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Exposure to pesticides in present-day use, diabetes mellitus and lung function impairment

PhD dissertation

Martin Rune Hassan Hansen

Health
Aarhus University

The National Research Center for the Working Environment

2020

The thesis has been assessed to be acceptable for oral public defense. When this document was published on-line (April 29, 2020), the oral public defense had not yet taken place, and the PhD degree had not yet been awarded.

Cover illustration

Data collection team in the “Pesticide Exposure, Asthma and Diabetes in Uganda” project.

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Preface

This thesis concludes the PhD project entitled "Exposure to pesticides in present-day use, diabetes mellitus and lung function impairment" that was conducted at the research unit for Environment, Work and Health at the Department of Public Health at Aarhus University, and at the National Research Center for the Working Environment.

I became affiliated with the research unit for Environment, Work and Health while I was a medical student. In 2012-2013, I was enrolled as a research year student and carried out a study on health effects of long-term insecticide exposure among publically employed spraymen in Bolivia, mainly under the supervision of Professor Vivi Schlünssen (then Assistant Professor).

After graduating as a medical doctor and two years of clinical work, I was enrolled as a PhD student in 2017, again with Vivi Schlünssen as main supervisor. The original focus of the PhD project was the possible link between pesticide exposure and diabetes mellitus; as my supervisors and I developed the project further, we decided to also investigate respiratory effects of pesticide exposure. To investigate these issues, we have conducted a cross-sectional study in the general population of a semi-urban area of Nepal, and a follow-up study among smallholder farmers in Uganda. Both projects were carried out in close collaboration with partners from other research units at Aarhus University, as well as NGO and academic partners from Nepal and Uganda.

The thesis provides an overview of the existing evidence on diabetes-related and respiratory effects of insecticide exposure, summarizes findings from the epidemiological studies conducted as part of the PhD project, and discusses the methods applied. It is my hope that the findings will be useful for the cost-benefit analyses that are necessary for the rational use of pesticides.

Martin Rune Hassan Hansen

Martin Rune Hassan Hansen, January 2020

Acknowledgements

When I started my PhD three years ago, I could not imagine how it would proceed in the end. Through circuitous routes, I ended doing a study on another continent and more than a year later than expected. I am grateful for my experiences during the past three years, and I wish to thank all the people who helped me succeed in my endeavors.

I would like to thank my main supervisor Vivi Schlünssen for all the support she has given me since I became affiliated with the department in 2012. I am grateful for her trust and for how free I have been to work on high-risk research projects. I have benefitted greatly from the experience of my co-supervisor Erik Jørs; without his advice on how to conduct studies in low- and middle-income countries, I would not have succeeded. I would also like to thank my other supervisors - Flemming Lander and Annelli Sandbæk - for their support, helpful insights and constructive criticism.

I wish to thank Bishal Gyawali, Dinesh Neupane and Per Kallestrup of the COBIN-D project for letting me become part of the study group and collecting data on my behalf in a challenging environment.

The PEXADU project was planned and carried out in close collaboration with partners from Uganda National Association for Community and Occupational Health (UNACOH) and Makerere University School of Public Health (MakSPH) in Kampala, Uganda. I owe my thanks to all of those who have collaborated with me, but especially Daniel Sekabojja, Victoria Nabankema, Aggrey Atuhaire and Dr. Deogratias Sekimpi from UNACOH, as well as Dr. John Ssempebwa and Ruth Mubeezi from MakSPH.

I would like to thank my team of data collectors from Uganda, who worked hard to ensure the success of the PEXADU project. In alphabetical order, they are Amusa Wamawobe, Betty Kateregga, Brenda Wagaba, Evans Twin, Grace Lubega, Jonathan Mugweri, Imelda Namatovu, Joviah Gonza, Lydia Yariwo and Timothy Masaba.

I am grateful to Philipp Staudacher and Samuel Fuhrimann of the “Pesticide Use in Tropical Settings” (Pestrop) project that they agreed to collaborate on the PEXADU project and let me reuse parts of their data collection tools. I also owe my thanks to Marie Frederiksen and Jörg Schullehner for a fruitful collaboration.

I am also grateful to all the participants in both the COBIN-D and the PEXADU projects, without whom the project would have been impossible. I wish to thank the Nepal Development Society, Diálogos,

Caritas Uganda, the Agency for Integrated Rural Development and the Wakiso District Farmer's Association for their invaluable help during the projects.

I am grateful to my parents for all they have taught me and for their moral support during my project. Finally, I would like to thank my wonderful wife Wajd who has supported me throughout my PhD. She has put up with listening to my frustrations caused by never-ending changes to the project plans, has accepted life as a grass widow while I spent seven months collecting data in Uganda, reminded me to (sometimes) have a life apart from the project, and even became part of the project team. None of this could be taken for granted, and I am deeply grateful.

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Abstract

Background and aims

Epidemiological studies have associated exposure to pesticides with increased risk of diabetes mellitus and decreased lung function, but many of the previous studies are cross-sectional and with inadequate confounder control. We aimed to investigate the correlations between exposure to cholinesterase inhibitor insecticides (organophosphates and carbamates), blood sugar levels and lung function while accounting for important confounders.

Methods

From October 2016 to April 2017, we conducted a nested cross-sectional study as part of the Community Based Intervention for Management of Diabetes in Nepal (COBIN-D) trial. We measured fasting plasma glucose among 2,310 persons from the general population of a semi-urban area of Nepal, and collected questionnaire-based subjective information on pesticide exposure and confounders. Odds of diabetes mellitus were analyzed in logistic regression models.

Next, we conducted the "Pesticide Exposure, Asthma and Diabetes in Uganda" (PEXADU) study: a short-term follow-up study among 364 smallholder farmers in Uganda. At baseline in September-October 2018 and at follow-up in November-December 2018 and January-February 2019, we measured each participant's glycated hemoglobin A (HbA_{1c}), a measure of average blood sugar level in the last 8-12 weeks. We quantified exposure to cholinesterase inhibitor insecticides using red blood cell acetylcholinesterase activity normalized by hemoglobin concentration (AChE/Hb). Lung function was quantified by spirometry, and spirometric indices converted to Z-scores using the Global Lung Function Initiative equations. Information on confounders was collected by questionnaire. We analyzed data in linear mixed effect models accounting for family relationships and repeated measurements.

Results and discussion

In the COBIN-D population, the risk of diabetes mellitus was lower among subjects who reported that they had ever used pesticides – adjusted OR 0.68 [0.52; 0.90], but exposure levels were relatively low, and there were no clear exposure-response relationships. Large demographic differences between the exposed and non-exposed groups mean that residual confounding is likely.

In the PEXADU study, low acetylcholinesterase activity (indicating exposure to organophosphate and carbamate insecticides) was associated with decreased HbA_{1c}. Compared to reference subjects with

AChE/Hb 25.8 U/g (50th percentile), subjects with AChE/Hb 24.3 U/g (35th percentile) had HbA_{1c} that was 0.74 [0.17; 1.31] mmol/mol lower in the adjusted analysis, and subjects with ACHE/Hb 27.1 U/g (65th percentile) had HbA_{1c} 0.63 [0.12; 1.14] mmol/mol higher than the reference. Low acetylcholinesterase activity was also associated with decreased pulmonary function. In the adjusted analysis, Z-score for FEV_1 (forced expiratory volume in 1 second) was 0.045 [0.003; 0.087] lower for subjects with AChE/Hb 24.5 U/g (35th percentile) than for reference subjects with AChE/Hb 25.9 U/g (50th percentile), and subjects with AChE/Hb 27.3 U/g (65th percentile) had FEV_1 Z-score 0.043 [-.002; 0.087] higher than the reference. Demographic variables were similar between subjects with AChE/Hb below and above the median, making residual confounding less likely.

Conclusion

Our results do not support a causal link between exposure to cholinesterase inhibitor insecticides and diabetes mellitus. The association between low acetylcholinesterase activity and decreased HbA_{1c} in the PEXADU study may be due to reverse causality. We demonstrated an association between low acetylcholinesterase activity and decreased lung function. Evidence from human exposure studies with less toxic cholinesterase inhibitors indicate that the association between acetylcholinesterase and pulmonary function may represent a causal effect of exposure to cholinesterase inhibitor insecticides, and efforts to limit exposure should be strengthened.

Resumé

Baggrund og formål

Epidemiologiske studier har vist en sammenhæng mellem eksponering for pesticider og øget risiko for diabetes mellitus og nedsat lungefunktion, men mange af studierne var tværsnitsstudier og tog ikke tilstrækkelig højde for andre faktorer, der kan påvirke risikoen for diabetes og lungefunktionsnedsættelse. Formålet med dette projekt var at undersøge sammenhængene mellem eksponering for insektgifte, der virker ved at hæmme enzymet acetylkolinesterase (organofosfater og carbamater), blodsukkerniveau og lungefunktion, i et stærkt studiedesign og under hensyntagen til andre risikofaktorer.

Metoder

Fra oktober 2016 til april 2017 gennemførte vi et tværsnitsstudium som en del af et klinisk forsøg med titlen "Community-Based Intervention for Management of Diabetes in Nepal" (COBIN-D). Vi målte fasteblodsukker blandt 2.310 personer fra baggrundsbefolkningen i et semi-urbaniseret område i det centrale Nepal, og indsamlede subjektive informationer omkring brug af pesticider og andre risikofaktorer ved hjælp af spørgeskemaer. Odds for diabetes mellitus blev analyseret i logistiske regressionsmodeller.

Herefter gennemførte vi studiet "Pesticide Exposure, Asthma and Diabetes in Uganda" (PEXADU): Et korttids-opfølgningsstudium blandt 364 småbønder i Uganda. Vi målte deltagernes HbA_{1c} (glykosyleret hæmoglobin A, et mål for gennemsnits-blodsukkeret i de sidste 8-12 uger) ved hvert af tre besøg i september-oktober 2018, november-december 2018 og januar-februar 2019, og vi målte den hæmoglobin-justerede enzymaktivitet af acetylkolinesterase (AChE/Hb) i deltagernes blod som udtryk for eksponering for acetylkolinesterase-hæmmende insektgifte. Lungefunktion blev testet ved hjælp af spirometri, og spirometriske mål konverteret til Z-værdier ved hjælp af reference-ligninger fra Global Lung Function Initiative. Information omkring kendte risikofaktorer blev indsamlet ved hjælp af spørgeskemaer. Vi analyserede data i lineære *mixed effect* modeller, der tog hensyn til slægtskab mellem deltagere samt de gentagne målinger af både eksponering og helbredsudfald.

Resultater og diskussion

I COBIN-D population fandt vi en lavere forekomst af diabetes mellitus blandt personer, der havde brugt pesticider, end blandt personer der aldrig havde brugt pesticider: Justeret Odds Ratio 0,68 [0,52; 0,90], men eksponeringsniveauerne var relativt lave, og der var ingen klare eksponerings-

responssammenhænge. Vi fandt store demografiske forskelle mellem de eksponerede og ikke-eksponerede personer, og vi finder derfor residual-confounding sandsynlig.

I PEXADU-studiet fandt vi en statistisk sammenhæng mellem lav AChE/Hb (tydende på eksponering for organofosfater og carbamater) og nedsat HbA_{1c}. Sammenlignet med reference-personer med AChE/Hb på 25,8 U/g (50-percentil) havde personer med AChE/Hb på 24,3 U/g (35-percentil) 0,74 [0,17; 1,31] mmol/mol lavere HbA_{1c} efter justering for kendte risikofaktorer, og personer med AChE/Hb på 27,1 U/g (65-percentil) havde 0,63 [0,12; 1,14] mmol/mol højere HbA_{1c} end reference-personerne. Samtidig var lav AChE/Hb associeret med nedsat lungefunktion. Efter justering for kendte risikofaktorer var Z-værdien for FEV_1 (forceret udåndingsvolumen i første sekund) 0,045 [0,003; 0,087] lavere for personer med AChE/Hb 24,5 U/g (35-percentil) end for reference-personer med AChE/Hb 25,9 U/g (50-percentil), mens personer med AChE/Hb 27,3 U/g (65-percentil) havde en FEV_1 Z-værdi der var 0,043 [-0,002; 0,087] over reference-personernes. Personer med AChE/Hb over og under medianen havde stort set ens demografiske karakteristika, hvorfor residual-confounding næppe forklarer vores resultater.

Konklusion

Vores resultater understøtter ikke hypotesen om en årsagssammenhæng mellem eksponering for kolinesterase-hæmmende insektgifte og diabetes mellitus. Den statistiske sammenhæng mellem lavt niveau af acetylkolinesterase og nedsat HbA_{1c} i PEXADU-studiet kan muligvis skyldes, at blodsukkeret påvirker acetylkolinesterase, i stedet for omvendt. Vi fandt en statistisk sammenhæng mellem lav acetylkolinesterase-aktivitet (foreneligt med eksponering) og nedsat lungefunktion. Tidligere eksponeringsforsøg på mennesker, udført med det mindre giftige kolinesterase-hæmmende stof pyridostigmin, understøtter at den statistiske association mellem acetylkolinesterase og lungefunktion kan skyldes en årsagssammenhæng. For at beskytte helbredet hos bønder og sprøjtearbejdere bør eksponering begrænses mest muligt.

