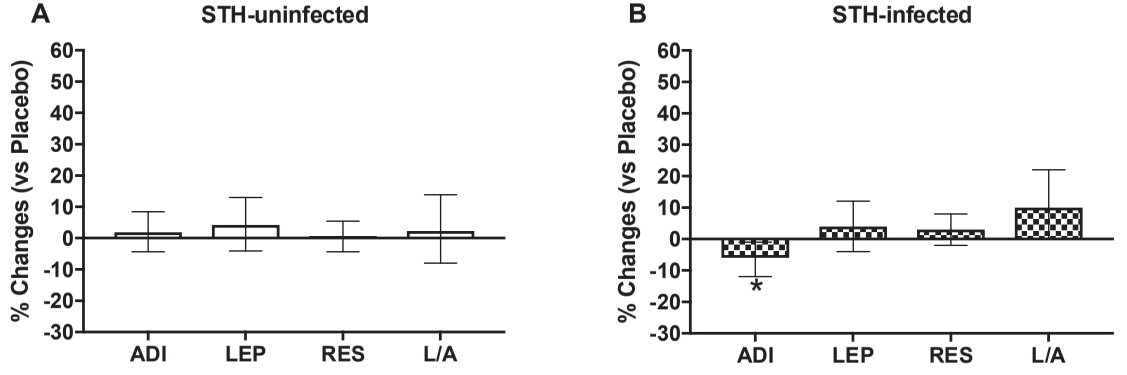


Figure S2. Effect of anthelmintic treatment on adiponectin, leptin, resistin, and leptin to adiponectin ratio in soil-transmitted helminth (STH)-infected and uninfected subjects, as assessed by PCR. The effects of anthelmintic treatment on adiponectin (ADI), leptin (LEP), resistin (RES), and leptin to adiponectin ratio (L/A) are presented as proportion of changes between pre and post treatment in the albendazole group compared to the placebo group which is set to zero. The effects of treatment are presented for each group of subjects: (**A**) STH-uninfected and (**B**) STH-infected subjects, as assessed by PCR. Adiponectin, leptin, resistin, and L/A ratio were log-transformed for analysis. Analysis was performed on 1387 subjects, after excluding 14 subjects with diabetes. Treatment effect estimates were the regression coefficient (β) obtained from mixed models indicating changes in log (ADI or LEP or RES or L/A); the treatment effect factors (10β) are proportional instead of additive. Thus, treatment effect factors indicate the proportional change in each variable in comparison to the placebo group. *p<0.05.

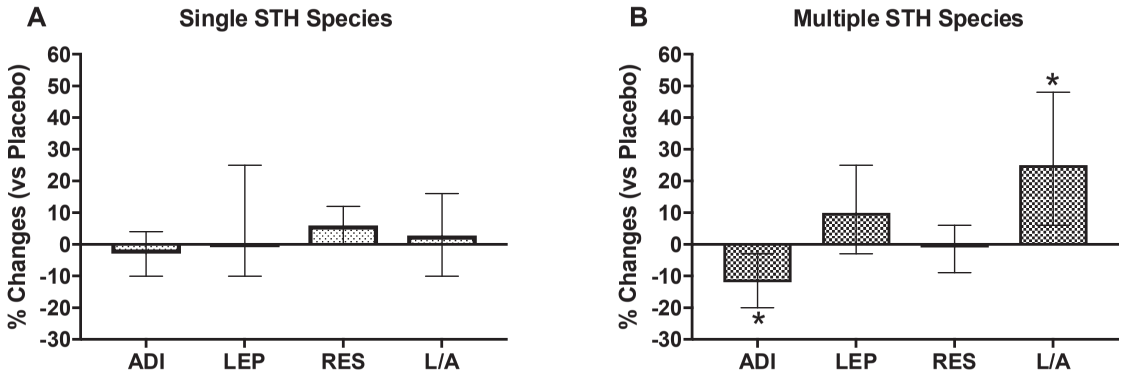


Figure S3. Effect of anthelmintic treatment on adiponectin, leptin, resistin, and leptin to adiponectin ratio stratified by number of helminth species a subject was infected with at baseline, as assessed by PCR. The effects of anthelmintic treatment on adiponectin (ADI), leptin (LEP), resistin (RES), and leptin to adiponectin ratio (L/A) are presented as proportion of changes between pre and post treatment in the albendazole group compared to the placebo group which is set to zero. The effects of treatment are presented for each group of STH-infected subjects with: (**A**) single STH species, (**B**) multiple STH species. Adiponectin, leptin, resistin, and L/A ratio were log-transformed for analysis. Analysis was performed on 1387 subjects, after excluding 14 subjects with diabetes. Treatment effect estimates were the regression coefficient (β) obtained from mixed models indicating changes in log (ADI or LEP or RES or L/A); the treatment effect factors (10^{β}) are proportional instead of additive. Thus, treatment effect factors indicate the proportional change in each variable in comparison to the placebo group. *$p<0.05$.

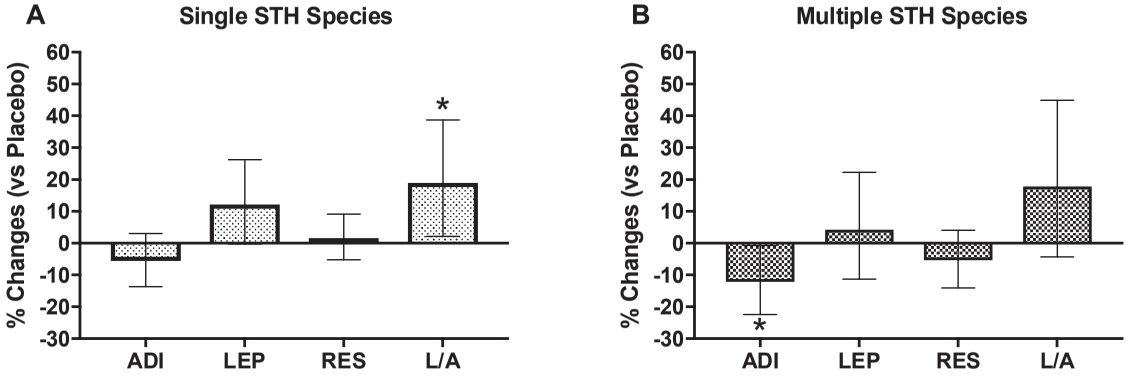


Figure S4. Effect of anthelmintic treatment on adiponectin, leptin, resistin, and leptin to adiponectin ratio stratified by number of helminth species a subject was infected with at baseline, as assessed by microscopy. The effects of anthelmintic treatment on adiponectin (ADI), leptin (LEP), resistin (RES), and leptin to adiponectin ratio (L/A) are presented as proportion of changes between pre and post treatment in the albendazole group compared to the placebo group which is set to zero. The effects of treatment are presented for each group of STH-infected subjects with: (**A**) single STH species, (**B**) multiple STH species. Adiponectin, leptin, resistin, and leptin to adiponectin ratio were log-transformed for analysis. Analysis was performed on 1183 subjects, after excluding 12 subjects with diabetes. Treatment effect estimates were the regression coefficient (β) obtained from mixed models indicating changes in log (ADI or LEP or RES or L/A); the treatment effect factors (10^{β}) are proportional instead of additive. Thus, treatment effect factors indicate the proportional change in each variable in comparison to the placebo group. *$p<0.05$

Table S1. Effect of Anthelmintic Treatment on Soil-transmitted Helminth Prevalence

	Placebo		Albendazole		
Method	**Baseline**	**Follow-up**	**Baseline**	**Follow-up**	**p-value***
Microscopy (%, n/N)	43.5% (270/620)	26.8% (166/497)	40.5% (233/575)	5.2% (24/466)	<0.0001
PCR (%, n/N)	53.8% (392/729)	45.0% (250/555)	54.4% (368/676)	10.4% (55/529)	<0.0001

*Analyzed using logistic model (lme4 package R software) with random household effects and random subject effects

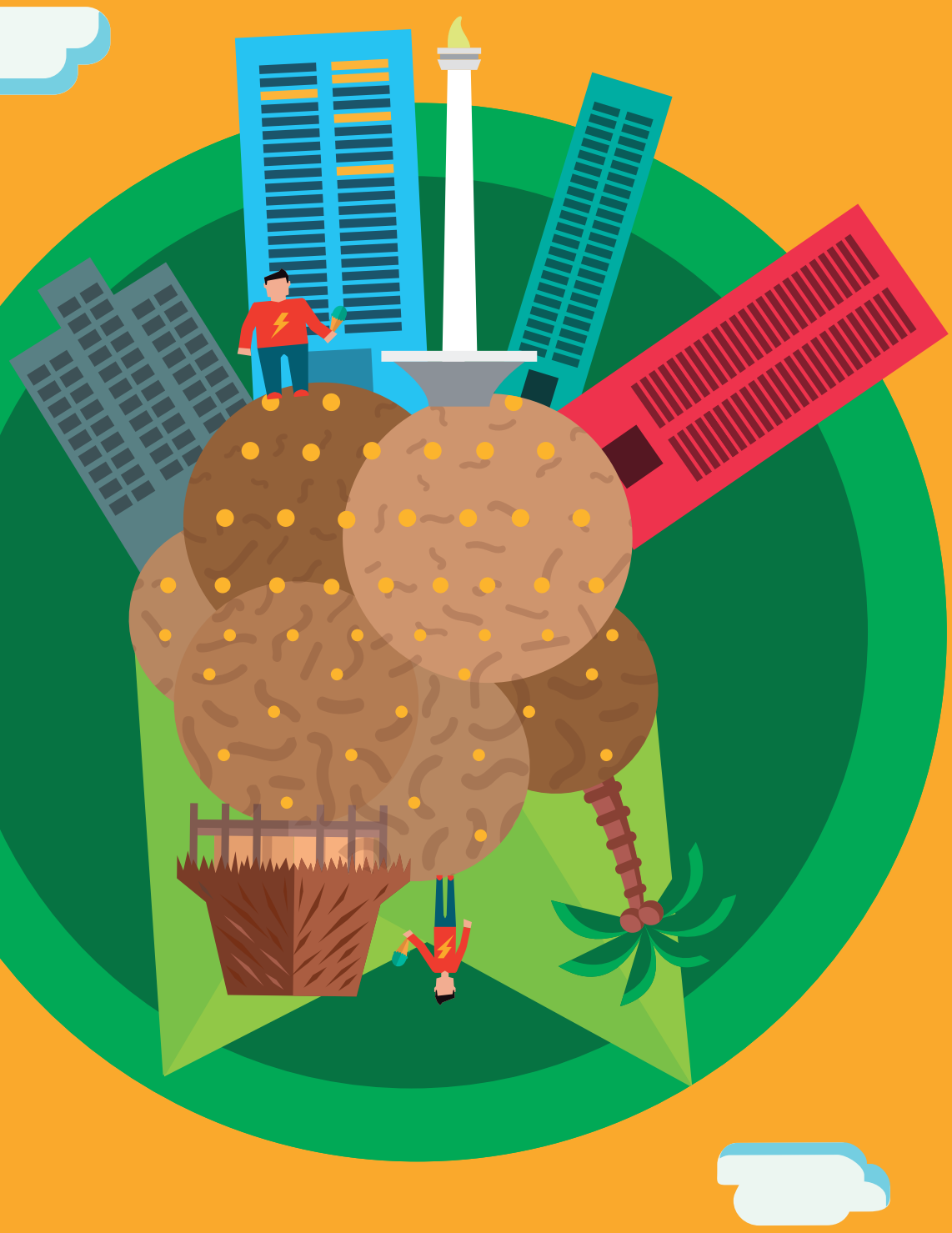
Table S2. Pathway analysis on the role of leptin to adiponectin ratio in the increased insulin resistance after anthelmintic treatment

	Crude	L/A Ratio	BMI	L/A Ratio + BMI
HOMA-IR*	1.07 (1.01 – 1.14) p=0.023	1.05 (0.99 – 1.11) p=0.075	1.06 (1.00 – 1.12) p=0.048	1.05 (0.99 – 1.11) p=0.075

*Analyses were performed using linear mixed model in unadjusted model (crude) and adjusted for leptin to adiponectin (L/A) ratio, BMI, or both.