List of tables

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Abbreviations

ACh	Acetylcholine
AChE	Red blood cell acetylcholinesterase
AChE/Hb	Red blood cell acetylcholine esterase normalized by hemoglobin concentration
ATS	American Thoracic Society
AU	Aarhus University, Aarhus, Denmark
BMI	Body mass index
BChE	Butyrylcholinesterase
CI	Confidence interval
COBIN-D	"Community-based Intervention for Management of Diabetes in Nepal" (project title)
DM	Diabetes mellitus
DDT	Dichloro-diphenyl-trichloroethane
eAG	Estimated average glucose
FEF_{25}	Forced expiratory flow at 25% FVC
FEF_{50}	Forced expiratory flow at 50% FVC
FEF_{75}	Forced expiratory flow at 75% FVC
FEF_{25-75}	Forced expiratory flow at 25-75% FVC
FEM	Fixed effect model
FEV_1	Forced expiratory volume in 1 second
FEV_1/FVC	FEV_1 divided by FVC
FPG	Fasting plasma glucose
FVC	Forced vital capacity
G6PD	Glucose-6-phosphate dehydrogenase
GDM	Gestational diabetes mellitus
G-IGT	Gestational IGT
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
HOMA-IR	Homeostatic model assessment of insulin resistance
HbA_{1c}	Glycosylated hemoglobin A
MakSPH	Makerere University School of Public Health, Kampala, Uganda
NGSP	National Glycohemoglobin Standardization Program (http://www.ngsp.org)
ODK	Open Data Kit (https://opendatakit.org)
PEXADU	"Pesticide Exposure, Asthma and Diabetes in Uganda" (project title)
$PM_{2.5}$	Particulate matter with an aerodynamical diameter < 2.5 µm
PM_{10}	Particulate matter with an aerodynamical diameter < 10 µm
PI	Prediction interval
RCM	Random coefficient model
SE	Standard error
SD	standard deviation
UNACOH	Uganda National Association of Community and Occupational Health
WHO	World Health Organization

List of original papers

During my enrolment at Aarhus University, I have contributed to the following papers as first author:

Paper I (published)

Hansen MRH, Jørs E, Sandbæk A, Kolstad HA, Schullehner J, Schlünssen V (2019). Exposure to neuroactive non-organochlorine insecticides, and diabetes mellitus and related metabolic disturbances: Protocol for a systematic review and meta-analysis. Environment international, 127, 664-670. https://doi.org/10.1016/j.envint.2019.02.074

Paper II (published)

Hansen MRH, Gyawali B, Neupane D, Jørs E, Sandbæk A, Kallestrup P, Schlünssen V (2019). Pesticide exposure and diabetes mellitus in a semi-urban Nepali population: a cross-sectional study [published online ahead of print December 14, 2019]. International Archives of Occupational and Environmental Health. https://doi.org/10.1007/s00420-019-01508-2

Paper III (submitted)

Hansen MRH, Schlünssen V, Sandbæk A. HemoDownloader: A utility for downloading data from HemoCue HbA1c 501 devices using a graphical user interface.

Paper IV (submitted)

Hansen MRH, Jørs E, Sandbæk A, Sekabojja D, Ssempebwa J, Mubeezi R, Staudacher P, Fuhrimann S, Burdorf A, Bibby BM, Schlünssen V. Correlation between low red blood cell acetylcholine esterase and low blood glucose level in a population of small-scale Ugandan farmers: A short-term follow-up study.

Paper V (submitted)

Hansen MRH, Jørs E, Sandbæk A, Sekabojja D, Ssempebwa J, Mubeezi R, Staudacher P, Fuhrimann S, Sigsgaard S, Burdorf A, Bibby BM, Schlünssen V. Organophosphate and carbamate insecticide exposure is related to decreased pulmonary function among smallholder farmers in Uganda: A short-term follow-up study.

Paper VI (submitted)

Hansen MRH, Schlünssen V. Pyridostigmine impairs pulmonary function in asthmatic subjects: Re-analysis of results from an observational study.

In addition, I have contributed as co-author to the three papers below that do not form part of this dissertation, but are provided as appendices. They give context for the findings in the dissertation itself, as the studies were conducted as part of the same projects.

Supplementary paper I (published)

Gyawali B, **Hansen MRH**, Povlsen MB, Neupane D, Andersen PK, McLachlan CS, Sandbæk A & Kallestrup P (2018). Awareness, prevalence, treatment, and control of type 2 diabetes in a semi-urban area of Nepal: Findings from a cross-sectional study conducted as a part of COBIN-D trial. PloS ONE, 13(11), e0206491. https://doi.org/10.1371/journal.pone.0206491

Supplementary paper II (published)

Gyawali B, Mishra SR, Ghimire S, **Hansen MRH**, Shah KJ, Subedee KC, Soti PB, Neupane D, Kallestrup P (2019). The burden and correlates of multiple cardiometabolic risk factors in a semi-urban population of Nepal: a community-based cross-sectional study. Scientific reports, 9(1):1-10. https://doi.org/10.1038/s41598-019-51454-9

Supplementary paper III (submitted)

Hansen WAH, Schlünssen V, Jørs E, Sekabojja D, Ssempebwa J, Mubeezi R, Staudacher P, Fuhrimann S, **Hansen MRH**. Vitalograph copd-6 mini-spirometer as more than a screening device: Validation in a healthy Ugandan population.

1 Introduction

The term "pesticide" is defined in the following manner by the European Commission:[1]

> *"A 'pesticide' is something that prevents, destroys, or controls a harmful organism ('pest') or disease, or protects plants or plant products during production, storage and transport."*[1]

By definition, pesticides are therefore bioactive compounds with detrimental effects on their target organism (e.g., weeds, insects, rodents). Depending on dose and route of exposure, some pesticidal compounds may also adversely affect the health of humans.

As the global population continues to grow,[2] so does the need for food and intensification of agriculture, and the use of pesticides is steadily increasing. From 1990 to 2017, the global consumption of agricultural pesticides grew ~80% from 2.3 million tons to 4.1 million tons of active compounds[3] – excluding residential use of pesticides and use in public health programs targeting insects that spread diseases such as malaria, dengue and Chagas' disease.

Rational use of pesticides in agriculture and public health programs requires careful consideration of the balance between possible costs (acute intoxications, chronic health effects of exposure, monetary costs) and benefits (increased agricultural production, better food security, and lower risk of insect-borne diseases). One of the prerequisites of such cost-benefit analyses is detailed knowledge of adverse health effects of exposure to specific pesticide compounds, and of exposure-response relationships.

As detailed below, there are indications that exposure to some currently used classes of pesticides may increase the risk of diabetes mellitus[4] and airway obstruction.[5] Due to the considerable morbidity and mortality associated with these diseases,[6] and the widespread use of pesticides in modern agriculture,[3] such effects could be of substantial importance for public health. The overall aim of this PhD project was therefore to investigate the possible link between exposure to currently used agricultural pesticides and the risk of diabetes mellitus and obstructive airway diseases, and to examine exposure-response relationships. Specific aims of individual studies are listed on page 20.

2 Background

2.1 Classification of pesticides

Due to the broadness of the term "pesticide",[1] a high number of chemical compounds can be classified as pesticides. On January 4, 2020, the European Union database of pesticides contained 1,417 separate active compounds.[7]

Pesticides may be classified based on their target organisms. E.g., herbicides target unwanted plants, insecticides target insects, fungicides target fungi, and rodenticides target rodents such as rats and mice. Due to inherent biological differences between the target organisms, different classes of pesticides have very different toxicodynamic modes of action; a few examples will be provided here. The herbicide glyphosate inhibits a plant enzyme involved in the synthesis of the amino acids tryptophan, tyrosine, and phenylalanine.[8] Pyrethroid insecticides such as permethrin, and the organochlorine DDT (dichloro-diphenyl-trichloroethane) are nerve toxins, slowing the closure of voltage-gated sodium channels in excitable cells.[9] Organophosphate and carbamate insecticides inhibit the enzyme acetylcholinesterase in the nervous system as further detailed on page 4.[9]

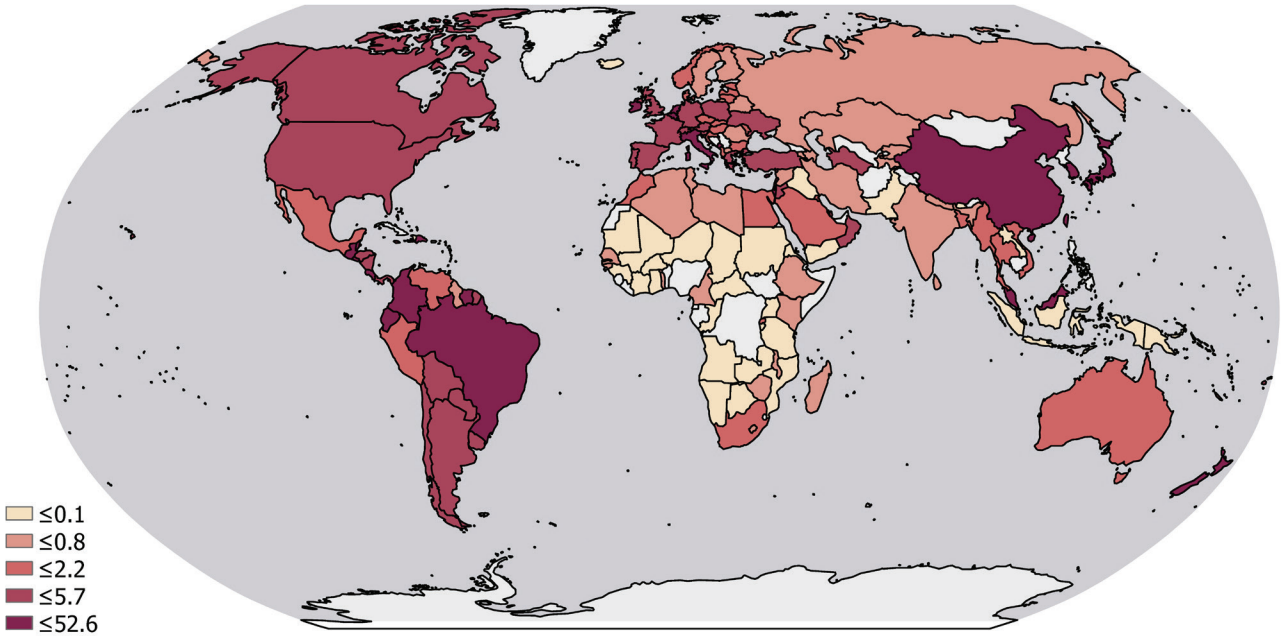
The sheer number of chemical compounds classified as pesticides, and their various modes of action, makes it meaningless to discuss health effects of "pesticides" as a group. Mechanistically, there is no reason to believe that e.g. glyphosate and organophosphate insecticides have similar effects in the human organism. In my PhD. project, I primarily focused on organophosphate and carbamate insecticides, because they share a well-known mode of action,[9] are widely used in agriculture[8] and public health programs targeting malaria,[10] and have well-established biomarkers.[11] Previous reports have suggested that exposure to these pesticides have detrimental health effects in humans, as described in sections 2.3, 2.5 and 2.7.

2.2 Global pattern of pesticide use

Pesticide use measured in kg of active compound per hectare of cropland varies between countries by orders of magnitude, as shown in Figure 2-1. E.g., the Maldives 52.6, mainland China 13.1, Brazil 5.9, Denmark 1.1, Nepal 0.2 and Uganda 0.01 kg/hectare in 2017.[3] It should be noted that farmers in low- and middle-income countries might have higher exposure levels than immediately expected based on such statistics, due to a lack of training on safe pesticide handling and limited use of personal protective equipment. This has been shown by previous studies in e.g. Nepal[12] and

Uganda,[13] [14] and is illustrated in Figure 2-2. Such exposure may put farmers at high risk of adverse health effects.

Figure 2-1: Consumption of agricultural pesticides in 2017



Countries color-coded by quintile of pesticide use, in kg active compound per hectare of cropland per year. Data unavailable for gray areas. Map created using Esri ArcGIS Pro (Esri, Redlands, California, USA). Credits: © EuroGeographics for the administrative boundaries. Data on pesticide use and cropland provided by the Food and Agriculture Organization of the United Nations.[3] Basemap provided by Esri, HERE, Garmin, © OpenStreetMap contributors, and the GIS user community.

Figure 2-2: Ugandan smallholder farmer applying pesticides while wearing minimal personal protective equipment

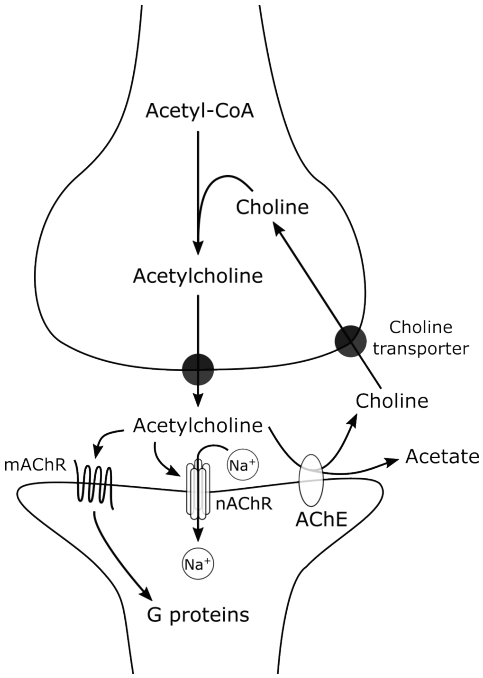
Photo by Martin Rune Hassan Hansen. Eastern Region, Uganda, December 2018.

2.3 Toxicology of cholinesterase inhibiting insecticides

The main toxicodynamic target of both organophosphate and carbamate insecticides is the nervous system enzyme acetylcholinesterase.[9] To aid the understanding of the possible health effects of exposure to these insecticides, the following section provides a brief description of normal cholinergic neurotransmission and the role played by acetylcholinesterase under physiological conditions.