PART II

What are the differences in metabolic profiles between populations in rural and urban areas?



Chapter 5

IMPACT OF RURAL-URBAN ENVIRONMENT ON METABOLIC PROFILE AND RESPONSE TO A 5-DAY HIGH-FAT DIET

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(Manuscript in submission)

ABSTRACT

Epidemiological studies have indicated that rural living might be protective against type 2 diabetes development. We compared the metabolic profile and response to a short-term high-fat high-calorie diet (HFD) of people with the same genetic background living in an urban and rural area of Indonesia. First, we recruited 154 Floresian male subjects (18-65 years old), of whom 105 lived in a rural area (Flores) and 49 had migrated and lived in urban area (Jakarta) for more than 1 year. The urban group had significantly higher whole-body insulin resistance (IR), as assessed by homeostatic-model-assessment of IR (HOMA-IR), [mean difference (95%CI), p-value: 0.10 (0.02 – 0.17), p=0.010]. Next, we recruited 17 urban and 17 rural age-and-BMI-matched healthy-young-male volunteers for a 5-day HFD challenge. The HOMA-IR increased in both groups similarly [-0.77 (-2.03 - 0.49), p=0.223]. Neither rural living nor factors associated with rural living such as current helminth infection and total IgE were associated with protection against acute induction of IR by HFD.

INTRODUCTION

The prevalence of obesity and type 2 diabetes (T2D) is increasing worldwide, especially in low and middle-income countries (LMIC) that are currently facing rapid rate of urbanization.[1, 2] Rural-to-urban migration has indeed been shown to be associated with increased obesity and other cardiovascular (CV) risk factors, such as dyslipidemia and hypertension,[3-11] suggesting that living in rural environment might be protective against T2D development.

In addition to changes towards a sedentary lifestyle and increased dietary fat intake, migration to an urban environment is also associated with a reduction exposure to microorganism and parasites, such as helminth infections, which are still endemic in many rural areas of LMIC.[12] There is data suggesting that helminth infections might confer a protection against the development of obesity and T2D,[13-16] presumably by promoting type-2 and regulatory immune responses and subsequent reduction in systemic inflammation.[17-19] However, it is worth mentioning that the relative contribution of helminth infections in comparison to the more established factors such as a sedentary lifestyle and diet remains to be clarified.

An increase in dietary fat intake, commonly observed upon rural-to-urban migration,[7, 20] has been reported to be associated with impaired insulin resistance (IR) and glucose homeostasis.[21] Mice on high-fat diet (HFD) have provided models to study obesity and the development of IR.[22, 23] Similarly, in humans, short-term HFD has been utilized to study the susceptibility to the development of IR,[24-28]. Using this model, it has been possible to show how risk of IR is dependent on ethnicity.[25, 28] Short-term HFD has also been shown to induce organ-specific and systemic inflammation as evidenced by the increase in plasma cholesteryl ester transfer protein (CETP) level,[24, 29] which is predominantly produced by Kupffer cells (KC),[30] as well as in plasma C-reactive protein (CRP) level [24].

Taken together, the chronic increase of energy rich diet, in addition to a more sedentary lifestyle, among people who migrate from a rural to urban areas,[20] might lead to the development of IR and T2D. However, there is still incomplete insight into the pathophysiology of the development of IR and T2D in rural-to-urban migration. In addition, there has been no study comparing the metabolic response towards a short-term HFD in terms of changes in glucose homeostasis and inflammation, between people living in urban and rural areas.

As some metabolic differences between subjects living in rural and urban area can be due to genetic differences, this study compared the metabolic profile between individuals with the same genetic background living in urban and rural areas, and examined their metabolic and inflammatory response to a 5-day high-fat high-calorie (HFD) diet. Furthermore, as rural areas often go hand in hand with helminth infections and associated IgE responses, we aimed to assess their contribution to metabolic profile. We hypothesized that individuals living in rural areas, in comparison to those living in urban areas, will have a better metabolic profile and will be relatively more protected from the induction of IR and inflammation by the HFD.

METHODS

Study Design and Population

The present study consisted of a cross-sectional and an interventional study. The cross-sectional study was performed in an urban (Jakarta) and a rural area (Nangapanda, Ende, Flores island) in Indonesia. We recruited 49 males (18-65 years old) with Floresian ethnical background who had migrated from Flores island and lived in Jakarta for more than 1 year (urban group). As their rural counterparts, we recruited 105 Floresian males with a similar age range, randomly selected from three villages in Nangapanda with age stratification, as described previously.[31]

For the HFD intervention study, 17 from urban and 17 from rural area, age-and-BMI-matched healthy young male volunteers (18-40 years old) were recruited via local healthcare workers who informed their community, in both Nangapanda and Jakarta, of the study. BMI-matching was performed to assess whether the difference between urban and rural in term of past or current exposure to STH infections affect the HFD-associated increase in IR, independent of adiposity. Exclusion criteria were T2D, recent body weight changes, intake of medication that could affect inflammation or IR.

The study was approved by the Medical Ethical Committee of the Faculty of Medicine, Universitas Indonesia (556/H2.F1/ETIK/2014) and performed in accordance with the principles of the revised Declaration of Helsinki. All volunteers gave written informed consent before participation.

Cross sectional Study

In the cross-sectional study, we invited all subjects to come to the Field Study Centre (FSC) in both rural and urban area to undergo clinical measurements and blood sample collections. Stool samples were also collected. All clinical measurements and blood sample collections were performed after an overnight fast. Anthropometric measurements of body weight, height, and waist circumference were performed. BMI was calculated as weight in kg divided by square of height in meter.

After collection of fasting blood samples, we performed an oral glucose tolerance test (OGTT), in which blood glucose levels were re-measured 2 hours after subjects were given 75g glucose dissolved in 200 mL of water (2h-BG). In this cross sectional study, we calculated HOMA-IR (homeostatic model assessment of insulin resistance), a well-validated measure of whole-body IR in humans (HOMA-IR = fasting serum insulin (mU/L) x fasting glucose (mmol/L) / 22.5)[32], as our primary outcome. We also measured HbA1c, fasting blood glucose (FBG), fasting insulin, 2h-BG, BMI, waist circumference, adiponectin, leptin, high-sensitive C-reactive protein (hsCRP), total IgE, and prevalence of soil-transmitted helminths (STH) as our secondary outcomes.

Intervention Study

Subjects were examined before and after a 5-day HFD intervention, consisting of the subject's regular diet supplemented with 375 mL cream (Greenfields™ Whipping Cream, Greenfields Indonesia Ltd, Jakarta, Indonesia) per day [1,500 kcal/day, 83% fat (60% saturated fat)]. After baseline measurements, each subject received three bottles of 125 mL cream per day for five consecutive days. Subjects were instructed to continue their regular diet, and to consume one bottle of cream after each meal (3 meals per day) to make sure they could adhere to their regular dietary habits.

Subjects were asked to keep a food diary before and during the HFD intervention to estimate normal dietary intake and to check for compliance and compensatory behavior. Dietary assessment, using a 24 hours food recall, was performed by a trained dietician. Compliance was further assessed by interviewing the subject and collecting the bottles every day. During the study, subjects were asked not to change lifestyle habits. Measurements of clinical parameters and blood drawing were done on the day before starting the HFD intervention (D-0) and one day after the fifth day of the HFD intervention (D-6).

In this intervention study, we had HOMA-IR as our primary outcome. As our secondary outcomes, we measured adipose-IR index, a measure of adipose tissue IR, which was calculated as the product of the fasting serum free fatty acid (FFA) and insulin (Adipose-IR index = FFA[mM] x Insulin [pM]).[33, 34] In addition, we also measured hsCRP, CETP, and lipid levels [total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C)]. Due to limited amount of sera after intervention, adiponectin and leptin level were measured only at baseline. All others measurements for the interventional study were performed pairwise (before and after intervention).

Laboratory measurements

Fasting blood glucose and 2h-post-load glucose were measured in capillary blood using Breeze®2 glucose meters (Bayer Health Care LLC, Basel, Switzerland) in the FSC. All sera, plasma and whole blood samples from rural area were frozen at −20°C in the FSC, and subsequently shipped and stored at −80°C in Faculty of Medicine Universitas Indonesia (FKUI), Jakarta, Indonesia and Leiden University Medical Centre (LUMC), Leiden, The Netherlands. All sera, plasma and whole blood samples from urban area were directly transported from FSC (Jakarta) to be stored at −80°C in FKUI, and subsequently shipped and stored at -80°C at LUMC.

Serum insulin concentrations were determined by a solid-phase, enzyme-labeled chemiluminescent immunometric assay, while HbA1c was measured using a cation-exchange chromatography (IC)-based high performance liquid chromatogtaphy (HPLC) assay. A latex-enhanced immunoturbidimetric method was used to measure hsCRP. Assays of TC, HDL-C, and TG were based on enzymatic colorimetric methods. These measurements have been described previously.[16] To convert from mmol/L to mg/dL, we multiplied the TC, HDL-C, and LDL-C level by factor of 38.67, while for TG levels we multiplied by 88.57.

Plasma CETP levels were measured with enzyme-linked immunosorbent assays (ELISA) kits according to the manufacturer's instructions (DAIICHI CETP ELISA, Daiichi, Tokyo, Japan). FFA were measured using ELISA kits according to the manufacturer's instructions (abcam ab 65341 FFA Quantification Assay Kit, Cambridge, UK). Adiponectin and leptin were also measured by using ELISA commercial reagents (DuoSet ELISA R&D System Europe Ltd, Abingdon, UK). The levels of total IgE, an important determinant of total IgE levels,[35] were measured using ELISA as described previously.[36] The presence of STH [hookworm

(Necator americanus, Ancylostoma duodenale), Ascaris lumbricoides, Trichuris trichiura, Strongyloides stercoralis] was assessed using PCR as described in detail elsewhere [36, 37]

Statistical Analysis

Normally distributed continuous variables were summarized as mean and standard deviation [mean (SD)], while non-normally distributed data were summarized as geometric mean and its 95% confidence interval [geomean (95% CI)]. In the cross-sectional study, sample size was calculated to aim at a difference in HOMA-IR between urban and rural group of 0.5. The SD of HOMA-IR from previous study was 0.84.[14] We used a significance level of 5% and a power of 80%, thus we needed at least 45 subjects for each group. For the interventional study, sample size was calculated to aim at a difference in changes of HOMA-IR between urban and rural group of 0.70. The SD of the HOMA-IR changes after HFD intervention from previous study was 0.68.[25] We used a significance level of 5% and a power of 80%, thus we needed at least 15 subjects per group or 30 subjects in total. Next, to assess STH effect on the metabolic response upon HFD intervention we used similar calculation, aiming at having at least 15 subjects per group.

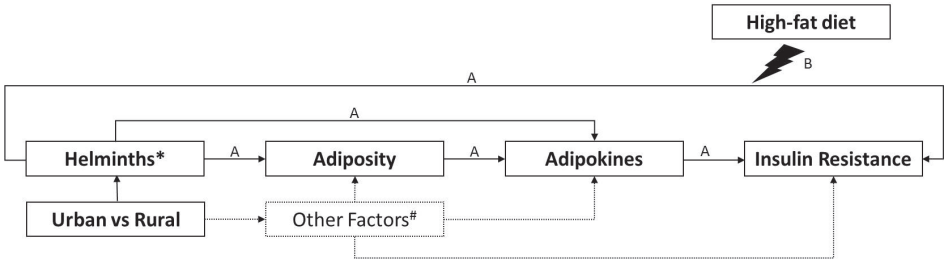

Figure 1. Conceptual framework. In the cross-sectional study (A), we assessed whether the differences in past or current exposure to helminths contribute to the difference in insulin resistance (IR) between subjects living in urban and rural area, and whether the observed difference in IR is independent from adiposity. In the high-fat diet (HFD) study (B), first, we assessed whether past or current exposure to helminths protect against the HFD-associated increase in IR, independent of adiposity. Next, we also assessed whether the presence of current helminth infection protect against the HFD-associated increase in IR. *Past and current exposure to helminths was assessed by measuring serum total IgE level, a general marker for Th2 responses, commonly induced by soil-transmitted helminth (STH). Current exposure to helminths was assessed using stool PCR. #Other factors that were not specifically assessed in this study.

The original plan for the linear regressions was based on a conceptual framework (**Figure 1**) of the proposed causal pathways. In the cross-sectional study (A), we assessed whether the difference between urban and rural subjects, in term of past or current exposure to STH, by using total IgE level as a proxy, contributes to the difference in insulin resistance (IR) between subjects living in urban and rural area, and whether this difference in IR is independent from adiposity. Next, we further stratified the urban and rural group based on their STH infection status. However, as the number of urban subjects with STH infections was very low and therefore was excluded from analysis, eventually we had three groups: rural subjects with STH infections, rural subjects without STH infections, and urban subjects without STH infections. We calculated variance inflation factors (VIFs) to check multicollinearity in our regression models and VIF values below 4 were considered appropriate. Due to multicollinearity between BMI and WC, we used WC as clinical marker for adiposity. In addition, we also assessed the association between length of stay in urban area and metabolic profiles (IR, adiposity, and leptin) among subjects living in urban area using age-adjusted linear regression model. Analyses were performed using IBM Statistics 23.

In the HDF intervention study (B), first, we assessed whether the difference between urban and rural in term of past or current exposure to STH infections affect the HFD-associated increase in IR, independent of adiposity, by matching both groups for BMI. To compare the parameter before and after the HFD intervention for each group, whenever appropriate, paired t-test or Wilcoxon-signed ranked test was performed. A mixed model was applied to assess mean differences before and after intervention between group. Groups were modelled as fixed effects, and to model correlation within subjects, random-specific intercept was used. Next, among subjects living in rural area, similar model was used to further assess whether the presence of current STH infections protect against the HFD-associated increase in IR. The mixed model analysis was performed using R software (lme4).

RESULTS

The metabolic profile of rural and urban study participants

The mean length of stay of urban subjects in Jakarta was 20.7 (range: 1 - 40) years. The differences in metabolic profile between subjects living in rural and urban are summarized in **Table 1**. Urban subjects had a significantly higher HOMA-IR compared to rural subjects [1.45 (1.06 – 1.90) vs 0.96 (0.80 – 1.13), respectively,

p=0.010]. Similarly, other metabolic parameters, such as 2h-blood glucose, HbA1c, BMI, waist circumference, and leptin level were significantly higher in urban subjects (**Table 1**). Interestingly, independent of age, increasing length of stay in urban area (in years) was positively associated with increasing BMI [estimate (95% CI), 0.152 (0.036 – 0.269) kg/m^2, p=0.012, **Figure 2A**], waist circumference [0.449 (0.135 – 0.762) cm, p=0.006, **Figure 2B**] and to a lesser extent with leptin [0.013 (-0.001 – 0.027), p=0.068], but not HOMA-IR [0.005 (-0.003 – 0.013), p=0.182].

The prevalence of STH was significantly lower in the urban compared to rural subjects [5% (2/42) vs 57% (52/92), respectively, p<0.0001]. Similarly, the levels of total IgE, often driven by STH infections,[35] were lower in the urban compared to rural subjects (168 (105 – 271) IU/mL vs 931 (702 – 1.235) IU/mL, respectively, p<0.0001) (**Table 1**).

Table 1. Comparison of metabolic profiles between subjects living in urban and rural area

Variables	Urban (n=49)	Rural (n=105)
Duration in urban (in years)	20.7 (1.0-40.0)	-
Age (in years)	39.3 (13.5)	44.5 (12.2)*
HOMA-IR	1.45 (1.06 – 1.90)	0.96 (0.80 – 1.13)*
Fasting Insulin (mU/L)	4.9 (3.8 – 6.4)	3.1 (2.5 – 3.8)**
Fasting Blood Glucose (mmol/L)	5.7 (1.4)	5.4 (0.9)
2h-Blood Glucose (mmol/L)	7.7 (3.2)	5.9 (1.9)**
HbA1c# (mmol/L)	37.9 (14.3)	32.3 (6.6)*
HbA1c# (%)	5.6 (1.3)	5.1 (0.6)*
Body Mass Index (kg/m^2)	24.3 (4.9)	22.7 (4.0)*
Waist Circumference (cm)	84.9 (13.8)	79.3 (11.9)*
Adiponectin (µg/mL)	4.38 (3.31 – 5.78)	3.54 (3.09 – 4.07)
Leptin (ng/mL)	5.62 (3.98 – 7.92)	2.64 (2.06 – 3.38)*
CRP (mg/L)	1.57 (1.17 – 2.05)	1.67 (1.29 – 2.11)
Total IgE (IU/mL)	168 (105 – 271)	931 (702 – 1,235)**
Prevalence of STH (%, n/N)	5 (2/42)	57 (52/92)**

All variables are presented as mean and its standard deviation, however, HOMA-IR, fasting insulin, adiponectin, leptin, CRP, and total IgE level are presented as geomean (95%CI) and were log transformed for analysis, while duration in urban is presented as mean (range). Analysis for the difference between urban and rural group was performed using independent t-test (*p<0.05, **p<0.0001) #Hba1c measurements were available in 42 and 95 of urban and rural subjects respectively. Abbreviation: HOMA-IR= the homeostatic model assessment of insulin resistance, CRP= C-reactive protein, STH=soil-transmitted helminth.

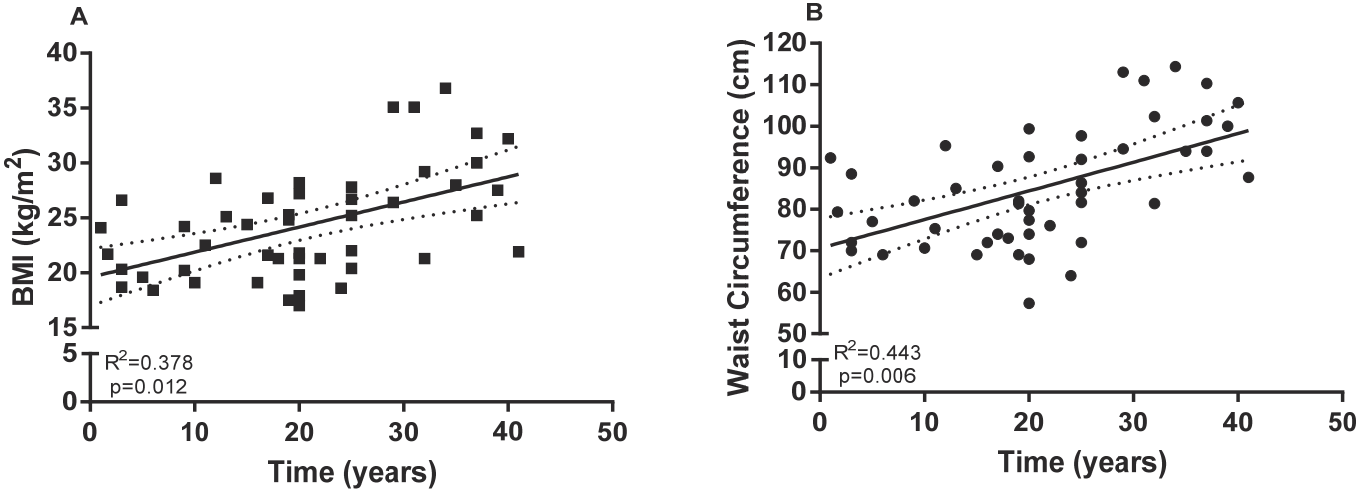

Figure 2. The association between length of stay in urban area with adiposity.The association between length of time in urban area with (A) body mass index (BMI) and (B)waist circumference are presented in scatter plot graphs (n=49), and analysed using age-adjusted linear regression. Each year increase of a time spent in urban area was associated with a significant increase in both (a) BMI [0.152 (0.036 – 0.269) kg/m^2, p=0.012] and (b) Waist Circumference [0.449 (0.135 – 0.762) cm, p=0.006].

Table 2. Associations between living in urban and rural area with HOMA-IR, leptin, and waist circumference

	Differences for each variable between urban and rural (rural group as the reference group)*					
Variables	**Crude**	**Model 1 (Age)**	**Model 2 (Age+Total IgE)**	**Model 3 (Age+Waist)**	**Model 4 (Age+Total IgE+Waist)**	**Model 5 (Age+Waist+Leptin)**
HOMA-IR[$]	0.10 (0.02 – 0.17) p=0.010	0.09 (0.02 – 0.17) p=0.016	0.08 (-0.00 – 0.17) p=0.061	0.02 (-0.04 – 0.08) p=0.545	0.04 (-0.03 – 0.11) p=0.294	0.01 (-0.06 – 0.07) p=0.774
Leptin (ng/mL)[$]	0.33 (0.14 – 0.51) p=0.001	0.36 (0.18 – 0.55) p<0.0001	0.10 (-0.03 – 0.24) p=0.137	0.11 (-0.01 – 0.22) p=0.076	0.08 (-0.05 – 0.21) p=0.216	-
Waist Circumference (cm)	5.6 (1.3 – 9.9) p=0.010	7.2 (3.0 – 11.3) p=0.001	4.2 (-0.5 – 8.8) p=0.077	-	-	-

*Beta coefficient (95% CI) from linear regression. [$]HOMA-IR and leptin level were log transformed for analysis. Model 1: adjusted for age. Model 2: adjusted for model 1 plus total IgE level. Model 3: adjusted for model 1 plus waist circumference. Model 4: adjusted for model 2 plus waist circumference. Model 5: adjusted for model 3 plus leptin level. Abbreviation: HOMA-IR= the homeostatic model assessment of insulin resistance.

As the number of subjects with current STH infections in urban area was very low (n=2), it was not possible to assess the contribution of current STH infections to the HOMA-IR difference between urban and rural subjects. Therefore, we used total IgE level as a proxy for past and current STH exposures. The age-adjusted difference in HOMA-IR between urban and rural subjects was slightly attenuated [from estimated mean differences (95% CI), 0.09 (0.02 – 0.17), p=0.0010 to 0.08 (-0.00 – 0.17), p=0.061] after further adjustment for total IgE level (Table 2). Further adjustment for total IgE level also attenuated the age-adjusted difference in waist circumference [from 7.2 (2.0 – 11.3), p=0.001 to 4.2 (-0.5 – 8.8), p=0.077] and leptin level [from 0.36 (0.18 – 0.55), p<0.0001 to 0.10 (-0.03 – 0.24), p=0.137] (Table 2). To assess the contribution of adiposity and leptin in the difference in HOMA-IR between urban and rural, adjustment with waist circumference [to 0.02 (-0.04 – 0.08), p=0.545] or both waist circumference and leptin level [to 0.01 (-0.06 – 0.07), p=0.774] strongly attenuated the difference in HOMA-IR (**Table 2**).

In addition, we stratified rural and urban subjects based on STH infection status into three groups, namely: urban group without STH infections, rural group without STH infections, and rural group with STH infections. The highest value of HOMA-IR, waist circumference, and leptin was observed in urban group without STH infections, followed by rural group without STH infections and the lowest among rural group with STH infections (**Figure S1**). The contrast was observed for total IgE level (**Figure S1**).