Acetylcholine (ACh) is a neurotransmitter found in the central and peripheral nervous systems, as well as at the neuromuscular endplate (i.e., the place of contact between motor neurons and skeletal muscle fibers).[15 16] Figure 2-3 shows a schematic presentation of neurotransmission at a cholinergic synapse. Upon arrival of an electrical impulse, vesicles containing ACh are released from the axon of the presynaptic nervous cell into the synaptic cleft. ACh diffuses across the synaptic cleft and binds to ACh receptors on the surface of the postsynaptic cell to trigger a response.[15] The enzyme acetylcholinesterase (AChE) is located in the postsynaptic membrane and breaks down ACh into acetate and choline to terminate the signal. Choline can then be re-absorbed by the presynaptic cell for synthesis of new acetylcholine.[15]

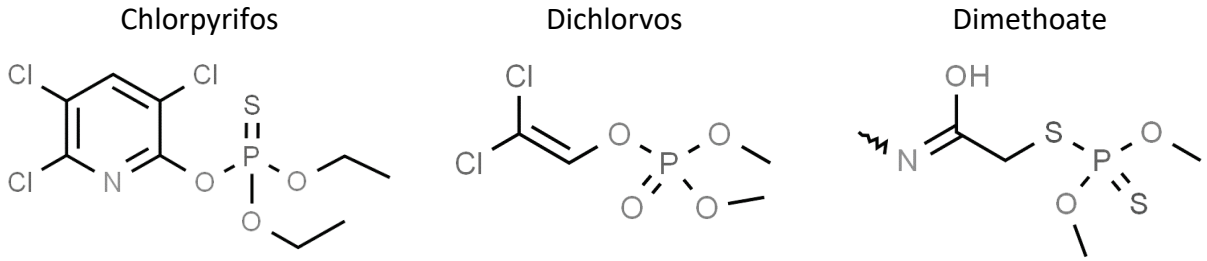
Figure 2-3: Schematic presentation of cholinergic neuronal transmission at a synapse



See text for details. Acetyl-CoA = acetyl-coenzyme A. mAChR = muscarinic acetylcholine receptor. nAChR = nicotinic acetylcholine receptor. Na^+ = sodium ion.

Organophosphate insecticides are chemical derivatives of phosphoric acid.[17] Structural formulas of some example organophosphate insecticides are shown in Figure 2-4. The compounds bind covalently to the active site of the AChE enzyme, inactivating the enzyme. This leads to a build-up of ACh in the synaptic cleft and over-stimulation of ACh receptors on the postsynaptic cell.[17] The AChE may undergo spontaneous reactivation, or it may undergo a chemical process known as "aging" that leads to permanent inactivation of the enzyme, after which enzyme activity can only be reestablished by the synthesis of new enzyme molecules.[17]

Figure 2-4: Structural formulas of some organophosphate insecticides



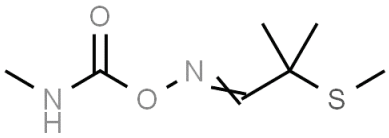
All formulas provided by ChemSpider.[18]

While inhibition of the AChE enzyme in the nervous system is responsible for the desired effect of organophosphate insecticides on target pests,[9] the same inhibition happens in humans who are exposed to the compounds. Acute high-dose exposure to organophosphate insecticides in humans leads to over-activity in the part of the nervous system that uses AChE as neurotransmitter. Signs of acute intoxication include increased secretions (salivation, bronchorrhea, diarrhea, etc.), bronchoconstriction, incontinence, bradycardia, muscle weakness, fasciculations, convulsions and coma. Respiratory failure may develop due to a combination of bronchoconstriction, bronchorrhea, pulmonary edema, muscle weakness and central respiratory depression.[17] The severity of symptoms and the risk of death depends on the compound, the dose and the route of exposure.[17]

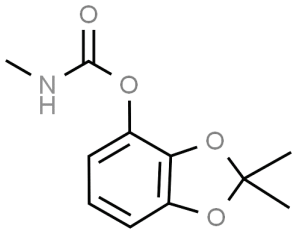
Carbamate insecticides are esters of N-methyl carbamic acid; example structures are shown in Figure 2-5. Carbamates also inhibit acetylcholinesterase, but the mechanism involved is different from the one of organophosphates. Carbamates are hydrolyzed by acetylcholinesterase, but in the process, they temporarily inactivate the enzyme. It spontaneously reactivates within minutes to a few hours, and "aging" of the enzyme does not occur. Because of this reactivation, and because many carbamates have short half-lives in the body and do not readily cross the blood-brain-barrier, acute carbamate intoxication in humans is seldom as severe as organophosphate poisoning.[17]

Figure 2-5: Structural formulas of some carbamate insecticides

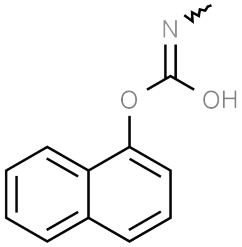
Aldicarb



Bendiocarb



Carbaryl



All formulas provided by ChemSpider.[18]

2.4 Diabetes mellitus

"Diabetes mellitus" is an umbrella term covering a heterogeneous group of diseases with considerable overlap in pathophysiology, the common denominator being hyperglycemia. In this thesis, the terms "diabetes mellitus" and "diabetes" will be used interchangeably; diabetes insipidus is an entirely different disease and will not be discussed.

According to the American Diabetes Association,[19] diabetes mellitus may be broadly categorized in the following manner:

1. Type 1 diabetes mellitus
2. Type 2 diabetes mellitus
3. Gestational diabetes mellitus
4. Other specific types of diabetes mellitus

Type 1 diabetes makes up 5-10% of all cases of diabetes mellitus. It is characterized by an absolute lack of insulin from the endocrine pancreas. Most, but not all cases are mediated by autoimmunity.[19] Type 2 diabetes mellitus makes up > 90% of all cases of diabetes mellitus, and is caused by a relative insulin deficiency, due to a combination of insulin resistance and decreased insulin production.[19] Gestational diabetes mellitus is any diabetes diagnosed in the second or third trimester of pregnancy in a woman who did not have overt diabetes before pregnancy.[19] In addition to these three main categories, lower numbers of diabetes mellitus are caused by the specific factors such as diseases of the exocrine pancreas,[19] monogenic diseases,[19] other endocrine diseases,[20] and some chemical exposures.[19]

Some risk factors for diabetes mellitus are well known. The risk of type 1 diabetes is elevated among individuals with specific Human Leukocyte Antigen (HLA) genotypes.[19] Type 2 diabetes mellitus is

often termed a “lifestyle disease”, as the risk of the disease increases with overweight/obesity and physical inactivity. In addition, genetic background plays a large role for type 2 diabetes.[19] Gestational diabetes shares many of its risk factors with type 2 diabetes, and a woman diagnosed with gestational diabetes has an elevated risk of developing type 2 diabetes later in life.[19]

As the common denominator of all forms of diabetes is hyperglycemia, quantification of blood glucose levels is central to diagnosis. Fasting plasma glucose (FPG), oral glucose tolerance test (OGTT) and glycated hemoglobin A (HbA_{1c}) are all considered acceptable examinations for the diagnosis of diabetes mellitus,[19] even though the different tests may not classify the same persons as diabetic or non-diabetic.[19] Current diagnostic cut-offs are listed in Table 2-1. Clinical diagnosis of diabetes mellitus normally requires that an abnormal test be repeated at least once, unless there are clear clinical signs and symptoms compatible with hyperglycemia.[19] However, in epidemiological studies participants are often classified as diabetic or healthy based on a single test.[21]

Table 2-1: Current diagnostic cut-offs for the diagnosis of diabetes mellitus and prediabetes

Parameter	Unit	Normal	Prediabetes	Diabetes
Fasting plasma glucose	mmol/L	$x \leq 6.9$	$5.6 \geq x \leq 6.9$	$x \geq 7.0$
	g/dL	$x \leq 99$	$100 \geq x \leq 125$	$x \geq 126$
2-hour plasma glucose during 75g OGTT	mmol/L	$x \leq 7.7$	$7.8 \geq x \leq 11.9$	$x \geq 11.1$
	g/dl	$x \leq 139$	$140 \geq x \leq 199$	$x \geq 200$
HbA_{1c}	mmol/mol	$x \leq 38$	$39 \geq x \leq 47$	$x \geq 48$
	% NGSP	$x \leq 5.6$	$5.7 \geq x \leq 6.4$	$x \geq 6.5$

Source: American Diabetes Association.[19] The World Health Organization sets the cut-off for impaired fasting glucose higher, at 6.1 mmol/L = 110 mg/dL.[21] Gestational diabetes mellitus is diagnosed only based on OGTT, and with different cut-offs.[19] NGSP = National Glycohemoglobin Standardization Program.[22] OGTT = oral glucose tolerance test.

2.5 Cholinesterase inhibitor insecticides as risk factors for diabetes mellitus

A previous systematic review has shown a statistically significant correlation between exposure to pesticides and the risk of diabetes mellitus: OR 1.58 [1.32; 1.90] for the top vs. the bottom tertile of exposure to any pesticides.[4] However, almost all studies included in the meta-analysis investigated only organochlorine insecticides[4] that have a very small share of the pesticide market.[23] Important evidence might have been missed due to a search strategy that was too narrow (e.g., lacking search terms for individual pesticidal compounds), and because the review focused only on outright diabetes mellitus and did not include studies on blood glucose levels as a continuous metric. Therefore, we planned a new systematic review on the association between exposure to neuroactive non-organochlorine insecticides, diabetes mellitus and related metabolic disturbances (hyperglycemia, insulin resistance and decreased insulin production). To ensure transparency, the protocol for the systematic review was peer-reviewed and published (Paper I) before the review started. At the time of writing, the systematic review is still ongoing. In the following sections, I will briefly describe the methods employed in the systematic review, followed by a presentation of preliminary findings.

2.5.1 Scoping review of epidemiological evidence

2.5.1.1 Methods

As described above, the term “pesticides” covers a huge amount of different compounds, often with very different modes of action. Therefore, it does not make sense to review them as one group. We decided to focus on non-organochlorine neuroactive insecticides because they have an 85% share of the insecticide market and target the same organ system.[23]

Studies eligible for the systematic review were any human epidemiological or exposure studies (except ecological studies) presenting a measure of association between exposure to any neuroactive non-organochlorine insecticide and the risk of diabetes mellitus, or continuous measures of glycemic regulation. Studies on unspecified “insecticides” or “pesticides” were not included. In the protocol (Paper I), we specified that we would exclude studies in populations with co-exposure to irrelevant classes of pesticides; during the review process we decided to discard this exclusion criterion, as it would have excluded practically all studies.

A comprehensive search strategy was developed. Search terms for insecticide exposure included generic terms such as “insecticide” and “pesticide”, names of classes of compounds such as

"organophosphate" and names of specific compounds such as "malathion". Search terms for outcome included "diabetes", "blood glucose", "hba1c" and numerous synonyms. A complete list of search terms is provided in the online appendix of Paper I. We searched several scientific databases without any language restriction: PubMed, Embase, Scopus, Web of Knowledge, and LILACS (a Latin American database), and hand-searched the reference lists of included papers.

Two separate reviewers (post.doc. Jörg Schullehner and myself) screened articles at the title/abstract level and then at the full-text level. As the justification for the systematic review was to summarize evidence that had been missed by previous reviews on the subject, the search strategy was optimized for maximum sensitivity, at the cost of specificity. After de-duplication, we screened 19,665 entries at the title-abstract level and 560 entries at the full-text level. In total, 55 relevant full-text papers were identified. Study characteristics for these 55 papers have been extracted, and the papers are currently undergoing assessment of risk of bias according to standardized criteria.

As a systematic presentation of all 55 papers is outside the scope of this thesis, I will limit my presentation to the 27 papers that specifically investigated effects of cholinesterase inhibitor insecticides and provided an objective measure of exposure. These studies are most relevant for Paper IV that focuses on the association between red blood cell acetylcholinesterase activity and blood glucose levels among smallholder farmers in Uganda.

Characteristics and results of each of the 27 studies are presented in the appendix on page 89. Risk of bias due to confounding has been estimated for each study, and rated as "low", "probably low", "probably high", or "high". For studies on type 1 diabetes, studies were rated as "low" risk of bias if they adjusted or otherwise accounted for ethnicity, HLA genotype or family history of DM. For studies on unspecified, gestational or type 2 diabetes, and for studies on blood glucose levels as a continuous variable, important confounders were ethnicity, age , sex, family history of diabetes, weight status and physical activity level. Such studies were rated as "high" risk of bias if they did not account for any of the important confounders, or if they only accounted for age and/or sex. "Low" risk studies were defined as studies accounting for all the important confounders. Studies rated as "probably low" risk accounted for age, sex and some of the other important confounders, and I deemed it unlikely that considerable residual confounding existed. "Probably high" risk studies also adjusted for age, sex and some other confounders, but I suspected considerable residual confounding.

2.5.1.2 Results

Most of the 27 identified studies had "high" or "probably high" risk of bias due to confounding. Table 2-2 and Table 2-3 provide an overview of characteristics and results from 11 identified studies with "probably high", two studies with "probably low", and one study with "low" risk of bias. The remaining 13 studies all had "high" risk of bias due to confounding; they are summarized in the appendix on page 89 and will not be further discussed. Most identified studies investigated continuous measures of glycemic regulation. A few investigated unspecified diabetes mellitus or gestational diabetes as dichotomous outcomes. Only one study on type 1 diabetes was identified. As shown in Table 2-2 and Table 2-3, study findings were heterogeneous. Selected studies are presented in more detail below.

Velmurugan *et al*[24] conducted a cross-sectional study among 802 farmers and non-farming villagers in southern India. Exposure to organophosphate and carbamate insecticides was quantified using BChE (plasma cholinesterase) and plasma levels of organophosphates, while objective glycemic regulation was assessed using HbA_{1c}. Authors found no statistically significant difference in BChE between diabetic and non-diabetic subjects, but they demonstrated significant positive correlations between plasma levels of organophosphates and odds of diabetes: E.g., OR 1.70 [0.86; 1.37] for fourth quintile vs. first quartile of monocrotophos (p for trend 0.032) after adjustment for sex, age, family history of diabetes, and weight status.

Ranjbar *et al*[25] used data from 2227 persons in a cross-sectional sample of the general non-institutionalized US population (National Health and Nutrition Examination Survey) and compared levels of FPG, HOMA-IR and HbA_{1c} between groups, defined by urine concentrations of organophosphate metabolites below or above the limit of detection. After adjustment for confounders (age, sex, smoking, weight status, ethnicity, socioeconomic status, fasting duration and urinary creatinine), there were no statistical or clear numerical associations between any of the glycemic outcomes and any of the metabolites.

In a hospital-based follow-up study among 1195 pregnant Canadian women, Shapiro *et al* [26] demonstrated inverse associations between first-trimester urinary metabolites of organophosphates and the risk of being diagnosed with gestational diabetes mellitus (GDM) or gestational impaired glucose tolerance (G-IGT) later in pregnancy: OR 0.5 [0.3; 0.9] for 4^{th} vs. 1^{st} quartile of the sum of dimethyl-phosphate and diethyl-thiophosphate ($p < 0.01$ for trend). Results were adjusted for age, weight status, ethnicity, education and urine-specific gravity. Shapiro *et al* [26] themselves suggested

that the inverse correlation might be due to confounding from health benefits of intake of fruit and vegetables, as diet was assumed the main source of insecticide exposure in the study population. Shapiro *et al* had excluded ⅓ of otherwise eligible women due to missing outcome data, as these women were followed at hospitals that only tested high-risk individuals for GDM/G-IGT. While authors thought that differences between included and excluded women was of "small magnitude",[26] I find that there is a high risk of bias due to selection problems: Excluded women were considerably less likely to be obese than included women were (10% vs. 15%, p = 0.005), and exposure profiles might also have differed.

Garcia-Garcia *et al*[24] described a cross-sectional study among greenhouse workers exposed to different pesticides (of which some were carbamate insecticides) and unexposed controls, supplemented with follow-up limited to the greenhouse workers. Workers were examined in both low- and high-exposure seasons, while controls were examined in the low-exposure season only. Trends in FPG across phases and groups were analyzed in a mixed effect model, and results were conflicting. On one hand, during the low-exposure season greenhouse workers had lower FPG than controls (ΔFPG = -0.91 [-1.21; -0.62] mmol/L), but greenhouse workers' FPG increased from the low-exposure to the high-exposure season (ΔFPG = 0.16 [0.01; 0.31] mmol/L). Analyses accounted for age, sex, smoking, and weight status. While many different agrochemicals were used in the greenhouses, biomarkers showed that a substantial portion was carbamates: AChE/Hb was 10.4 [9.1; 11.7] µmol/min/g lower for greenhouse workers than controls in the low-exposure period and decreased by a further 1.1 [0.4; 2.7] µmol/min/g in the high-exposure season. In a similar follow-up study among pesticide mixers and pesticide applicator pilots in Venezuela,[27] Rojas *et al* demonstrated a concomitant increase in FPG and decrease in AChE in the spraying season, compared to the pre-season examination.

Nascimento[28] *et al* conducted a study among 54 children aged 5-16 years from an agricultural area of Brazil; the children were examined in both a low-exposure and a high-exposure period. Environmental exposure to cholinesterase inhibitor insecticides was quantified using AChE and BChE, and glycemic regulation was assessed by FPG. There was no change in AChE between the exposure periods, but BChE was 34% lower in the high-exposure than in the low-exposure period. At the same time, a paired *t*-test showed that FPG was significantly higher in the high-exposure period (5.33 vs. 4.85 mmol/L, p < 0.001). A cross-sectional analysis limited to the high-exposure period showed a

negative correlation between BChE and FPG after adjustment for sex, age, BMI and pubertal stage ($r = -0.509$, $p < 0.001$).

In addition to the studies just described, several cross-sectional studies have demonstrated significantly higher FPG and lower ChE activity among highly exposed persons, compared to less- or non-exposed controls.[29-31] However, the studies were small and all had "probably high" risk of confounding. Furthermore, a number of other cross-sectional studies with similar risk of bias showed non-significant or no effects on glycemic regulation.[32-35]

El-Morsi *et al*[36] conducted the only identified study on type 1 diabetes mellitus, which was also the only study rated as "low risk" of confounding. In a hospital-based case-control design, they compared serum levels of organophosphate insecticides between 75 diabetic children and 35 healthy controls. Results were conflicting. Odds of having malathion > the limit of detection were significantly higher in cases than controls (OR 4.11 [1.74; 9.69]), but odds of having detectable levels of profenofos and chlorpyrifos-methyl were lower (OR 0.13 [0.02; 0.69] and 0.16 [0.05; 0.49], respectively). The study accounted for genetic risk factors for diabetes by excluding any children with a positive family history of diabetes.

In conclusion, existing evidence regarding the association between cholinesterase inhibitor insecticides and diabetes is only suggestive. Half of the 27 identified studies had high risk of confounding, as they failed to account for the most basic confounders (age and sex). Out of the other half, most had "probably high" risk of confounding. Furthermore, results are conflicting, with both significant positive, significant negative and null associations reported.

2.5.2 Biological plausibility

In this PhD thesis, I have focused on reviewing and generating *epidemiological* evidence on the possible associations between cholinesterase inhibitor insecticides and perturbed glycemic regulation. While biological plausibility is a classic criterion for a statistical association to be deemed causal,[37] elucidating possible biological mechanisms responsible for an association has not been a focus of the project. However, a causal effect of cholinesterase inhibitor insecticides on glycemic regulation *is* biologically plausible. Other authors have proposed numerous mechanisms by which the effect could be mediated. These include changes in gluconeogenesis and glycogenolysis,[38] impaired glucose sensitivity in pancreatic β-cells due to over-stimulation of ACh receptors,[38] insulin resistance due to pro-inflammatory disturbances[38] and oxidative stress,[38] and changes in the gut microbiome.[24]

Table 2-2: Previous studies on cholinesterase inhibitor insecticides and diabetes mellitus (occupational exposure)

Reference	Country	Study design	Study population	Exposure biomarker(s)	Outcome	n	Exposure metric	Effect on glucose	Effect on biomarker	Risk of confounding
Rojas[27] 1996	Venezuela	Combination of cross-sectional and follow-up	Pesticide mixers, pesticide applicator pilots, unexposed pilots	AChE (not Hb-adjusted)	FPG	73	Group-based	+	-	Probably high
Patil[29] 2009	India	Cross-sectional	Healthy pesticide applicators and healthy unexposed controls	BChE	FPG	90	Group-based	+	-	Probably high
Tsatsakis[32] 2011	Greece	Cross-sectional	Farmers, farmworkers and rural residents	Hair OP metabolites	DM	220	Metabolites (continuous)	(+)	N/A	Probably high
Bayrami[33] 2012	Iran	Cross-sectional	Farmers and unexposed workers from same village	BChE	FPG	80	Group-based	(-)	-	Probably high
Abbassy[30] 2014	Egypt	Cross-sectional	Farmers	BChE	FPG	NR	Group-based	+	-	Probably high
Garcia-Garcia[39] 2016	Spain	Combination of cross-sectional and follow-up	Greenhouse workers and healthy unexposed controls	AChE/Hb and BChE	FPG	280	Group-based	+/-	+/-	Probably high
Marrero[31] 2017	Venezuela	Cross-sectional	Organophosphate applicators and unexposed controls (lab technicians)	BChE	FPG	30	Group-based	+	-	Probably high
Velmurugan[24] 2017	India	Cross-sectional	Farmers and non-farming villagers	Plasma OPs	DM	802	Metabolites (continuous)	+	N/A	Probably high
Arevalo-Jaramillo[34] 2019	Ecuador	Cross-sectional	Exposed rural women (from high- and low-exposure areas) and unexposed controls (women from city)	BChE	FPG	115	Group-based	(-)	(+)/-	Probably high

Please see legend on page 15.

Table 2-3: Previous studies on cholinesterase inhibitor insecticides and diabetes mellitus (environmental or unclear mode of exposure)

First author	Country	Study design	Study population	Exposure biomarker(s)	Outcome	n	Exposure metric	Effect on glucose	Effect on biomarker	Risk of confounding
Cecchi[35] 2012	Argentina	Cross-sectional	Pregnant women from area with intensive agriculture	AChE/Hb BChE	FPG	97	Group-based	0	-	Probably high
El-Morsi[36] 2012	Egypt	Case-control	Children with DM type 1 and healthy controls (= siblings of other pediatric patients)	Serum OPs	DM type 1	110	Cases vs. controls: Metabolite levels	+/-	N/A	Low
Ranjbar[25] 2015	USA	Cross-sectional	General non-institutionalized US population	Urine OP metabolites	FPG HbA_{1c} HOMA-IR	2227	Metabolites (< vs. ≥ LOD)	0	N/A	Probably low
Shapiro[26] 2016	Canada	Follow-up	Pregnant women	Urine OP metabolites	GDM or G-IGT	1195	Metabolites (continuous)	-	N/A	Probably high
Nascimento[28] 2018	Brazil	Combination of cross-sectional and follow-up	Children from agricultural area	AChE (not Hb-adjusted) BChE	FPG	54	Comparison of high-exposure and low-exposure period. BChE as continuous variable in high-exposure period.	+	-	Probably low

Mode of exposure unclear for Cecchi[35] 2012. Environmental exposure in remaining studies.

Legend for Table 2-2 and Table 2-3:

- + = statistically significant positive relationship, (+) = statistically non-significant positive relationship, 0 = no clear association, (-) = statistically non-significant negative relationship, - = statistically significant negative relationship. Note that when the biomarker is cholinesterase, we expect to find a negative relationship between exposure and biomarker.
- Exposure metric: Studies with "group-based" modelling strategies compare the outcome in different groups, e.g. farmers and unexposed controls.
- Abbreviations: GDM = gestational DM, G-IGT = gestational impaired glucose tolerance, N/A = not applicable, NR = not reported, OP = organophosphate insecticide.

2.6 Obstructive airway diseases and spirometry

2.6.1 Asthma and chronic obstructive pulmonary disease

Asthma and chronic obstructive pulmonary disease (COPD) are two related, but distinct diseases affecting a total of 533 million individuals in the world,[40] and causing a total of 3.6 million deaths per year.[40] The common denominator between the two diseases is airway obstruction, i.e. a narrowing of the lower airways, leading to an increased resistance to airflow out of the lungs.[41 42]

Asthma is a chronic inflammatory disease with intermittent attacks of airway obstruction caused by bronchoconstriction, edema of mucous membranes and mucus hypersecretion in response to irritant (e.g., cold air, physical exertion, air pollution, chemical compounds), or allergenic stimuli (e.g., animal dander, pollen).[41] Known risk factors for asthma include family history of the disease,[43] allergy/atopy,[41] passive smoking[44] and preterm birth.[45]

In COPD, airway obstruction is caused by varying combinations of chronic inflammation with edema of mucous membranes, hypersecretion of mucus, and destruction of septa between alveoli (emphysema) that leads to loss of elastic recoil of the lungs.[42] While COPD is often colloquially referred to as "smoker's lung", not all patients with COPD are current or former smokers. Globally, it is estimated that 27% of the Disability-Adjusted Life Years lost to COPD are caused by tobacco smoking, 2% by second-hand smoke, 17% by ambient air pollution, 14% by household air pollution, 9% by occupational exposures, and 4% by ozone exposure.[40] However, the relative importance of the causes varies considerably between highly developed and less developed countries (defined by the Social Development Index, a composite metric that takes into account education, income and fertility rate). In highly developed countries, smoking and second-hand smoke exposure are the most important causes of COPD, while air pollution and occupational exposures cause more of the COPD burden in less developed countries.[40] Individuals with α-1-antitrypsin deficiency (a rare genetic condition) have increased risk of developing COPD.[42]

2.6.2 Spirometry

Spirometry is central to the diagnosis of airway obstruction. Simply put, spirometry is a measure of the amount of air that a person can exhale, and how fast.[46] Spirometry does not measure the lungs' ability to exchange gases. While a number of different indices can be calculated from a spirometric examination, the two central metric are the forced expiratory volume in 1 second (FEV_1) and the forced vital capacity (FVC). The FEV_1 is the amount of air that can be forcefully exhaled from the lungs

in the first second after a maximal inhalation, and the FVC is the total amount of air that can be exhaled forcefully.[46] Pulmonary function is strongly influenced by age, sex, height and ethnicity.[47] Correct interpretation of spirometry results presupposed that these factors are accounted for. All other things equal, men generally have higher lung function than women, young persons have higher lung function than older individuals, and tall persons have higher values than persons of short stature have. African Americans generally have lower lung function than persons of European descent.[47]

Obstruction is defined by the ratio between FEV_1 and FVC (FEV_1/FVC), and often a fixed cut-off of $FEV_1/FVC < 0.7$ is used.[41 42 48] However, FEV_1/FVC decreases physiologically with age, meaning that using a fixed cutoff for FEV_1/FVC can lead to over-diagnosis of obstruction in elderly and under-diagnosis in young individuals.[49] Alternatively, obstruction can be defined as FEV_1/FVC below the Lower Limit of Normal, defined as the 5th percentile of values for non-smoking individuals with the same age, sex, height and ethnicity.[49]

An important difference between asthma and COPD is that asthmatics can have normal or near-normal lung function in-between attacks, while patients with COPD typically have fixed airway obstruction.[41 42] In clinical medicine, patient whose spirometry shows obstruction are therefore re-tested after administration of bronchodilator medication. Considerable reversibility of obstruction indicates that the condition is asthma, though patients with COPD might also show some improvement. [41 42]

2.7 Cholinesterase inhibitor insecticides as risk factors for pulmonary disease

Respiratory failure is a well-known complication of acute intoxication with organophosphate insecticides, and is caused by a combination of bronchoconstriction, increased mucus production in the airways, pulmonary edema and central respiratory depression.[50] As early as 1963, an epidemiological study suggested that longer-term, lower-dose exposure to organophosphates might also lead to lung function impairment due to bronchoconstriction.[51] In the following section, I will provide a brief overview of the current evidence for a causal link between pulmonary impairment and exposure to cholinesterase inhibitor insecticides at levels too low to cause acute intoxication.