Comparison of metabolic responses after a short-term HFD intervention between subjects living in an urban and rural area

Among subjects who were included in the interventional part of the study (n=34), we observed no significant differences between the age-and-BMI-matched urban (n=17) and rural group (n=17) in terms of HOMA-IR, adipose-IR index, CRP, and lipid levels at D-0 (Pre HFD). At this time point, serum CETP levels were significantly lower in the urban group [1.96 (0.58) µg/mL vs 2.59 (0.64) µg/mL, in urban and rural group respectively, p=0.006]. Both groups showed a good compliance in terms of dietary intervention, all participants consumed all the cream provided and maintained their regular diet, resulting in a mean daily calorie intake that was ~60% higher compared to their regular diet, and ~56% of energy was derived from fat.

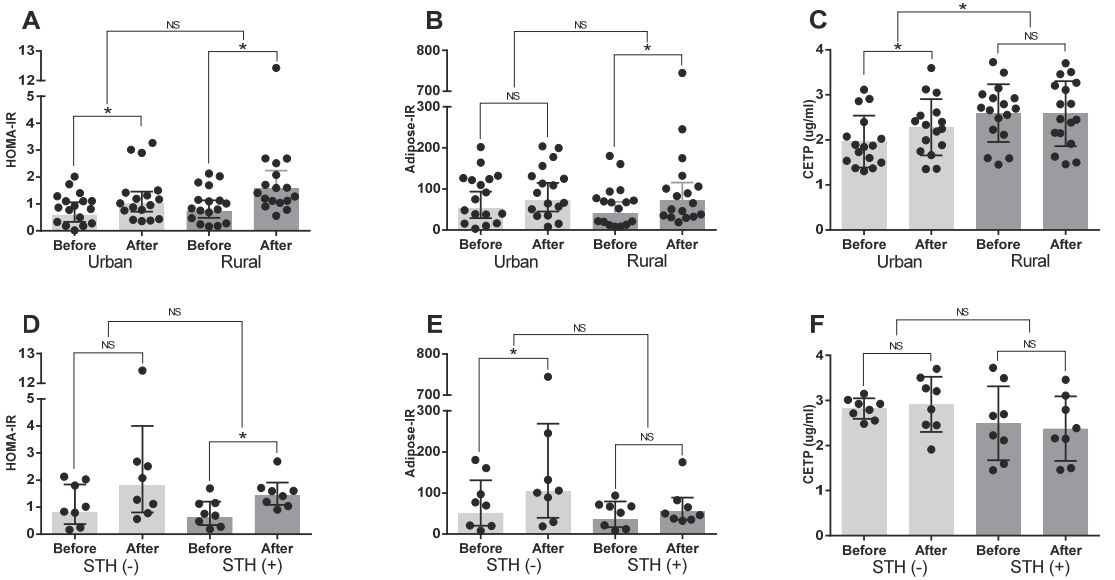

Figure 3. Comparison of Metabolic Responses to High-Fat Diet. HOMA-IR and adipose-IR index are presented as geometric mean and its corresponding 95% confidence interval, while CETP levels are presented as mean with its standard deviation. There were no significant differences in the increase of HOMA-IR (A), adipose-IR index (B) between urban and rural group, however, the increase in CETP level (C) was higher in the urban group. Furthermore, in rural group, there were no significant differences in the increase of HOMA-IR (D), adipose-IR index (E), and CETP level (F) between STH-infected and uninfected group. The difference between before and after intervention for each group was analysed using paired t-test, while the difference in the magnitude of changes for each parameter was analysed using linear mixed model (*p<0.05, NS: p>0.05).

Intervention with a 5-day HFD resulted in a significant increase of HOMA-IR in both the urban [from 0.78 (0.51 – 1.09) to 1.13 (0.78 – 1.57), p=0.03] and rural group [from 0.87 (0.59 – 1.21) to 1.69 (1.01 – 2.45), p=0.001] (**Figure 3.A**, **Table S1**), which was mainly driven by the increase in fasting insulin level in both urban [from 4.05 (2.98 – 5.52) to 5.59 (4.18 - 7.47), p=0.02] and rural group [4.63 (3.42 – 6.26) to 7.68 (5.70 – 10.34), p=0.001] (**Table S1**). Comparing the changes in IR before and after intervention between urban and rural group, we observed no significant differences for either HOMA-IR [estimated mean differences (95% CI), -0.77 (-1.95 – 0.41), p=0.21] (**Figure 3.A**, **Table S1**) or adipose-IR index [-41.20 (-115.12 – 32.73), p=0.28] (Figure 3.B, Table S1).

Interestingly, we observed a significant increase in CETP levels after HFD intervention in the urban group only [from 1.96 (0.58) to 2.28 (0.63), p=0.004 in urban group vs from 2.59 (0.64) to 2.58 (0.72), p=0.93 in rural group) (**Figure 3.C**). Therefore, in comparison to the rural group, the increase in CETP level was significantly higher in urban group [0.33 (0.06 – 0.60), p=0.02] (**Figure 3.C**, **Table S1**). However, as indicated above, the CETP levels were already much higher in the rural group at D-0 (Pre HFD), even higher than the D-6 (post-HFD) CETP level in the urban group. Intervention with HFD also did not significantly increase hsCRP in the two groups (**Table S1**). In terms of HFD effects on lipid levels, whereas we observed no significant difference in changes in TC, LDL-C, and TG levels between urban and rural groups, the increase in HDL-C after intervention was significantly higher in the urban group in comparison to rural group [3.34 (0.19 – 6.50), p=0.04] (**Table S1**).

The effect of current STH infections on the metabolic responses upon short-term HFD intervention

Next, due to the very low prevalence of STH infections in the urban group [6% (1/17)], the effect of current STH infections on the metabolic response towards a short-term HFD intervention was only assessed in the rural group of which 50% was positive for STH infection (8/16). Thus, our study was underpowered (power of 56%) to detect any differences in metabolic responses between STH-infected and uninfected subjects.

Despite a significantly lower baseline body weight in STH-infected subjects [51.1 (11.0) kg vs 63.3 (10.2) kg, p=0.037], there was no significant difference in the magnitude of increase in HOMA-IR in STH-infected and STH-uninfected subjects [-1.08 (-3.38 – 1.22), p=0.36] (**Figure 3.D**), adipose-IR [-87.82 (-222.08 – 46.44), p=0.21] (Figure 3.E), or CETP level [-0.21 (-0.62 – 0.20), p=0.32] (**Figure 3.F**) after

intervention in comparison to uninfected subjects. Interestingly, we observed a significantly higher increase in LDL-C level [10.49 (1.99 – 18.99), p=0.03] after intervention among STH-infected subjects in comparison to STH-uninfected subjects (Table S2). However, the LDL-C level were much lower in STH-infected group at D-0 in comparison to STH-uninfected group [85.8 (11.9) vs 114.9 (24.7), p=0.013], and the LDL-C level at D-6 in STH-infected group [94.5 (10.8)] did not reach the LDL-C level in the STH-uninfected group at D-0 (**Table S2**).

DISCUSSION

Our study showed that, in comparison to individuals living in a rural area, those living in an urban area had higher whole-body IR, as assessed by HOMA-IR. This higher whole-body IR was mainly mediated by the higher adiposity and leptin levels. To a lesser extent, the differences in exposures to STH infection between urban and rural individuals, might to a small extent contribute to the differences in whole-body IR, adiposity or leptin level. Intervention with a short-term HFD increased whole-body IR in both the urban and rural group. In comparison to rural group, CETP level was lower in the urban group, and HFD intervention induced a stronger increase in CETP in this group. The presence of STH infections did not seem to have a protective effect on acute induction of IR from short-term HFD, however it has to be noted that our study was underpowered to detect an STH effect.

Our study found that the higher whole-body IR in individuals living in urban area was mediated by the higher adiposity, as well as a higher leptin level, a pro-inflammatory adipokine, which has been previously reported to be associated with glucose metabolism.[8, 38] The increase of adiposity and, to a lesser extent, leptin level, was positively associated with the duration of time spent in the urban environment. This suggests that a higher degree of acculturation in terms of urban lifestyle, drifting away from their traditional lifestyle,[11] could lead to a positive energy balance,[20] hence increasing adiposity over time. In addition, reduced exposures to environmental factors, such as to STH infections, which have been shown to have beneficial metabolic effects,[13] partly through the induction of type-2 and regulatory immune response,[18, 19] might contribute to the difference in whole-body-IR, adiposity, and leptin level between urban and rural individuals. This was supported by our finding that the difference in whole-body IR, adiposity, and leptin level between urban and rural individuals was attenuated, but only slightly, after adjustment for total IgE level, a general marker for type-2 immune responses, and a proxy for past and current STH exposures.

Next, whereas, as expected the overall metabolic profile of individuals living in a rural area, in term of adiposity and whole-body IR, was better, in comparison to those living in an urban area, in contrast to our hypothesis, a short-term 5-day HFD intervention induced a similar increase of IR in both urban and rural individuals. As both groups were BMI-matched, these findings suggest that the direct protective metabolic effect of a combined past and current environmental exposures to helminths,[13] independent of their effect on adiposity, might be relatively weak in comparison to the strong induction of IR by the HFD intervention. Indeed, our group has recently reported that the increased IR in STH-infected subjects after deworming was mainly mediated by the increased adiposity.[16] Thus, adjusting for adiposity, in a way, remove the possible main pathway for STH-associated protection against the development for IR.

Although our study was underpowered to assess the effect of current STH infection, it is possible that the presence of current STH infections might not be sufficient to protect against a strong induction of IR by short-term HFD, as in rural subjects, the increase in IR after HFD in STH-infected subjects was similar in comparison to STH-uninfected subjects. However, it is possible that the HFD intervention in STH-infected subjects with lower body weight would have a stronger impact than in STH-uninfected subjects, masking any protective effects of STH infections.

Interestingly, we observed that the baseline serum CETP level was significantly lower in urban subjects. As CETP is mainly produced by KCs, higher CETP level may represent an increase in hepatic macrophage (KC) content, hence liver inflammation.[30] Also, environmental factors in the rural area, mainly exposure to various infectious agents, such as microorganisms and parasites, may explain the increased CETP level. For instance, it has been shown that subjects with chronic hepatitis C virus infection have elevated serum CETP levels.[39] Supporting this, the prevalence of hepatitis in our rural study area was higher than our urban study area (4.3% vs 0.8%).[40] However, currently, there are no available data connecting macrophage polarization status to CETP level and therefore further studies are needed.[41]

In contrast to what is seen in urban subjects,[24, 29] we found no increase in CETP levels in rural subjects after the HFD intervention. It is possible that the lack of an increase in CETP level in rural subjects was caused by the CETP levels that were already high, thus precluding its further increase after HFD intervention.

Our results suggest an inflammation-independent mechanism of short-term HFD-associated induction of IR[23] there was no significant increase in CRP following HFD. Studies on the role of inflammation in HFD-associated induction of IR have shown conflicting results. In one study an increase in CRP and expression of M1 macrophage markers in skeletal muscle was reported,[24] while in another, no increase was seen in circulating pro-inflammatory cytokines.[42]

In terms of lipid levels, while no significant changes in lipid levels were observed in rural group, HFD intervention significantly increased HDL-C level in urban group. As it has been reported that urban subjects had a higher fat intake than rural subjects[20] at baseline and the fact that both groups received the same type of HFD intervention, differences in the relative changes of dietary composition before and after intervention[43-45] between urban and rural might potentially contribute to the difference in HDL-C level changes after intervention. In rural group, while no significant changes were observed in STH-uninfected subjects, HFD intervention resulted in a significant increase in LDL-C in STH-infected subjects, which might be related to the lower baseline LDL-C level and body weight in STH-infected subjects.