2.7.1 Epidemiological evidence

In a recently published systematic review and meta-analysis, Ratanachina *et al* summarized the evidence for a link between exposure to cholinesterase inhibitor insecticides and objective decrease in lung function.[5] They found “tentative evidence” for an effect on FEV_1/FVC, with a pooled difference

in FEV_1/FVC of -0.22 [-0.46; 0.01] in FEV_1/FVC when comparing exposed farmers to non-exposed controls. Most studies investigating effects on FEV_1 also showed a decrease, but Ratanachina *et al* found that the estimates were too heterogeneous to calculate a pooled effect estimate. Many of the studies were cross-sectional and failed to adequately adjust for potential confounders.[5] Two of the highest-quality studies are described in more detail below.

Raanan *et al*[52] conducted a follow-up study among 279 children born to mothers in an agricultural community. Exposure was assessed by levels of organophosphate metabolites in maternal urine during pregnancy, and in the children's urine from age 0 to 6 years. The children underwent spirometry at age 7. Analyses showed no clear association between metabolites in maternal urine, and the children's pulmonary function. However, each 10-fold increase in post-natal, creatinine-adjusted level of dialkylphosphate metabolites was associated with a -0.16 [-0.30; -0.02] L change in FEV_1, -0.17 [-0.34; 0.01] L change in FVC and 0.01 [-0.02; 0.03] change in FEV_1/FVC, after confounder adjustment.

Ye *et al*[53] examined 4,446 adolescents and adults from the general Canadian population in a cross-sectional design. Creatinine-adjusted urinary levels of organophosphate metabolites were measured, and participants underwent spirometry. No clear association was seen between metabolite levels and pulmonary function for adolescents: Adjusted difference in FEV_1 –2.4 [–35.7, 31.0] mL, FVC 13.9 [–24.4, 52.2] mL, and FEV_1/FVC –0.3 [–0.9, 0.2] %, respectively, when the sum of dialkylphosphate metabolites increased by 171%. For adults, statistically significant decreases in both FEV_1 and FVC were demonstrated when metabolite levels increased: FEV_1 –32.7 [–59.0, –6.3] mL, FVC –32.6 [–57.2, –8.1] mL, FEV_1/FVC –0.2 [–0.6; 0.2] %.

2.7.2 Biological plausibility

A link between exposure to cholinesterase inhibitor insecticides and lung function impairment is biologically plausible. Due to the high acute toxicity of the compounds, it would be unethical to investigate their pulmonary effects in human exposure studies. However, some researchers have carried out human experimental studies with the carbamate pyridostigmine that can be used as a prophylactic drug against poisoning with some chemical warfare agents.[54] As pyridostigmine has the same mode of action (inhibition of cholinesterase) as carbamate and organophosphate insecticides,[54] effects of pyridostigmine may provide clues for possible effects of these insecticides.

Two randomized, double-blinded, placebo-controlled, crossover trials on pulmonary effects of pyridostigmine have been conducted. Roach *et al* examined 20 healthy individuals and 10 subjects with mild asthma. Participants performed spirometry after one day of taking either pyridostigmine 30 mg or placebo orally three times per day. During pyridostigmine treatment, red blood cell acetylcholinesterase decreased by an average of 21.4%, but according to Roach *et al*, there was no effect on FEV_1 and FVC (numeric results not reported).[55] Ram *et al* examined 12 healthy and 13 asthmatic subjects. Healthy subjects received either a single dose of pyridostigmine 60 mg or placebo, while asthmatics received a dose of 30 mg or placebo. Spirometry was performed before drug/placebo administration and two hours after. Pyridostigmine 60 mg caused a mean decrease of 0.13 liters in FEV_1 in the healthy subjects ($p = 0.015$), and the degree of whole blood cholinesterase inhibition and FEV_1 decrease was strongly correlated ($r^2 = 0.88$, $p = 0.0001$). On the other hand, Ram *et al* found no effect of pyridostigmine 30 mg on asthmatics.[56]

Gouge *et al* conducted a non-randomized, non-blinded, non-placebo-controlled clinical trial among 10 asthmatics and 6 healthy subjects who all received 30 mg of pyridostigmine orally. FVC was measured before and after drug administration. The original study authors concluded there were "no changes in forced vital capacity".[54] However, I disagreed with their methods of statistical analysis, and when I re-analyzed the study data as described in Paper VI, I found that FVC of asthmatics decreased by 6.8 [2.8, 10.9] percentage-points of the predicted value, while the FVC of healthy subjects was unaffected.

Caution is of course warranted before extrapolating effects of acute and relatively high-dose exposure to long-term effects of exposure to lower doses of the same or similar compounds. Nevertheless, the preceding section shows that it is biologically plausible for cholinesterase inhibitor compounds to cause lung function impairment.

3 Aims and overview of papers

As outlined in the previous chapter, epidemiological studies have suggested that the risk of diabetes mellitus and obstructive airway disease is increased among persons exposed to cholinesterase inhibitor insecticides. We hypothesized that these associations might represent causal effects, and aimed to investigate the associations in two new epidemiological studies with objective outcome metrics that accounted for important confounders.

The specific aims of the PhD study were:

1. Summarize existing evidence on neuroactive non-organochlorine insecticides as risk factors for diabetes in a systematic review and meta-analysis, using a comprehensive search strategy and standardized quality assessment criteria.
2. Investigate the link between self-reported pesticide use and diabetes mellitus in a cross-sectional sample of the general adult population of a semi-urban area in Nepal.
3. Investigate the association between objectively quantified exposure to cholinesterase inhibitor insecticides and objective measures of glycemic regulation and pulmonary function in a short-term cohort study among smallholder farmers in Uganda. As part of this analysis, we also evaluated exposure-response relationships.

Table 3-1 provides an overview of the papers included in this dissertation. Paper I is a protocol for the systematic review that is still ongoing. Preliminary findings were presented in the scoping review on page 8. To answer aim number 2, Paper II presents results for data collected as part of the cross-sectional “Community-based Intervention for Management of Diabetes in Nepal” (COBIN-D) project. Answers to aim number 3 are presented in Paper IV and Paper V, based on data collected in the study entitled “Pesticide Exposure, Asthma and Diabetes in Uganda” (PEXADU). Paper III describes a piece of open-source software that I developed to support data collection for Paper IV. Paper VI presents results from a reanalysis of published data from a previously conducted clinical trial on pulmonary effects of the cholinesterase inhibitor medicine pyridostigmine. The re-analysis was conducted as an effort to assess the biological plausibility of the statistical associations demonstrated in Paper V. Paper II, Paper IV and Paper V are the main studies in this thesis and will therefore be described in greater detail than Paper I, Paper III and Paper VI.

Table 3-1: Schematic overview of papers included in the dissertation

Paper	Study type	Country	Subjects	Number of subjects	Exposure	Outcome
I	Systematic review protocol	N/A	N/A	N/A	Neuroactive non-organochlorine insecticides	Diabetes mellitus and blood glucose levels
II	Cross-sectional study	Nepal	General population (COBIN-D)	2,310	Self-reported pesticide use	Diabetes mellitus and blood glucose levels
III	Open-source software	N/A	N/A	N/A	N/A	N/A
IV	Follow-up study	Uganda	Smallholder farmers (PEXADU)	364	Cholinesterase inhibitor insecticides (objectively quantified)	Blood glucose levels
V						Lung function
VI	Clinical trial	Saudi Arabia	American soldiers	16	Pyridostigmine	Lung function

N/A = not applicable.

4 Methodology

4.1 Methods used in Paper I

Paper I is a protocol for a systematic literature review on exposure to neuroactive non-organochlorine insecticides, diabetes mellitus and related metabolic disturbances such as hyperglycemia. The review is still ongoing. A brief description of the methods and a summary of preliminary findings have been provided on page 8.

4.2 Methods used in Paper II

The subject of this paper is the possible link between self-reported use of any pesticides and risk of diabetes in a sample of the general population from a semi-urban area in Nepal.

4.2.1 Design and study population

Paper II is based on a cross-sectional study nested in the COBIN-D project, which was an intervention study on the effect of education on glycemic controls among diabetes patients in the former Lekhnath Municipality, a semi-urban area in Western Nepal. The primary investigator of the COBIN-D study was Bishal Gyawali, then a PhD student at the Department of Public Health at Aarhus University. My supervisors and I entered a collaboration with the primary COBIN-D investigators, and questions on pesticide exposure were added to the COBIN-D questionnaire before baseline data collection started.

Data were collected from October 2016 to April 2017. A random sample of 2,815 persons from the general adult population of the former Lekhnath Municipality had participated in a previous phase of the COBIN project that focused on hypertension.[57 58] 87.4% of these individuals could be reached and were willing to participate in a new study, giving a study population of 2,310 persons for Paper II.

4.2.2 Exposure assessment

Participants underwent a structured interview, based on the "World Health Organization STEPwise approach to surveillance (STEPS)" questionnaire for assessment of classic risk factors for con-communicable disease,[59] supplemented with questions on pesticide use. The latter included information on ever-use of any pesticide (dichotomous), number of years of pesticide use (continuous), number of weeks of use per year (continuous), number of hours per week (continuous), names of pesticides used, and which months spraying took place.

4.2.3 Outcome assessment

Fasting plasma glucose was measured in capillary blood for all participants. If a participant was not fasting, a new appointment was made.

4.2.4 Ethics

The study was approved by the Nepal Health Research Council, Kathmandu, Nepal (reg. no. 263/2016, see page 111) and conducted in accordance with the Declaration of Helsinki. Subjects gave informed consent before inclusion.

4.2.5 Statistical analyses

FPG was classified as non-diabetic (≤6.9 mmol/L) or diabetic (≥ 7.0 mmol/L) as defined by the American Diabetes Association (ADA)[19] and the WHO[21] (see page 7). Each of the continuous exposure metrics (years, weeks/year and hours/week of pesticide use) was categorized as low, medium or high to *a priori* defined cut-points to yield three approximately equal-sized groups. The possible association between pesticide exposure and diabetes mellitus was investigated in logistic regression models that adjusted for age, sex, BMI, waist-to-hip-ratio, physical activity level, family history of diabetes mellitus, and previous diagnosis of cardiovascular disease. Possible confounders were selected *a priori*, because they were known risk factors.

4.3 Methods used in Paper III

This paper describes a piece of open-source software (HemoDownloader) that I developed to aid in data management during the operative phase of the study described in Paper IV. As described in details below (page 25), the main outcome metric in that study was glycated hemoglobin A (HbA_{1c}), and this was determined using the point-of-care device “HemoCue HbA1c 501" (HemoCue AB, Ängelholm, Sweden). While the device had an internal memory where results were saved, they could not be exported in digital format. I developed the HemoDownloader software to allow my PC to interface with the HemoCue HbA1c 501 device over a serial (RS232) connection and save the data on the hard drive. The software has a graphical user interface and made it possible to create a structured database of HbA_{1c} results immediately at the end of each working day. The HbA_{1c} results could thus be backed up daily to avoid data loss while I was in the field in Uganda.

While the HemoCue HbA1c 501 normally cannot export results in digital format, it does support printing all results in memory using a special printer. To develop the HemoDownloader software, I connected my PC to the printer port of the HemoCue HbA1c 501 and recorded the raw binary data

that was transmitted from the device when its printer function was activated. The data format was deciphered by opening the raw binary data in a text editor and manually inspecting its structure. Once the data format had been determined, I developed code to parse the binary data into a structured format, and implemented existing open-source libraries for exporting the structured data in the desired format, e.g. Microsoft Office Excel or Comma-Separated Values.

To enable the use of the HemoDownloader software by other researchers, I decided to publish the program as open source. Paper III describes the software, and the source code is also available as an appendix.

4.4 Methods used in Paper IV and Paper V

Paper IV and Paper V describe studies on the associations between exposure to cholinesterase-inhibiting insecticides, pulmonary function and glycemic regulation. The studies were conducted as part of the PEXADU project in collaboration with partners from Makerere University School of Public Health and from the Uganda National Association of Community and Occupational Health. I was the primary investigator of the project.

4.4.1 Design and study population

The PEXADU project was a follow-up study among 364 smallholder farmers from the Wakiso District in central Uganda. In an attempt to maximize exposure contrast while minimizing confounding from demographic variables, participants were recruited from two local farmers' organizations - one organization for conventional farmers and one organization for farmers working to get organic certification for some of their crops. Both exposure, confounders and outcomes for each participant were determined in each of three project phases: Baseline in September-October 2018, follow-up in November-December 2018 and again in January-February 2019.

4.4.2 Exposure assessment

Neuronal acetylcholinesterase (AChE) is the main toxicodynamic target of organophosphate and carbamate insecticides,[9] as described on page 4. The enzyme isoform in erythrocytes (red blood cells) is affected by exposure to the same compounds, and it is much more available to sampling than the neuronal forms. Measurement of erythrocyte AChE is therefore used as a biomarker of exposure.[11] The most valid estimate of exposure is obtained by normalizing AChE by the hemoglobin (Hb) concentration in the sample to account for anemia, and for dilution during sampling and analysis.[60] It is important to note that because exposure impairs the activity of the AChE enzyme, *low* levels of

AChE/Hb indicate *high* exposure. A similar plasma enzyme called butyrylcholinesterase (BChE) can also be used to quantify exposure,[11] but was not measured in the PEXADU study.

At each visit to the examination center, participants gave a capillary blood sample for analysis of AChE/Hb. The analysis was performed using a point-of-care device (Test-mate ChE Cholinesterase Test System Model 400, EQM Research Inc., Cincinnati, Ohio, USA) according to the manufacturer's instructions.[60]

We also collected detailed subjective information on pesticide exposure in a questionnaire-based structured interview. The questionnaire was modified from the questionnaire used in the "Pesticides in the tropics" (Pestrop) project during a survey in the same area of Uganda in 2017,[61] supplemented with questions from another survey in Ethiopia.[62] The questionnaire contained detailed information on the specific compounds used, the number of years and intensity of using pesticides, use of personal protective equipment etc.