Our study is the first to compare the metabolic profile between people with the same genetic background, living in different environments (urban and rural) and to assess the metabolic responses to an intervention with a standardized short-term HFD. However, our study has several limitations. Due to the low prevalence of STH in urban area, our study could only assess the effect of current STH infections on HFD-induced IR in rural subjects. In addition to using a calculated HOMA-IR instead of the gold standard glycemic clamp to assess IR, physical activity assessment and biopsies on specific metabolic tissues (liver, muscle, adipose tissue) were not available.

In conclusion, in comparison to their rural ethnic counterparts, individuals living in an urban area had a higher whole-body IR, which was mainly mediated by their higher adiposity. The differences between urban and rural individuals in terms of past and current exposures to STH might have a relatively small contribution to the difference in whole-body IR. Contrary to our hypothesis, intervention with a short-term HFD induced similar increase in IR, in urban and rural individuals and in helminth infected and uninfected subjects. However, well-powered larger studies will be needed to determine which factors in terms of urbanization contribute to IR.

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Author Contributions Statement

DLT is a medical doctor who developed the study, in charge of the field study, involved in setting up the study, supervising gathering of data, intervention, follow up of the study subjects, analyzed the data and wrote the paper. KR is a medical biologist who developed the study, involved in setting up the study, supervising gathering of data, and involved in setting up the laboratory in the rural study area, and critically reviewed the manuscript. FK is a medical doctor in charge of the field study in urban area, involved in setting up the study, supervising gathering of data, intervention, follow up of the study subjects, and critically reviewed the manuscript. YD is a medical doctor involved in supervising the laboratory measurements and advised on the immunological and parasitological aspects of the study. YW is a medical biologist who performed the CETP measurements and advised on the metabolic aspects of the study. SMEN is a nutritionist who performed analysis on the food recall data. EI is a medical doctor involved in the gathering of data in both urban and rural area and follow up of the study subjects. DM is a health care officer from Flores who contributed to the development of both field study centre in Flores and Jakarta. EY is an endocrinologist who is involved in coordinating the study and advised on the metabolic aspects of the study. BG is a medical biologist who advised on the metabolic aspects of the study. TS is a parasitologist involved in supervising the laboratory measurements and advised on the immunological and parasitological aspects of the study. PCNR is a lipidologist who advised on the metabolic aspects of the study, and critically reviewed the manuscript. ES is an immunoparasitologist who is involved in coordinating the study and advising on parasitological and immunological aspects of the study and supervised the writing of the manuscript. PS is an endocrinologist who advised on the metabolic aspects of the study, supervised the writing of the manuscript and is the scientific coordinator of this study. DSH is an endocrinologist who developed the study, supervised the writing of the manuscript, and is the principal investigator of this study. JWAS is an endocrinologist who developed the study, critically reviewed and supervised the writing of the manuscript, and is scientific coordinator of this study. MY is an immunologist who developed the study, critically reviewed and supervised the writing of the manuscript and is the scientific coordinator of this study. All authors reviewed the manuscript.

Declaration of interests

All authors declare no competing interests. The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

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SUPPLEMENTARY MATERIALS

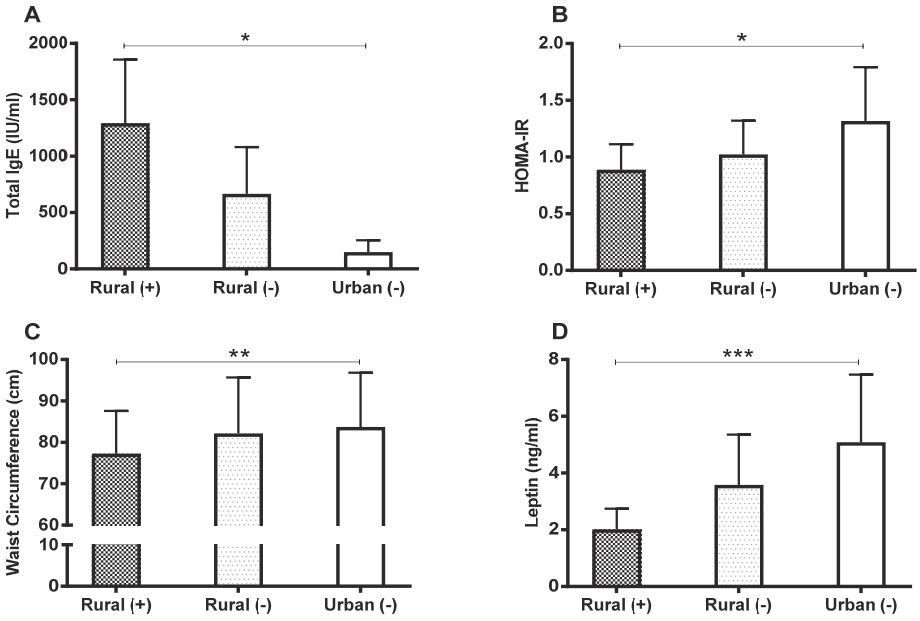

Figure S1. The comparison of metabolic profile of rural and urban subjects stratified by helminth infection status. The levels of total IgE, HOMA-IR, waist circumference, and leptin on different group of living area and soil-transmitted helminth (STH) infection status are presented as geometric mean and its 95% confidence interval, except for waist circumference which are presented as mean (SD). The number of urban subjects with helminth infections was very low (2/42) and was not included in this graph. Trend analysis was performed between three groups, namely: (1) rural subjects with STH infections [Rural (+)], (2) rural subjects without STH infections [Rural (-)], and (3) urban subjects without STH infections [Urban (-)]. Total IgE level was the lowest in Urban (-) group and progressively become higher in Rural (-) and Rural (+) groups (**A**). The contrary was observed for HOMA-IR (**B**), waist circumference (**C**), leptin level (**D**). *p<0.05 in unadjusted model, **p<0.05 in age-adjusted model, ***p<0.05 in age-waist circumference-adjusted model.

Table S1. Comparison of metabolic responses towards a short-term HFHC diet between subjects living in an urban and rural area

	Urban n=17			Rural n=17			Estimated differences in the magnitude of changes between urban and rural subjects **
Variables	**Pre HFHC Diet**	**Post HFHC Diet**	**p-value***	**Pre HFHC Diet**	**Post HFHC Diet**	**p-value***	
Age (years)	30.1 (6.4)	-	-	29.5 (8.0)	-	-	-
Body Mass Index (kg/m^2)	23.1 (4.7)	-	-	21.6 (3.6)	-	-	-
HOMA-IR	0.78 (0.51 – 1.09)	1.13 (0.78 – 1.57)	**0.03**	0.87 (0.59 – 1.21)	1.69 (1.01 – 2.45)	**0.001**	-0.77 (-1.95 – 0.41), p=0.21
Fasting Blood Glucose (mmol/L)	5.15 (0.44)	5.23 (0.50)	0.59	4.96 (0.19)	5.46 (0.73)	**0.005**	**-0.42 (-0.82 - -0.03), p=0.04**
Fasting Insulin (mU/L)	4.05 (2.98 – 5.52)	5.59 (4.18 - 7.47)	**0.02**	4.63 (3.42 – 6.26)	7.68 (5.70 – 10.34)	**0.001**	-2.35 (-6.55 – 1.84), p=0.28
Adipose-IR Index	51.6 (28.5 – 93.3)	71.9 (45.0 – 114.7)	0.23	40.5 (24.0 – 68.4)	72.0 (44.8 – 115.7)	**0.006**	-41.20 (-115.12 – 32.73), p=0.28
Free Fatty Acid (mmol/L)	3.93 (3.37 – 4.59)	3.43 (2.91 – 4.04)	0.29	2.83 (2.42 – 3.30)	2.65 (2.27 – 3.09)	0.39	-0.32 (-1.24 – 0.59), p=0.49
CETP (µg/mL)#	1.96 (0.58)	2.28 (0.63)	**0.004**	2.59 (0.64)	2.58 (0.72)	0.93	**0.33 (0.06 – 0.60), p=0.02**
CRP (mg/L)	2.04 (0.94 – 4.74)	2.24 (1.21 – 3.75)	0.82	1.14 (0.59 – 1.89)	1.06 (0.58 – 1.71)	0.81	-0.31 (-3.61 – 2.98), p=0.85
Total Cholesterol (mg/dL)	159.1 (23.6)	159.9 (22.8)	0.84	169.2 (23.7)	170.8 (27.6)	0.65	-0.84 (-11.10 – 9.42), p=0.87
Triglyceride (mg/dL)	114.3 (46.6)	116.2 (47.9)	0.83	120.0 (31.5)	109.6 (38.2)	0.37	12.30 (-14.73 – 39.32), p=0.38
HDL-C (mg/dL)	41.9 (9.5)	45.8 (8.9)	**0.01**	45.9 (9.6)	46.5 (9.3)	0.60	**3.34 (0.19 – 6.50), p=0.04**
LDL-C (mg/dL)	94.5 (22.9)	91.0 (22.1)	0.251	99.3 (23.6)	102.5 (22.8)	0.21	-6.66 (-13.91 – 0.58), p=0.08

All variables are presented as mean and its standard deviation, however, HOMA-IR, Fasting Insulin, Adipose-IR Index, Free Fatty Acid, and CRP levels are presented as geomean (95%CI). *The difference between before and after HFHC diet intervention were analysed using paired t-test. **The difference in changes (before and after HFHC-diet) of different parameters between urban and rural were analysed using linear mixed model and are presented as [Estimated Differences in Changes (95%CI), p-value]. #CETP measurements were only available for 33 subjects. Abbreviation: HOMA-IR= homeostatic model assessment of insulin resistance, CETP= cholesteryl ester transfer protein, CRP= C-reactive protein, HDL-C= high-density lipoprotein cholesterol, LDL-C= low-density lipoprotein cholesterol.

Table S2. Comparison of metabolic responses towards a short-term HFHC diet between STH-infected and uninfected subjects living in rural area

	STH-infected n=8			STH-uninfected n=8			Estimated differences in the magnitude of changes between STH-infected and STH-uninfected subjects**
Variables	**Pre HFHC Diet**	**Post HFHC Diet**	**p-value***	**Pre HFHC Diet**	**Post HFHC Diet**	**p-value***	
Age (years)	27.0 (9.6)	-	**-**	32.0 (6.3)	-	**-**	-
Body Mass Index (kg/m^2)	20.1 (3.5)	-	**-**	23.1 (2.4)	-	**-**	-
HOMA-IR	0.73 (0.37 – 1.19)	1.47 (1.08 – 1.93)	**0.002**	1.00 (0.45 – 1.75)	2.03 (0.72 – 4.34)	**0.06**	-1.08 (-3.38 – 1.22), p=0.36
Fasting Blood Glucose (mmol/L)	5.03 (0.22)	5.69 (0.88)	**0.04**	4.90 (0.17)	5.26 (0.57)	0.13	0.30 (-0.30 – 0.90), p=0.34
Fasting Insulin (mU/L)	4.04 (2.60 – 6.29)	6.80 (5.17 – 8.95)	**0.008**	5.12 (2.89 – 9.08)	8.97 (4.69 – 17.17)	**0.04**	-3.74 (-11.67 – 4.18), p=0.36
Adipose-IR Index	36.6 (16.9 – 79.6)	55.8 (34.9 – 89.0)	0.22	51.7 (20.4 – 73.4)	103.2 (39.6 – 268.8)	**0.02**	-87.8 (-222.1 – 46.4), p=0.21
Free Fatty Acid (mmol/L)	2.88 (2.41 – 3.45)	2.44 (1.94 – 3.09)	0.21	3.03 (2.42 – 3.83)	2.99 (2.34 – 3.81)	0.87	-0.37 (-1.18 – 0.44), p=0.37
CETP (µg/mL)	2.49 (0.82)	2.38 (0.72)	0.46	2.82 (0.23)	2.91 (0.61)	0.59	-0.21 (-0.62 – 0.20), p=0.32
CRP (mg/L)	0.96 (0.28 – 2.00)	1.60 (1.48 – 3.57)	0.13	1.32 (0.29 – 3.17)	0.71 (0.39 – 1.11)	0.25	2.27 (-0.63 – 5.18), p=0.13
Total Cholesterol (mg/dL)	157.2 (6.4)	163.7 (12.9)	0.20	184.6 (26.4)	182.1 (34.8)	0.69	8.89 (-4.73 – 22.51), p=0.21
Triglyceride (mg/dL)	117.1 (24.6)	101.1 (40.5)	0.40	123.8 (40.4)	121.9 (36.1)	0.92	-14.17 (-59.15 – 30.81), p=0.54
HDL-C (mg/dL)	48.1 (9.5)	49.1 (10.8)	0.50	45.1 (10.0)	44.8 (7.8)	0.88	1.21 (-2.61 – 5.03), p=0.54
LDL-C (mg/dL)	85.8 (11.9)	94.5 (10.8)	**0.01**	114.9 (24.7)	113.0 (28.6)	0.65	**10.49 (1.99 – 18.99), p=0.03**

All variables are presented as mean and its standard deviation, however, HOMA-IR, Fasting Insulin, Adipose-IR Index, Free Fatty Acid, and CRP levels are presented as geomean (95%CI). *The difference between before and after HFHC diet intervention were analysed using paired t-test. ** The difference in changes (before and after HFHC-diet) of different parameters between STH-infected and STH-uninfected were analysed using linear mixed model and are presented as [Estimated Differences in Changes (95%CI), p-value]. Abbreviation: HOMA-IR= homeostatic model assessment of insulin resistance, CETP= cholesteryl ester transfer protein, CRP= C-reactive protein, HDL-C= high-density lipoprotein cholesterol, LDL-C= low-density lipoprotein cholesterol.

Chapter 6

SUMMARIZING DISCUSSION

Adapted from:

Helminths, hygiene hypothesis and type 2 diabetes.

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SUMMARY OF WHAT WAS ALREADY KNOWN

- The prevalence of obesity and type 2 diabetes is increasing, especially in urban areas of LMIC.
- Rural to urban migration is associated with increased risk of obesity
- In rural area, helminth infections are still highly prevalent, while the prevalence of T2D is low
- Studies in animal models have shown a protective effect of helminth infections on glucose metabolism
- Cross sectional studies have shown that past and current chronic helminth infections are associated with lower adiposity and insulin resistance.
- Recent animal studies reported an important role of adipose tissue in helminth-mediated protective metabolic effects.

WHAT WAS ALREADY KNOWN ABOUT HELMINTH INFECTIONS, URBANIZATION, AND TYPE 2 DIABETES?

The prevalence of T2D is increasing worldwide, especially in urban areas of LMIC, whereby socioeconomic changes leads to increased adiposity and development of T2D.[1, 2] In animal models, a protective role for helminth infections on glucose metabolism was suggested.[3-8] In most rural areas of LMIC, where helmints are still highly prevalent, the prevalence of T2D is low, showing an inverse ecological association between STH and T2D.[9, 10] In line with this, several cross-sectional studies from different populations reported on the possible protective metabolic effects of past[11, 12] or current helminth infections[13, 14]. Despite the fact that a recent meta-analysis confirmed the protective metabolic effects of helminths[15], all available evidence from human studies were cross-sectional, thus, no causal relation could be drawn.

In general, the prevalence of T2D in rural areas is lower,[2] suggesting that living in rural area might give a relative protection against the development of T2D, in comparison to living in an urban area. It has been suggested that, in addition to the better known changes in diet and lifestyle, reduced biodiversity, commonly seen in urban areas, might also play some role in the increasing prevalence of inflammatory diseases,[16] including T2D. Furthermore, current deworming programs in many LMICs,[17, 18] might also contribute by removing helminth-associated beneficial effects,[9] which in turn alleviates helminth-associated induction of type 2 immune responses and regulatory network,[19] thus increasing the risk of inflammatory disorders, including T2D.[9, 10, 20-22]

Chronic low grade inflammation seems to be a key characteristic of obesity and T2D.[23] Whereas in obesity-associated development of IR, AT inflammation plays an important role,[23-26] experimental studies in animal model have shown that helminth infections cause changes in AT.[4-7] These changes are not only in terms of lowering fat mass, but also involve the immune cell composition in AT, shifting towards a more anti-inflammatory milieu, and better glucose homeostasis.[4-7]

SUMMARY OF THE FINDINGS

- Repeated three-monthly treatment with triple dose of 400mg albendazole over 12 months reduced STH prevalence and intensity, thus to reach a substantial reduction in STH, a more intensive deworming than the current policy is needed.
- At community level, 12 months of anthelmintic treatment reduced total IgE level and eosinophil count, but did not affect insulin resistance.
- In STH-infected subjects, as assessed by microscopy, 12-month anthelmintic treatment increased insulin resistance, which was mediated by an increase in BMI and leptin to adiponectin ratio (L/A ratio), as well as reduction in eosinophil count.
- In comparison to those living in rural area, individuals living in urban area had higher whole body insulin resistance, which was mainly mediated by the higher adiposity and leptin level, which were progressively increased with increased duration of time spent in urban area.
- Different environmental factors (including past or current exposure to STH) did not seem to affect the metabolic response to HFD intervention, independent from adiposity.

HOW DID OUR STUDIES ADVANCE THE FIELD?

Through designing a randomized controlled trial (RCT) of anthelmintic treatment in a rural area of Indonesia[27], endemic for STH[28], as detailed in **Chapter 2**, it was possible, for the first time, to study the effect of anthelmintic treatment on the host metabolic homeostasis. Four rounds of three-monthly albendazole treatment given for three consecutive days lead to a significant reduction in infection intensity and prevalence but not elimination of STH.[29] Our triple doses of albendazole treatment regimen resulted in lower prevalence of helminth compared to single dose albendazole treatment previously given in the same study area.[28] Although not a major questions in our study, our data are important for the global deworming programs, as they indicate that the current annual single dose treatment[18] is unlikely to have an impact on the control, and in particular the elimination of STH. The issue of re infection needs to be considered,[30, 31] and in addition to intensive treatment, interruption of transmission by decontaminating the environment[32] will be essential for any global impact to be achieved.

Besides reduction in prevalence and intensity of STH infections, anthelmintic treatment also led to a significant reduction of Th2 responses at the community level, as measured by total IgE and eosinophil counts[29], which is in line with a previous report in Ecuador[33]. However, it did not affect insulin resistance nor any other metabolic parameter at the community level. However, in STH-infected subjects, anthelmintic treatment led to a significant increase in IR[29], providing the first evidence on the causal association between helminth infections and IR in humans (**Chapter 3**), further strengthening the evidence for the helminth-associated protective metabolic effects documented in cross-sectional studies[11-15] and animal models[3-8].

Interestingly, infection intensity and number of helminth species seems also to play a role. The increase in IR after anthelmintic treatment was higher in helminth-infected subjects, as assessed by microscopy, in comparison to helminth-infected subjects, as assessed by PCR, the later detecting less clinically relevant infections[34]. Moreover, subjects infected with multiple helminth species showed a more pronounced increase in IR after anthelminthic treatment, which suggests the importance of, not only infection intensity, as these subjects infected with multiple helminth species had a higher infection intensity in comparison to those infected with single species, but also the importance of polyparasitism for the down modulatory effect of helminth[35]. Previous cross- sectional studies in the

same population also support this notion, as increasing number of STH species was also associated with a progressively lower IR[14].

Following the evidence of a causal relation between helminth and IR, it would be important to be able to understand the pathways involved. Despite the significant reduction in Th2 responses, as assessed by serum total IgE level and eosinophil count, this pathway does not seem to play a role in the increased IR, in particular the total IgE. Although the presence of STH is an important determinant for total IgE level,[33] other environmental exposures or conditions can also affect total IgE. Our trial showed that the increased IR after anthelmintic treatment seems to be mainly mediated by the increased adiposity, as assessed by BMI, which is not surprising as adiposity has been closely associated with nutrition and the development of IR.[36] However, a role for eosinophils was shown when additional adjustment for eosinophil counts further attenuated the increased IR. This suggests that eosinophils, if anything, might be involved in the causal pathway. The possible role of eosinophils in affecting glucose homeostasis has been shown in animal models[3-6] and a human study[37].

Although our trial has shown that anthelmintic treatment increased adiposity, this only gives us limited information on the pathways involved in mediating the beneficial effects of helminths in humans. It is interesting to study whether the increase in adiposity is solely responsible for the increased IR, or it is also associated with changes in the physiology of AT. Studies in animal models of diet-induced obesity have shown that indeed chronic helminth infection is associated with less fat mass gain,[4] of which associated with increased white AT M2 macrophages and eosinophils, as well as less IR.[4] Interestingly, helminth-derived molecules also induce a similar type 2 responses and beneficial metabolic effects without any significant changes in body fat.[4, 5]

In human, however, it was not possible to perform AT biopsies. We therefore assessed the effect of anthelmintic treatment on adipokines, mediators secreted by human AT, especially leptin and adiponectin, two major adipokines which have been shown to have pro and anti-inflammatory properties, respectively, as well as resistin, which has been reported to be increased in helminth infected subjects[38] (**Chapter 4**). Indeed, anthelmintic treatment in helminth-infected subjects increased the leptin to adiponectin ratio, and through mediation analysis we showed that this increase may be involved in the anthelmintic-associated increase in IR. The shift toward a more proinflammatory, higher leptin to adiponectin ratio,

after anthelmintic treatment was mainly caused by the significant reduction in adiponectin level, an anti-inflammatory adipokine [39], and to a lesser extent, through an increase in leptin level, a pro-inflammatory adipokine [39]. No causal association between helminth infection and resistin, a pro-inflammatory adipokine, was observed in our trial, contrasting the previously reported finding.[38]

Furthermore, the increase in leptin level after anthelmintic treatment in STH-infected subjects might also suggest that STH suppresses leptin level, as levels recovered up to the levels in STH-uninfected subjects when STH were removed by anthelmintic treatment. Low leptin levels has been associated with higher susceptibility to infection[40-42] and lower autoimmunity.[43] Furthermore, as leptin can increase Th1 and suppress Th2 cytokine production,[40] and can act as a negative signal for the proliferation of human regulatory T cells [44], we may speculate that the supressed leptin level in helminth-infected subjects might contribute to helminth-associated induction of modified type 2 and regulatory immune responses,[21] and if anything, contribute to a reduced helminth clearance from the host.[45]

Next, as available evidence suggests that living in rural areas might provide relative protection against the development of T2D, in comparison to living in urban areas, we designed a study to compare the metabolic profile and responses to a short-term HFD intervention between individuals with similar genetic background living in rural and urban areas. **Chapter 5** describes that subjects living in the urban area have a significantly lower STH infections, as well as lower total IgE levels. These subjects had higher whole-body IR, which was mainly mediated by higher adiposity and leptin level. Past or current exposure to STH, as assessed by total IgE, albeit small, contributed to the differences in whole-body IR, adiposity, and leptin level. However, neither rural living nor current STH infections protected against acute induction of IR by high-fat diet intervention. Thus, living in rural area, as well as having helminth infections, might only give a relatively weak protection against the strong induction of IR by short-term HFD, independent of the effect on adiposity.