Two further methods were employed to collect objective information on insecticide exposure. A random subsample of participants were selected using a pseudo-random number generator and asked to provide spot urine samples for the analysis of urinary metabolites of pesticides. In addition, at baseline all participants were given a passive sampling device in the form of a silicone wristband and asked to wear it until they came back in phase 2. A random subsample of the persons selected for urine sampling also wore silicone wristbands from phase 2 to 3. Passive sampling using silicone wristbands is a relatively recent technique for the quantification of insecticide exposure, but has been successfully used among farmers in settings similar to the PEXADU project.[63]

Unfortunately, obtaining permission to export the silicone wristbands and urine samples from Uganda has proved very difficult. At the time of writing (January 2020), these samples were still stored in a biobank in Kampala, and no data were yet available.

4.4.3 Outcome assessment

4.4.3.1 Outcome in Paper IV

The outcome of interest in Paper IV is objectively measured glycemic regulation, expressed as glycosylated hemoglobin A (HbA_{1c}) and fasting plasma glucose. HbA_{1c} is a measure of a person's average blood glucose in the last 8-12 weeks.[64] HbA_{1c} was measured in venous blood for each participant in each phase. It was not logistically feasible to ask all participants to come fasting in the morning, nor to randomize who should come fasting. In each phase, capillary FPG was therefore

measured in the non-random subsample of participants who were able and willing to come fasting in the morning. On page 65, I discuss the potential for FPG to be biased by selection problems.

Both HbA_{1c} and FPG were analyzed at the examination center using point-of-care equipment – HemoCue HbA1c 501 and HemoCue Glucose 201 RT (HemoCue AB, Ängelholm, Sweden), respectively.

4.4.3.2 Outcome in Paper V

The outcome of interest in Paper V is objectively measured pulmonary function, expressed by pre-bronchodilator spirometry as measured by a diagnostic-quality spirometer (MicroDL, Micro Medical, Rochester, Kent, England). Participants performed spirometry in each phase of the project, unless they fulfilled exclusion criteria. For ethical reasons, these criteria were selected to exclude anyone with risk of adverse events due to spirometry,[65] and persons with contagious respiratory diseases. A list of exclusion criteria is provided in Paper V.

Forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC) and the ratio between the two (FEV_1/FVC) were converted to Z-scores using the Global Lung Function Initiative (GLI-2012) equations[47] with African-American as the reference ethnicity.

Participants who underwent spirometry in phase 1 were also tested using the copd-6 mini-spirometer (Vitalograph, Ennis, Ireland) to validate the latter instrument. For reference, this validation study is presented in Supplementary paper III, but the study does not form part of the thesis and will not be discussed further.

4.4.3.3 Covariate data collection

Height and weight were measured in a standardized manner[66] using a medical weighing scale and a stadiometer, respectively. Weight measurements were adjusted to take into account that the strength of the gravitational acceleration in Uganda is slightly different from Central Europe where the scale had been calibrated (see details in appendix, page 98). Subjective information on classic risk factors for non-communicable diseases were collected using an interviewer-administered questionnaire containing items from a number of standardized questionnaires created for use in developing countries: WHO STEPS[59] for demographics, smoking and alcohol consumption, WHO Global Physical Activity Questionnaire[67] for physical inactivity and World Health Survey[68 69] questions on biofuel smoke exposure. Custom questions had been added where needed. Graphical showcards were used to help participant understand selected questions.

4.4.4 Sample size

The sample size for the PEXADU project was convenience-based. I estimated that in each phase we could examine 450 persons, of which half would be conventional and half semi-organic farmers. Before data collection, I calculated the sizes of the effects on blood glucose and pulmonary function that could be detected with this sample size and 80% power, and compared the estimates to existing literature to see it was feasible to do the study with this sample size.

4.4.4.1 Statistical power for glycemic regulation

Based on previous studies in Uganda, I assumed that the standard deviation of both fasting[70] and random plasma glucose[71] would be 1.6 mmol/L. I used Stata to calculate the smallest effect size on fasting blood glucose that could be demonstrated in a group of 450 people with 80% power.

Stata code 4-1

```
power twomeans 0, sd(1.6) power(0.8) n(450)
```

The output showed that we could demonstrate a difference in mean FPG = 0.4 mmol/L. This is roughly equivalent to the effect sizes shown in previous studies among farmers in Iran[72] and a combination of farmers and pesticide shop-keepers in India.[73]

4.4.4.2 Statistical power for pulmonary function tests

A previous study in Ethiopia showed that FEV_1 was 140 ml lower among pesticide-exposed persons than among non-exposed controls. [62] The SD for FEV_1 was 560 mL for pesticide-exposed males and 500 mL for non-exposed males.[62] I calculated the smallest effect on FEV_1 that could be demonstrated in a group of 450 people with 80% power:

Stata code 4-2

```
power twomeans 0, sd1(0.5) sd2(0.56) power(0.8) n(450)
```

The output showed that we could detect a difference = 140 ml, the same difference as shown in Ethiopia.[62] The SD for FEV_1 among females in the Ethiopian study was lower than the SD for males, so this estimate is conservative.

4.4.4.3 Difference between planned and final sample size

As described above, it was our plan to include 450 persons in the PEXADU project. However, we experienced some setbacks and started baseline data collection three weeks later than expected. For

this reason, we were unable to collect baseline data for as long as we wished. When baseline data collection ended in early October 2018, we had reached 364 participants.

4.4.5 Ethics

The studies described in Paper IV, Paper V and Supplementary paper III were approved by the Higher Degrees Research and Ethics Committee at Makerere University School of Public Health, Kampala, Uganda (reg.no. 577) and the Uganda National Council for Science and Technology, Kampala, Uganda (reg.no. HS234ES), see page 112. The studies were conducted in accordance with the Declaration of Helsinki. Participants gave informed consent before inclusion and received their own biochemical and spirometry results at the end of each visit. They were financially compensated for lost earnings on project days.

4.4.6 Statistical analyses

For almost all participants in the project, we had three separate observations listing exposure, outcome and confounder levels in a particular phase. Furthermore, almost a third of participants were genetically related to at least one other participant. If we failed to take this interdependence of data into account in our analyses, the standard errors of our estimates might be too small,[74] leading to a risk of false positive results. Data were therefore analyzed in linear mixed effect models with random effect terms for family and participant. Details about the mathematical models are provided in Paper IV and Paper VI.

4.5 Methods used in Paper VI

Paper VI presents results from a non-blinded, non-randomized clinical trial on pulmonary function among sixteen volunteers given pyridostigmine, a cholinesterase inhibitor medicine that can be used prophylactically to protect against some chemical warfare agents.[54] The trial was carried out in 1994 and the dataset made public by the original authors.[54] Because of the shared mode of action, pulmonary effects of pyridostigmine could provide indirect evidence for effects of cholinesterase inhibitor insecticides.

The authors of the original paper measured forced vital capacity in 10 asthmatic subjects and 6 healthy subjects, followed by administration of 30 mg of pyridostigmine. Forced vital capacity was measured again after 2, 4, 6 and 8 hours. Unfortunately, the original study authors chose a suboptimal statistical approach, analyzing study data in an "analysis of variance" (no further details provided in the paper) and using un-paired *t*-tests. The authors concluded that there was no statistically significant difference in FVC before and after pyridostigmine administration, but I got the clear impression from the table of raw data in the paper that FVC was systematically lower 2 hours after pyridostigmine administration compared to baseline. I therefore re-analyzed the data in a linear mixed effect model with a fixed effect for each time of measurement and a random effect for each person. Details of the mathematical model are provided in Paper VI.

5 Summary of results

5.1 Key results from Paper I

The systematic review planned in Paper I is still ongoing, and preliminary results have been presented as part of the introduction on page 8. No further results related to the systematic review will be presented in this chapter.

5.2 Key results from Paper II

Out of 2,310 persons in the current phase of the COBIN-D project, 36% reported that their main occupation was farming. The majority of both farmers (70%) and non-farmers (58%) reported that they had ever used pesticides. However, exposure intensity levels were relatively low, considering that most of the pesticide use was agricultural. The median number of years of exposure was 9, the median number of weeks of spraying per year was 2, and the median number of hours of spraying per week was 0.5.

Overall, 271 (11.7%) of the participants were diabetic. Contrary to our hypothesis, we found a significantly lower prevalence of diabetes in the exposed population (9.9%) compared to non-exposed controls (14.7%) – unadjusted OR 0.64 [0.49; 0.82] and OR 0.68 [0.52; 0.90] after confounder adjustment. However, there was no clear association between continuous exposure metrics (years, weeks/year and hours/week of pesticide use) and odds of diabetes. Because of considerable demographic differences between exposed subjects and non-exposed controls, residual confounding is likely and may explain the results demonstrated.

Our original plan was to conduct a more in-depth study on health effects of pesticide exposure in the COBIN-D population after the study described in Paper II. However, because exposure levels in the COBIN-D population were lower than expected, we decided to conduct our second study on health effects of pesticides in another population with higher exposure levels. That is why the studies in Paper IV and Paper V were conducted in Uganda.

5.3 Key results from Paper III

As Paper III describes the HemoDownloader software, there are no results to present.

5.4 Key results from study IV and V

In the PEXADU study, 364 individuals participated at baseline. We followed up among 356 and 354 persons in phase 2 and 3, respectively. We summarized demographics at baseline, stratified by AChE/Hb below or above the median (26.3 U/g) to check for obvious imbalances that could introduce confounding. There were fewer males (27.1%) in the low AChE/Hb group than in the high AChE/Hb group (35.5%), but the groups were otherwise similar in terms of ages, educational level, and lifestyle factors.

AChE/Hb decreased significantly from phase to phase, mean change -0.74 [-0.85; -0.63] U/g/phase. HbA_{1c} increased non-significantly across phases, mean change 0.41 [-0.03; 0.85] mmol/mol/phase. There was no clear pattern in FPG; mean change -0.03 [-0.11; 0.04]. Most spirometric indices decreased across phases; e.g., mean change -0.10 [-0.13; -0.07] $phase^{-1}$ for FEV_1 Z-score, -0.06 [-0.09; -0.03] $phase^{-1}$ for FVC Z-score, and -0.08 [-0.13; -0.03] $phase^{-1}$ for FEV_1/FVC Z-score.

Figure 5-1 presents exposure-response relationships between AChE/Hb and each of the primary outcome metrics: HbA_{1c}, FPG, FEV_1 Z-score, FVC Z-score and FEV_1/FVC Z-score. The graphs are copied from figure 2 in Paper IV and figure 3 in Paper V. All results in Figure 5-1 are adjusted. For HbA_{1c} and FPG, the covariates were age, sex, alcohol consumption in the last week, tobacco consumption in the last week, MET-minutes of physical activity in the last week and servings of fruits and vegetables consumed per day in the last week. For spirometric indices, analyses are adjusted for age, sex, pack-years of smoking and cumulated lifetime hours of cooking. In addition, spirometric analyses are implicitly adjusted for height, as height it one of the factors included in the GLI 2012 equations.[47] Unadjusted analyses and analyses adjusted for an extended set of confounders (also including BMI and educational level) gave very similar results (see Paper IV and Paper V).

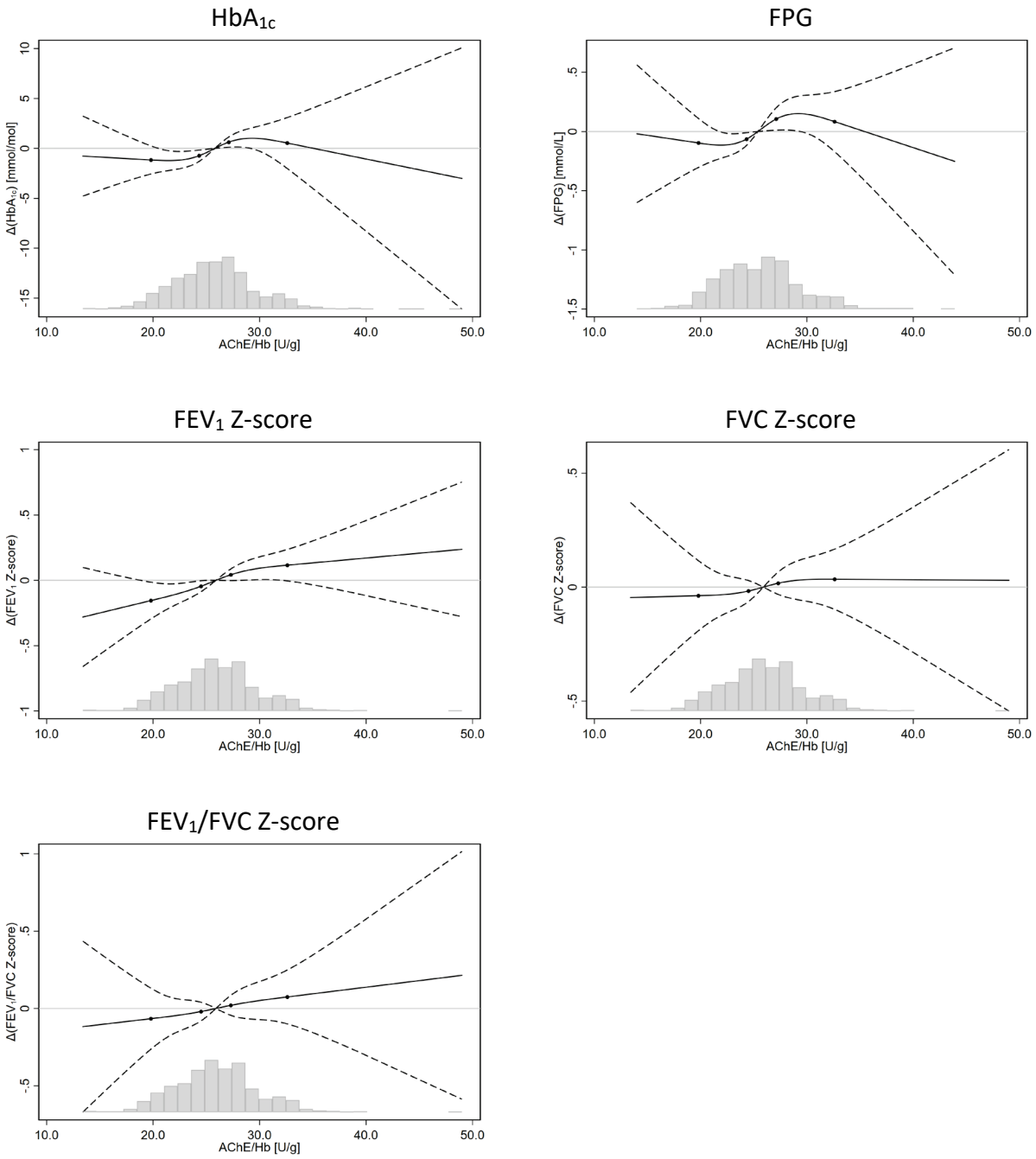
Figure 5-1 shows that low AChE/Hb is correlated with low HbA_{1c} and low FPG. Relative to individuals with AChE/Hb 25.8 U/g (50^{th}) percentile, individuals with AChE/Hb 24.3 U/g (35^{th} percentile) have an HbA_{1c} that is 0.74 [0.17; 1.31] mmol/mol lower, and individuals with ACHE/Hb 27.1 U/g (65^{th} percentile) have HbA_{1c} that is 0.63 [0.12; 1.14] mmol/mol higher than the reference. Sensitivity analyses gave similar results as the main analysis. When phase was entered as a separate fixed effect in the mixed effect model, the association between AChE/Hb and HbA_{1c} persisted, and it also persisted after including hemoglobin concentration as a covariate in the models.

As shown in Figure 5-1, low AChE/Hb was associated with low FEV_1 Z-score. Relative to AChE/Hb 25.9 U/g (50^{th} percentile), persons with AChE/Hb 24.5 U/g (35^{th} percentile) had a mean FEV_1 Z-score that was 0.045 [0.003; 0.087] lower, and persons with AChE/Hb 27.3 U/g (65^{th} percentile) had FEV_1 Z-score that was 0.043 [-0.002; 0.087] higher than the reference. Z-scores of FVC and FEV_1/FVC showed similar patterns, but the differences were numerically smaller and not statistically significant. While results from most sensitivity analyses were unchanged, I found no association between FEV_1 Z-score and AChE/Hb in a sensitivity analysis that included project phase as a covariate. However, in this sensitivity analysis, significant associations were seen between FEV_1 Z-score and project phase. Relative to phase 1, FEV_1 Z-score was -0.107 [-0.161 ; -0.052] in phase 2 and -0.207 [-0.268 ; -0.146] in phase 3, after adjustment for AChE/Hb and the other covariates.

5.5 Key results from Paper VI

The original authors of the pyridostigmine trial (Gouge *et al*) had concluded that there was no effect of pyridostigmine on FVC among neither asthmatics nor healthy volunteers.[54] In contrast, my re-analysis of the same data in a mixed effect model showed that 2 hours after pyridostigmine administration, mean FVC was 4.3 [0.9; 7.8] percentage-points of predicted lower than at baseline. This was driven exclusively by a difference of 6.8 [2.8; 10.9] percentage-points of predicted for asthmatics, while there was no difference in FVC for healthy subjects. This is illustrated in Figure 5-2.

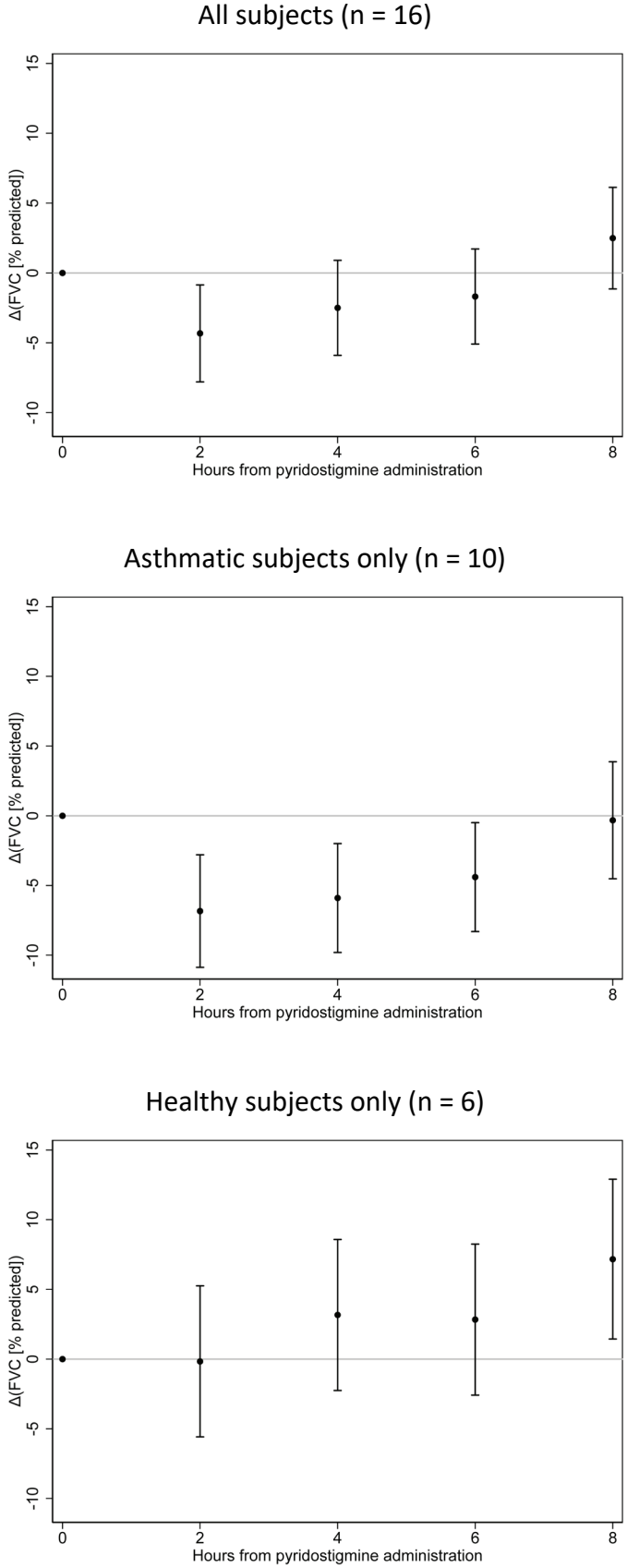
Figure 5-1: Main results from Paper IV and Paper V (health outcomes vs. AChE/Hb)



All results are from mixed effect models that include data from all three project phases. Solid black line shows estimated value of health outcome, relative to value at the median AChE/Hb in the model. Dashed black lines show 95% confidence interval. Black dots show location of knots for restricted cubic splines. Distribution of AChE/Hb values in each model indicated by overlaid histogram.

Analyses of glycemic regulation are adjusted for age, sex, alcohol consumption in last week, tobacco consumption in the last week, MET-minutes of physical activity in the last week, servings of fruits and vegetables consumed per day in the last week. Analyses of spirometric indices are adjusted for age, sex, pack-years of smoking, cumulated lifetime hours of cooking (and implicitly adjusted for height).

Figure 5-2: Results from re-analysis of pyridostigmine trial data



Dots represent point estimates of mean FVC in % predicted, relative to the mean at baseline. Bars represent 95% confidence intervals for the estimates.

6 Critical evaluation of methodology

6.1 Evaluation of methods in Paper I

As described above, Paper I is a peer-reviewed protocol for a systematic review on neuroactive non-organochlorine insecticides, diabetes mellitus and related metabolic disturbances. A systematic review can be defined as a "systematic assembly, critical appraisal and synthesis of all relevant studies on a specific topic".[74] Conducting a systematic review, as opposed to a narrative review, does not remove the need for subjective judgement, but has the purpose of making the subjective judgements consistent, transparent and reproducible.[75]

While the Navigation Guide methodology for systematic reviews normally includes integration of all available evidence on a topic (epidemiological, animal, *in vitro*, and *in silico*),[75] we decided to focus on peer-reviewed epidemiological evidence, as we deemed it infeasible to conduct a comprehensive review that included all streams of evidence, as well as gray literature. We might have missed some studies, but our justification for a new systematic review was that previous reviews could have missed important epidemiological evidence due to narrow search strategies, so we deemed it more important to conduct a very sensitive search for peer-reviewed epidemiological studies.

In the scoping review on page 10, I have only appraised the risk of bias due to confounding and not e.g. the risk of bias due to conflicts of interest. However, since most identified studies had a “high” or “probably high” risk of bias due to confounding, and since the studies had somewhat conflicting results, I concluded that there was only suggestive evidence for a causal link between exposure to cholinesterase inhibitor insecticides and diabetes mellitus. I find it unlikely that this conclusion would have been changed, had I also appraised the risk of bias from other sources than confounding.

6.2 Evaluation of methods in Paper II

6.2.1 Design and study population

The study described in Paper II was conducted as part of the COBIN-D trial, which was originally not intended as an occupational or environmental health study. However, the decision to conduct a study on pesticide exposure and diabetes mellitus as a part of the COBIN-D project seemed very attractive, as

1) In a previous phase of the COBIN project,[57 58] 35% of participants reported that their main occupation was agriculture, so we assumed that many of the participants would be exposed to various classes of pesticides.
2) The COBIN population was relatively large (2,815 persons in the previous phase[57]), and had been sampled from the general population in a random manner.
3) There were plans to measure fasting plasma glucose (FPG) in all participants.
4) It is difficult to establish new, large-scale epidemiological studies, especially in developing countries.

We used Paper II as a pilot study to assess the feasibility of conducting a second, more in-depth study in the non-intervention clusters of the COBIN-D population. The second study would include more detailed subjective exposure information, biomarkers of pesticide exposure, and measurement of HbA_{1c} in addition to FPG. However, as described on page 30, we discovered that while pesticide exposure prevalence was high in the COBIN-D population, the intensity of pesticide use was low. Therefore, we decided to conduct the second study among smallholder farmers in Uganda instead.

6.2.2 Information problems

6.2.2.1 Exposure assessment

Exposure assessment in this paper was entirely done by self-report. The relatively coarse exposure metrics could lead to non-differential misclassification of exposure, and therefore bias towards the null. To introduce bias away from the null, exposure misclassification would have to be differential, i.e. recall bias would have to be contingent on outcome status. If participants were aware of the subject of the study, diabetics might tend to over-report their pesticide exposure, which could introduce a spurious statistical association between pesticide exposure and diabetes, even if there was no causal link between the two. However, this is the opposite of the observed association (see

page 30). Therefore, I do not think that information problems related to exposure assessment pose any serious threat to the internal validity of the findings in the paper.

6.2.2.2 Outcome assessment

All participants were classified as diabetic or non-diabetic according to standardized criteria (see page 7), based on a fasting plasma glucose test. Due to financial constraints of the project, the device used to test participants was not a diagnostic-grade glucometer, but rather a device intended to monitor blood glucose levels in already diagnosed diabetics. Hence, it may not have had optimum accuracy or precision. Imprecision would lead to bias towards the null and cannot explain demonstrated associations. Inaccuracy might bias prevalence estimates for diabetes in the study population, but unless the inaccuracy is also related to exposure status, it cannot bias analyses of exposure vs. diabetes.

6.2.3 Selection problems

Participation rates in the COBIN study were high. In the previous phase of the COBIN project, 98% out of the 2,882 eligible individuals agreed to participate,[76] giving a study population of 2,815 persons. At baseline for Paper II, 172 (6%) of these individuals had either died or migrated out of the study area. Of the remaining 2,643 individuals, 2,310 persons (87.4%) consented to participate in the new study.

In the first phase of the COBIN project, participants were not tested for diabetes, but they were asked if they had ever been diagnosed with the disease. Compared to individuals who said that they had never been diagnosed, individuals who self-reported diabetes in the first phase of the COBIN project had a raw odds ratio = 1.68 [0.99; 2.85] for participation in this second phase, indicating that known diabetics were more likely than others to participate, and the difference was borderline statistically significant. This could bias our analyses of pesticide exposure vs. diabetes if participation rates also depended on exposure status. However, it is difficult to assess the potential for this to happen, as I have no information on pesticide use from the previous phase of the COBIN project.

6.2.4 Confounding

As Paper II is a (cross-sectional) observational study, caution is warranted before interpreting any statistical associations as causal effects, rather than due to confounding. While we did adjust our analyses for a number of possible confounders defined *a priori* based on literature, there were large demographic differences between exposed and non-exposed participants (see Paper II). It is

reasonable to assume that the two groups might also have differed in ways on which we do not have information (residual confounding), and this is a potential source of bias. I have also considered whether results could be biased by over-adjustment. E.g., if a hypothetical causal link between pesticide exposure and diabetes was mediated by BMI, adjustment for BMI would be inappropriate. However, as results from unadjusted and adjusted analyses were numerically similar, over-adjustment is unlikely to pose a problem for our analyses.

6.3 Evaluation of methods in Paper III

Exporting HbA_{1c} results digitally is not supported by the manufacturer of the HemoCue HbA1c 501 device, and the HemoDownloader software was developed after manually reviewing binary data from the HemoCue HbA1c 501 that were intended to be sent to a specialized printer, not to a computer for storage. To ensure integrity of the parsed HbA_{1c} data, extensive consistency checks were implemented in the HemoDownloader software. The software was used at the end of each working day while collecting data for Paper IV, and any bugs in the code were fixed when discovered. However, despite my efforts to make the software as reliable as possible, I cannot rule out the existence of additional software bugs, or that the software fails to account for unidentified details about the data format used by the HemoCue HbA1c 501. Hence, the software should only be used in epidemiological studies, and *not* for the diagnosis or monitoring of diabetes mellitus in a clinical setting. If the software was used clinically, it would be classified as a "medical device",[77] and it has *not* been approved for such use.