However, it is important to note that HFD did induce increased liver inflammation, as assessed by CETP level,[46-49] in individuals living in urban, but not in rural areas. The lack of increase in CETP level in rural subjects might be related to the fact that, in these subjects, the CETP levels were already high, thus precluding any further increase. Environmental factors in rural area may explain this high CETP levels, for instance, the higher prevalence of hepatitis in our rural study area may

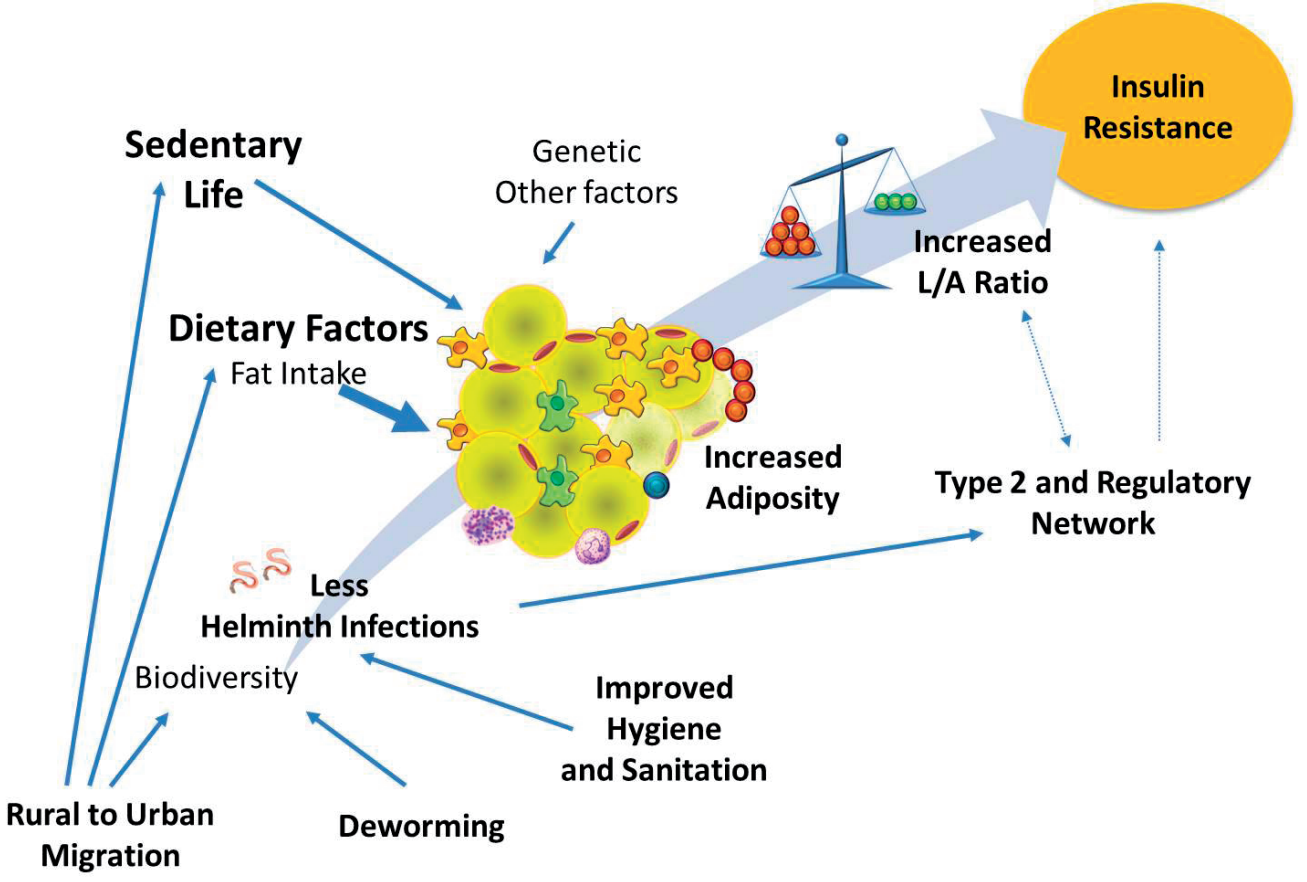

Figure 1. Simplified Schematic Overview on The Contribution of Helminths and Urbanization on The Development of Insulin Resistance. L/A: leptin to adiponectin

also contribute.[50] However, the currently available data shows inconsistent results of the effect of different infection or inflammatory exposures on CETP level,[50, 51] thus further studies are needed to assess this. Next, as CETP also play a role in lipoprotein metabolism[47, 52-54], the questions whether this high CETP level in rural will also affect lipoprotein metabolism and subsequent development of atherosclerosis remains.

Our data, from the RCT and from the HFD study, have shown that adiposity seems to play a central role in the development of IR, as well as in mediating the helminth-associated beneficial effects. Helminth parasites, through affecting food intake or absorption, could indeed influence adiposity and thereby IR. Taken together, a simplified schematic overview on the contribution of helminth infections on the development of IR in the light of urbanisation is summarized in **Figure 1**.

DIRECTIONS FOR FUTURE RESEARCH

Controlled Human Infections

Experimental infections with helminths or helminth-derived molecules in diet-induced obese mice have shown to improve glucose tolerance and increase insulin sensitivity compared to controls [4-7]. Importantly, these experimental studies enable us to investigate the potential mechanisms by which helminths can influence metabolic outcomes. Therefore, controlled human infection (CHI) models will be an excellent approach to unravel the mechanism by which helminth infections can improve metabolic profiles in human.

Human Fat Biopsies

It is important to note that, in helminth-infected subjects, anthelmintic-associated increased IR seems to be mainly mediated by the increase in adiposity, as well as an increase in leptin to adiponectin ratio. Moreover, the difference in IR between subjects living in urban and rural areas is also mainly mediated by the difference in adiposity. Thus, assessments of human adipose tissue biopsies[55-57] could give a powerful grip on the underlying mechanism that mediate the effect of helminths and urbanization on the development of IR.

Gut Microbiome

Intestinal helminths and bacteria reside in the same niche, the human intestine, and might interact with each other and affect the host immune system, nutrition, and metabolism.[58] Interestingly, emerging evidence suggests that the composition of gut microbiota plays a role in the pathogenesis of obesity-associated insulin resistance by affecting energy homeostasis and inflammation [59]. Therefore, alteration of the gut microbiome in the presence of helminth parasites,[60] might contribute to the observed difference in IR. Interestingly, whereas less bacterial diversity was commonly observed among obese people or diabetics [59, 61], greater bacterial diversity was recently reported among subjects infected with *Schistosoma haematobium* [62] and STHs [63]. However, the findings on the effects of STH infections on gut microbiota has not been consistent, as some studies showed them to be associated with a reduced bacterial diversity[64].

The effect of deworming on gut microbiota has also been inconsistent, whereas two studies reported no changes in gut microbiota composition[62, 64], one study reported alteration in gut microbiota composition, most notably, reduced levels of protective bacteria, *Clostridiales*[65]. Furthermore, human experimental

hookworm infection failed to induce changes in bacterial diversity, however it did induce a minor increase in the richness of the microbial species [66]. Interestingly, in mice, helminth infections are associated with an increase of intestinal short chain fatty acids (SCFA) [67], the end products of dietary carbohydrate fermentation, which has been shown to play an important role in the control of body weight and insulin sensitivity [68].

Taken together, helminth-associated diversity of the gut microbiome and amount of SCFAs might mediate the effects of helminths on whole-body IR. However, further studies are needed to confirm these findings, and unravel the complex interaction between helminth infections, gut microbiome, the host immune system and metabolism.

Rural to urban study

With the increasing burden of NCDs, which are mostly reflect the increasing rate of urbanization, additional efforts should be invested in assessing urbanization-associated changes in metabolic homeostasis, immune responses, and inflammation. Specific focus should be put into urbanization-associated decreased biodiversity, which might be potentially related to the emergence of inflammatory diseases,[16] including T2D. This is supported by two studies showing different immune activation profiles between subjects living in rural and urban areas,[69, 70] which suggests that environmental differences may contribute to immunological footprints, thus affecting the development of inflammatory diseases. However, the contribution of these differences in the immune system to the development of IR, hence T2D, remain to be clarified.

DIRECTIONS FOR FUTURE HEALTH POLICY AND CARE

In terms of health policy, control measures for communicable diseases, such as elimination of STH, need to go hand in hand with providing education, prevention, and monitoring the development of major NCDs, especially obesity and T2D. More attention to urbanization-related changes in metabolic health, which is currently lacking, is needed. In terms of health care, it is important to develop locally or nationally practical and sensitive diagnostic tools to detect the presence of NCDs, such as T2D, applicable to many resource-limited rural areas of Indonesia. This, hopefully, will enable us to monitor the development of obesity and T2D, as well as enabling us to reduce the high number of undiagnosed T2D in Indonesia.[71]

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APPENDIX

SUMMARY

In the past few decades, there has been a substantial increase in the number of people with type 2 diabetes (T2D) worldwide, especially in low and middle income countries. In these countries, rapid socioeconomic developments and urbanization have led to changes in diet, lifestyle, and environmental factors. This in turn is associated with complex changes in disease pattern, evident from the increasing prevalence of non-communicable diseases, including T2D, as well as the reduction in communicable diseases. Despite the strong evidence that T2D is associated with increased risk of infectious diseases, and that the presence of infections will worsen the host glucose metabolism, recent evidence suggests that helminth infections might give protection against the development of T2D. In this thesis, we reported our investigations on the impact of helminth infections, in light of increasing urbanization, on the development of T2D in Indonesia.

Chapter 1

Here we discussed the increasing prevalence of non-communicable diseases, especially T2D, in Indonesia, which is largely attributed to the rapid pace of socioeconomic development and urbanization. As a result, there is great geographical variation where high prevalence of T2D is seen in urban areas but low prevalence in rural regions of the country. This might suggest that traditional living environments in rural areas might give a relative protection against the development of T2D. This protection against the development of T2D has been reported to be mainly related to the more physically active lifestyle and healthier traditional diet, however other environmental factors might play a role as well. Several epidemiological studies, conducted in different populations, suggest that helminth infections, which are still endemic in many rural areas of low and middle income countries, including Indonesia, might confer protection against the development of T2D.

Chapter 2

Here we described in detail our study protocol to investigate whether helminth infections have a direct effect on insulin resistance, a marker and strong predictor for the development of T2D. To achieve this, we designed a large-scale randomised population-based, household-clustered, double-blind placebo-controlled trial of anthelmintic treatment in an area endemic for soil-transmitted helminth (STH), on Flores Island, Indonesia. The 12-months anthelmintic treatment regimen consisted of four rounds of three monthly 400mg albendazole or matching placebo given

for three consecutive days under direct supervision. We performed clinical anthropometry measurements, as well blood, urine, and stool sample collections before and after the anthelmintic treatment to assess any changes in insulin resistance (and other metabolic parameters), as well as immune responses, after reduction in STH infection prevalence and intensity.

Chapter 3

This chapter reported the main outcomes of an intensive 12-months anthelmintic treatment. Our intensive treatment significantly reduced STH prevalence and intensity, but did not eliminate these infections. Importantly, anthelmintic treatment in helminth-infected subjects lead to a significant but slight increase in insulin resistance, providing the first causal evidence on the protective effect of helminths on insulin resistance, hence T2D. Furthermore, the increase in insulin resistance was mainly mediated by the increase in body mass index, suggesting the importance of human adipose tissue in helminth-associated beneficial metabolic effects.

Chapter 4

Here we provided additional evidence on the importance of human adipose tissue in helminth-associated beneficial metabolic effects. Anthelmintic treatment in helminth-infected subjects lead to significant changes in two hormones secreted by adipose tissue, namely leptin and adiponectin. We observed an increase in leptin to adiponectin ratio which may contribute to the increase in insulin resistance. While leptin has been associated with inflammation and insulin resistance, the converse has been reported for adiponectin. Thus, leptin to adiponectin ratio can be regarded as a marker of a balance between inflammation and anti-inflammation, and has, in some studies, been reported to be positively associated with insulin resistance.

Chapter 5

In order to assess the contribution of different living environments on the development of T2D, we compared the metabolic profiles between people living in rural and urban area with similar genetic background. People who moved from Flores island to Jakarta had a higher degree of insulin resistance, which was mainly due to the higher adipose tissue mass and leptin level. Increasing time spent in urban area was positively associated with higher adipose tissue mass. Next, to assess whether rural living environments protect against diet-induced insulin resistance, we compared the metabolic response on a high-fat high-calorie diet

(HFD) between rural and urban participants with the same amount of adipose tissue mass. Living in a rural area or having current helminth infection, did not protect against the induction of insulin resistance by short term HFD intervention.

Chapter 6

This chapter summarizes and discusses the main findings of this thesis. First, we have proven that helminth infections do have a causal effect on the development of insulin resistance. Thus, the currently on going deworming programs might accelerate the development of T2D, which advocates that the current deworming program should go hand in hand with efforts to monitor and prevent the development of obesity and T2D. Second, the effect of helminths on the development of T2D seems to be mainly explained by reduction of adipose tissue mass, as well as modulating secretion of adiponectin and leptin. Third, living in urban area is also associated with higher insulin resistance, hence a higher risk to develop T2D, which is mainly explained by a progressive increase in adipose tissue mass with increasing duration of time spent in an urban area. In addition, although it is not a major question in our study, the findings in our deworming trial suggests that the current World Health Organization deworming treatment regimen might need to be intensified to reach helminth elimination.

SAMENVATTING

In de laatste decennia is het aantal mensen met type 2 diabetes (T2D) wereldwijd toegenomen, met name in landen met een laag of gemiddeld inkomensniveau. In deze landen hebben snelle sociaal economische ontwikkelingen en urbanisatie geleid tot veranderingen in dieet, leefstijl en omgevingsfactoren. Dit gaat gepaard met veranderingen in ziekte patronen, zoals blijkt uit de toenemende prevalentie van niet-overdraagbare ziekten zoals T2D, en tegelijkertijd een afnemende prevalentie van overdraagbare ziekten. Hoewel T2D wordt geassocieerd met een vergroot risico op infectieziekten, waarbij de aanwezigheid van infecties het glucose metabolisme van de patiënt verslechtert, hebben recente studies aangetoond dat worminfecties mogelijk bescherming bieden tegen de ontwikkeling van T2D. In dit proefschrift wordt het effect van worminfecties op de ontwikkeling van T2D in Indonesië beschreven in de context van toenemende urbanisatie.

Hoofdstuk 1

In dit hoofdstuk wordt de toenemende prevalentie van niet-overdraagbare ziekten, met name T2D, in Indonesië beschreven. Deze toename wordt grotendeels toegeschreven aan snelle sociaal economische ontwikkelingen en urbanisatie, veranderingen die leiden tot grote geografische variatie waarbij de prevalentie van T2D in stedelijke gebieden hoog en in de landelijke gebieden laag is. Dit zou erop kunnen wijzen dat de traditionele leefomgeving in landelijke gebieden wellicht bescherming biedt tegen de ontwikkeling van T2D. Hoewel deze bescherming met name toe te wijzen is aan de verschillen in leefstijl en dieet, kunnen andere omgevingsfactoren zoals worminfecties ook een rol spelen. Worminfecties komen veel voor in landelijke gebieden van landen met een laag of gemiddeld inkomensniveau zoals Indonesië, en een aantal epidemiologische studies heeft in verschillende populaties aangetoond dat worminfecties wellicht bescherming bieden tegen de ontwikkeling van T2D.

Hoofdstuk 2

Hier beschrijven we het studieprotocol dat is gebruikt om te onderzoeken of worminfecties een direct effect hebben op insuline resistentie, een indicator en voorspeller voor de ontwikkeling van T2D. Deze studie vond plaats op Flores, een eiland in Indonesië, waar veel mensen besmet zijn met wormen in het maag-darm kanaal. Gedurende één jaar is de helft van de onderzoeksgroep behandeld tegen worminfecties en de andere helft met een placebo. Deelnemers ontvingen hiervoor elke drie maanden, drie dagen achter elkaar medicatie tegen worminfecties of een

placebo. Naast het uitvoeren van antropometrische metingen (lengte, gewicht, heup- en tailleomvang), zijn er voor en na de behandeling bloed, urine en feces monsters verzameld om veranderingen in insuline resistentie (en andere metabole parameters) en de immuunrespons te kunnen meten.

Hoofdstuk 3

Dit hoofdstuk beschrijft de uitkomsten van de studie beschreven in hoofdstuk 2. Hoewel de behandeling tegen wormen er toe heeft geleid dat de prevalentie en intensiteit van worminfecties na één jaar sterk verlaagd was, waren er nog steeds enkele mensen besmet met wormen. Toen we het effect van de behandeling op insuline resistentie onderzochten, bleek dat er een kleine, maar significante toename in insuline resistentie was opgetreden in de groep mensen die voor aanvang van de behandeling geïnfecteerd waren. Dit is het eerste directe bewijs waaruit blijkt dat worminfecties een beschermend effect hebben op insuline resistentie, en dus de ontwikkeling van T2D. Daarnaast bleek dat de toename in insuline resistentie vooral was toe te wijzen aan een toename in Body Mass Index (BMI). Het lijkt er dus op dat het vetweefsel een belangrijke rol speelt in het gunstige effect dat worminfecties hebben op het glucose metabolisme.

Hoofdstuk 4

We hebben nader onderzocht hoe het vetweefsel betrokken is bij het gunstige effect van worminfecties op het glucose metabolisme, door te kijken naar het effect van anti-wormen behandeling op twee hormonen die worden uitgescheiden door het vetweefsel, namelijk leptine en adiponectine. Terwijl leptine wordt geassocieerd met inflammatie en insuline resistentie, geldt het tegenovergestelde voor adiponectine. Daarom kan de ratio tussen leptine en adiponectine worden beschouwd als een marker voor ontsteking, en eerdere studies hebben aangetoond dat deze ratio positief geassocieerd is met insuline resistentie. Na één jaar behandeling tegen wormeninfecties zagen we een toename in de ratio tussen leptine en adiponectine in de groep mensen die voorafgaand aan de behandeling geïnfecteerd waren, en dit zou kunnen bijdragen aan de eerder beschreven toename in insuline resistentie.

Hoofdstuk 5

Om te onderzoeken in hoeverre de leefomgeving bijdraagt aan de ontwikkeling van T2D, hebben we het metabole profiel vergeleken tussen mensen die in een landelijk (Flores) versus stedelijk (Jakarta) gebied van Indonesië wonen. De

genetische achtergrond van deze mensen was hetzelfde aangezien de mensen in het stedelijke gebied oorspronkelijk uit Flores kwamen. Het bleek dat de mate van insuline resistentie in de groep mensen in het stedelijke gebied hoger was in vergelijking met mensen in het landelijke gebied. Dit was voornamelijk toe te wijzen aan een hogere vetweefsel massa en een hoger leptine niveau. We zagen dat de tijdsduur die men in de stad had doorgebracht positief geassocieerd was met een hogere vetweefsel massa.

We hebben ook onderzocht of een landelijke leefomgeving bescherming biedt tegen een toename in insuline resistentie die veroorzaakt wordt door een dieet met veel vet en calorieën. Hiervoor zijn de metabole parameters in reactie op dit dieet gemeten in mensen die in een landelijk versus stedelijk gebied wonen, waarbij de deelnemers een vergelijkbare hoeveelheid vetweefsel hadden. De landelijke leefomgeving, inclusief de blootstelling aan wormeninfecties, bood geen bescherming tegen de toename in insuline resistentie die het kortdurende dieet veroorzaakte.

Hoofdstuk 6

In dit hoofdstuk worden de belangrijkste bevindingen van dit proefschrift samengevat en bediscussieerd. We hebben aangetoond dat worminfecties een direct effect hebben op de ontwikkeling van insuline resistentie. Dit betekent dat de huidige behandelprogramma's tegen worminfecties de ontwikkeling van T2D wellicht versnellen, en om die reden zou het goed zijn als er tegelijkertijd ook aandacht wordt besteed aan het monitoren en voorkomen van de ontwikkeling van obesitas en T2D. Daarnaast is gebleken dat het effect van worminfecties op de ontwikkeling van T2D grotendeels kan worden verklaard door een afname in vetweefsel massa en een verandering in de uitscheiding van adiponectine en leptine. Tot slot hebben we gezien dat het wonen in een stedelijk gebied geassocieerd is met een hogere mate van insuline resistentie, en dus een groter risico op de ontwikkeling van T2D. Dit is toe te wijzen aan een sterke toename in vetweefsel massa, gerelateerd aan de tijdsduur die een persoon in de stad heeft doorgebracht. Hoewel het niet de hoofdvraag van onze studie was, tonen de bevindingen in deze studie waarbij mensen één jaar lang behandeld zijn tegen worminfecties aan dat de huidige behandelprogramma's van de Wereldgezondheidsorganisatie (WHO) wellicht moeten worden geïntensiveerd om eliminatie van wormen te bereiken.

CURRICULUM VITAE

Dicky Levenus Tahapary was born in Surakarta, Central Java, Indonesia on the 30th of December 1980. He completed his medical doctor (MD) degree in August 2005 from the Faculty of Medicine, Universitas Indonesia (FKUI), Jakarta, Indonesia. Thereafter, he worked as a research assistant for 12 months under the supervision of Professor Pradana Soewondo at the Division of Metabolism and Endocrinology, Department of Internal Medicine FKUI, of which he was later on appointed as an academic staff after completing his training as an internal medicine specialist in January 2011.

In 2013, he was selected as a PhD candidate for a collaborative research project between FKUI and Leiden University Medical Centre (LUMC), funded by The Royal Netherlands Academy of Arts and Sciences (KNAW), specifically the Scientific Programme Indonesia-Netherlands (SPIN). The joint programme entitled "Helminth infections and type 2 diabetes mellitus in Indonesia: integrating parasitological, immunological and metabolic studies (SugarSPIN), with Professor Johannes W.A. Smits (Department of Internal Medicine LUMC and Radboud University Medical Centre), Professor Taniawati Supali (Department of Parasitology FKUI), and Professor Maria Yazdanbakhsh (Department of Parasitology LUMC) as the Dutch, Indonesian, and scientific coordinators, respectively.

After completing the field work in Nangapanda, Flores under the supervision of Professor Taniawati Supali and the rural-urban high-fat diet intervention study in both Flores and Jakarta under the supervision of Professor Pradana Soewondo, he worked at the Department of Parasitology LUMC under supervision of Professor Maria Yazdanbakhsh, Professor Johannes W.A. Smit and Dr. Erliyani Sartono. In the last 6 months of his PhD, he conducted additional research in the Department of Clinical Epidemiology LUMC.

His PhD fellowship was awarded by the Directorate of Higher Education, Republic of Indonesia (2013-2016) and Leiden University (2017). He also received research grants from Universitas Indonesia (2015), as well as from Ministry of Research, Technology, and Higher Education, Republic of Indonesia (2015 and 2016). After completion of his PhD, he will return to FKUI and combine clinical work with research and teaching. He also plans to complete his training to super specialise in endocrinology. Most of his research activities will be in the Indonesian Medical

Education and Research Institute, of which he was recently appointed as a vice chair of the Metabolic Disorders, Cardiovascular, and Aging Cluster. He plans to pursue research in his area of interest which is the field of obesity and T2D, especially in the context of epidemiological transition, and will continue to work closely with the Nangapanda Community Research Centre.

LIST OF PUBLICATIONS

Tahapary DL, de Ruiter K, Martin I, Brienen EAT, van Lieshout L, Djuardi Y, Djimandjaja CC, Houwing-Duistermaat JJ, Soewondo P, Sartono E, Supali T, Smit JWA, Yazdanbakhsh M. *Effect of anthelmintic treatment on leptin, adiponectin, and leptin to adiponectin ratio: a randomized controlled trial*. (submitted)

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