6.4 Evaluation of methods in Paper IV and Paper V

6.4.1 Study design and statistical analysis

In the PEXADU project, the same population sample was examined for both the exposure and outcomes of interest in three separate phases. It can therefore be discussed whether the PEXADU project is truly a follow-up study, or if the term "repeated measures study" would be a better description.

As described on page 28, we took the repeated measurements into account by analyzing data in multilevel linear mixed effect models. Our choice of model may be criticized, as any demonstrated associations between exposure and outcome might be due to reverse causality. This could especially be a problem for the analyses where both exposure and outcome are biochemical measurements (as in our analyses of AChE/Hb vs. blood glucose). Readers may suggest that it would have been more

appropriate to analyze our data in a way that explicitly accounted for the timing of measurements such that reverse causality was ruled out. E.g., one could fit a regression model of HbA_{1c} in phase 2 as a function of AChE/Hb in phase 1. However, such an analysis would be no guarantee against spurious statistical associations, as I will now demonstrate using Directed Acyclic Graphs (DAGs, a form of causal diagram).[78]

First assume that blood glucose levels causally influence AChE/Hb and not the other way around, and that both blood glucose and AChE/Hb are causally influenced by a set of confounders called C. The situation is depicted in the form of a DAG in Figure 6-1. Using standard rules for the analysis of DAGs,[78] we notice that to remove all spurious associations between AChE/Hb in phase 1 and blood glucose in phase 2, we need to adjust for both blood glucose in phase 1 and for the confounders called C. In DAG terminology, the adjustment will close the "backdoor paths" shown in purple. If we do that, we should find no association between phase 1 AChE/Hb and phase 2 blood glucose.

Figure 6-1: Directed Acyclic Graph assuming causal link from blood glucose to AChE/Hb

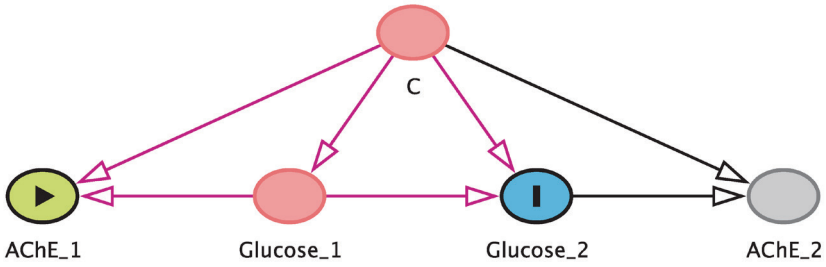


Figure created using the DAGitty software.[79] See text for details; color symbology is explained on page 68.

Now, assume instead that AChE/Hb causally influences blood glucose levels, but the effect is transient and does not last from one project phase to the next, as depicted in the DAG in Figure 6-2. In this case, adjusting for the same variables (blood glucose in phase 1 and confounders C), we would remove not only the spurious associations due to the backdoors through C. We would also miss the causal link between AChE/Hb and blood glucose, as we would have closed the causal path AChE_1 ➔ Glucose_1 ➔ Glucose_2.

Figure 6-2: Directed Acyclic Graph assuming causal link from AChE/Hb to blood glucose

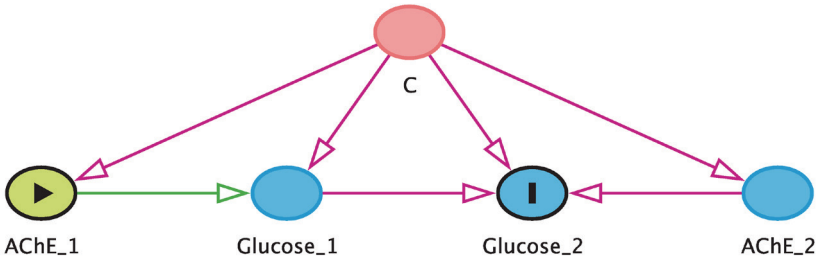


Figure created using the DAGitty software.[79] See text for details; color symbology is explained on page 68.

While the two previous examples are hypothetical, they do illustrate that analyzing data in an explicit follow-up model does not guarantee against bias. The price for avoiding a false positive result could be getting a false negative result instead.

Since we did not analyze our data in a model with an explicit follow-up structure, what is the advantage of our study design with repeated measurements in 364 persons, compared to a simple cross-sectional study with a study sample three times as large? One answer is that in studies with only a single measurement of exposure per person, exposure-response relationships will be biased toward the null, unless exposure is perfectly constant and perfectly measured. The degree of attenuation of the exposure-response relationship is given by Equation 6-1,[80] where β' is the observed regression coefficient that can be derived from study data, β is the true (unobserved) correlation between the exposure and outcome variables, ${\sigma^2}_{WP}$ is the within-person variance in exposure, ${\sigma^2}_{BP}$ is the between-person variance in exposure, and *n* is the number of times that exposure is measured for each person.[80]

Equation 6-1

$$\beta' = \beta \times \left(1 + \frac{\left(\frac{{\sigma^2}_{WP}}{{\sigma^2}_{BP}} \right)}{n} \right)^{-1}$$

Equation 6-1 shows that when the within-person variance in exposure grows relative to the between-person variance, the exposure-response relationship is attenuated (i.e., biased towards the null), but the degree of attenuation can be limited by increasing the number of exposure measurements per person (*n*). Hence, with multiple measurements of the exposure variable, results from regression analyses will be less biased.

Random measurement errors in the outcome variable will not attenuate the exposure-response relationship,[80] but effect estimates will be less precise and have wider confidence intervals, leading to a risk that demonstrated effects will be rejected as “statistically non-significant”. Our study design, with repeated measurements of both exposure and outcome, will therefore lead to effect estimates that are both more accurate and precise than a cross-sectional study would.

6.4.2 Information problems

Both exposure and outcome levels were objectively measured in the PEXADU population, but could still be biased or imprecise. In the following sections, I will present and discuss results from quality control of the measurements.

6.4.2.1 Temperature-sensitive materials

It was not possible to find a temperature-controlled facility for the storage of materials and participant examinations. The lack of temperature control could potentially have been a problem for the validity for measurements of both AChE/Hb and glycemic regulation, as the reagents were temperature-sensitive. The manufacturers' recommended temperature ranges for device operation and reagent storage are listed individually in Table 6-1 for the HemoCue Glucose 201 RT, HemoCue HbA1c 501 and Test-Mate ChE Cholinesterase System.

Table 6-1: Recommended temperature ranges for biochemical test equipment

Device	Operating temperature	Reagent storage temperature
HemoCue Glucose 201 RT	15-27 °C	0-30 °C
HemoCue HbA1c 501	17-32 °C	2-32 °C
Test-mate ChE	15-30 °C	15-30 °C

Data from operating manuals.[60 81 82]

AChE test materials were stored at room temperature at the UNACOH office in Kampala from February 2018 to August 2018. They were then moved to my private home in Kampala, and finally to the examination center. Reagents for the glucose and HbA_{1c} tests were stored at a temperature-controlled facility in Kampala. Shortly before use, they were delivered either to my private home in Kampala, or to the examination center in Wakiso, where they were stored at room temperature.

As Uganda has a tropical climate, it is highly unlikely that any reagents have been stored at temperatures lower than those recommended by the manufacturers. However, it was not immediately clear if any of the materials might have been stored at temperatures above the recommended ranges. I have no data on the temperature in my home in Kampala, but the building felt cool, and I find it unlikely that the indoor temperature was ever above 30 degrees. The temperature at the examination center was not continuously logged, but because of the large number of AChE tests carried out, and because the temperature at the time of each analysis was

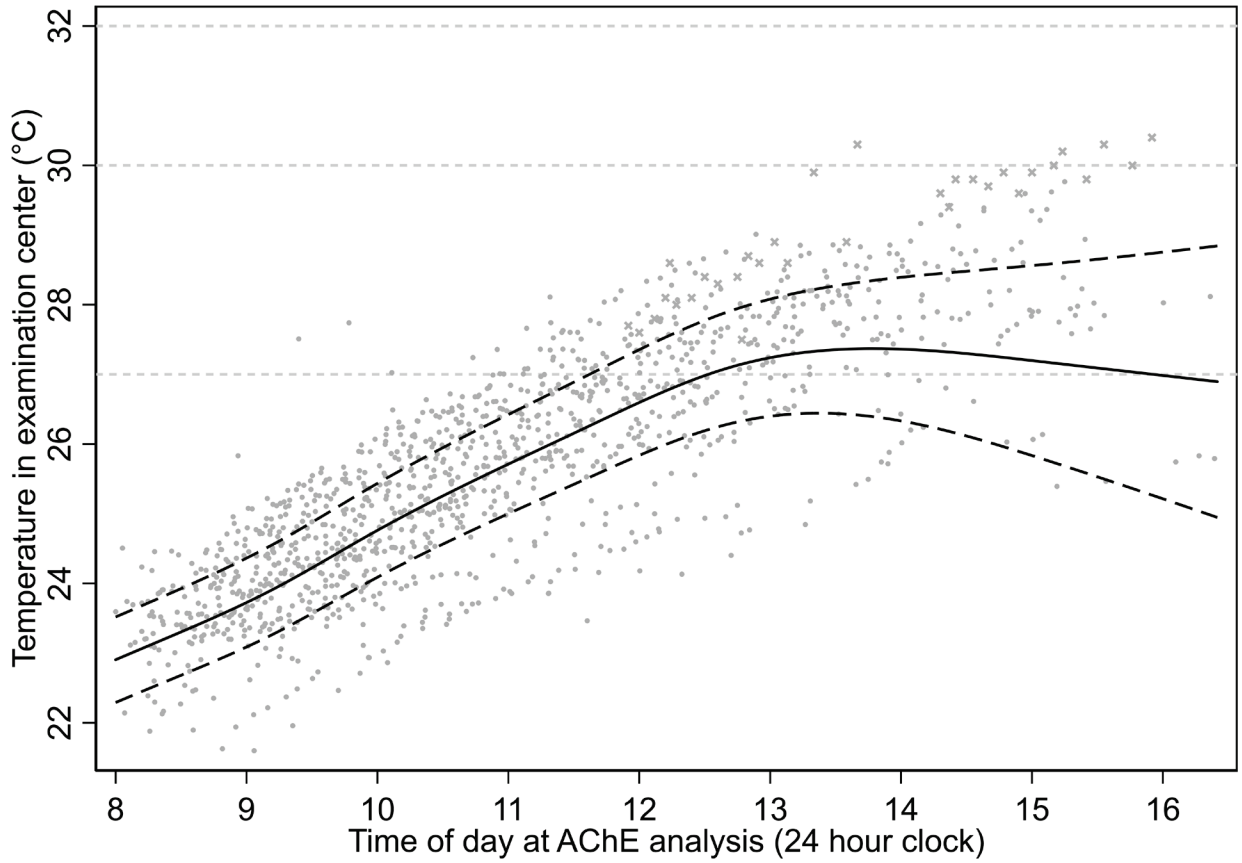
recorded, it was possible to get an overview of the temperature at the examination center across all working days (Figure 6-3).

Figure 6-3 shows that the recorded temperature at the examination center was only above 30 °C on one specific date (the points marked as crosses). While the temperature might have been higher on weekends, when the windows and doors of the examination center were closed, the results do indicate that high storage temperatures are unlikely to have influenced the reagents while at the examination center. However, we note that for a considerable amount of time, temperatures were above 27 °C, which is the upper limit of the recommended operating temperature for the HemoCue Glucose 201 device. To investigate if this could have influenced our results, I carried out descriptive analyses of the temperature at each FPG analysis. I linearly interpolated times and temperatures from the AChE analyses to obtain a best estimate of the temperature at each FPG analysis. The results are shown in Table 6-2. We notice that the percentage of analyses carried out at temperatures > 27 °C ranges from 3% to 15%, depending on the phase. As I could not rule out temperature-related errors in these measurements, my analyses of AChE/Hb vs. FPG included a sensitivity analysis where the high-temperature FPG analyses were excluded. Results from this sensitivity analysis was similar to the main analysis.

Table 6-2: Estimated temperature at time of FPG analysis

Project phase	Number of FPG measurements	Temperature OK (15-27 °C) n (%)	Temperature too high (>27 °C) n (%)
1	128	118 (92.2%)	10 (7.8%)
2	210	179 (85.2%)	31 (14.8%)
3	253	245 (96.8%)	8 (3.2%)
Total	591	542 (91.7%)	49 (8.3%)

Figure 6-3: Temperature at AChE examination as a function of time of day



Solid black line = trend from random coefficient model with restricted cubic splines of time (7 knots) and date as the level of the random terms. Dashed black lines = upper and lower limits of 95% confidence interval for the trend. Dashed gray lines = upper limits of temperature ranges (see text for details).
Dots are individual measurements of temperature. Crosses are measurements of temperature from analyses of AChE on venous blood samples that were carried out for quality control purposes (i.e., they are not from analyses of participant's primary AChE results).

6.4.2.2 Exposure assessment

6.4.2.2.1 Use of acetylcholinesterase as a biomarker of insecticide exposure

Red blood cell acetylcholinesterase (AChE) is *not* the primary toxicodynamic target of organophosphate and carbamate insecticides (the neuronal isoform of the enzyme is),[9 17] but AChE is readily available for sampling and can be used to quantify exposure. As described on page 4, carbamates reversibly inhibit AChE, while the inhibition caused by organophosphates is irreversible once the AChE-organophosphate complex has undergone a process known as "aging".[9 17] As red blood cells lack the biochemical machinery for protein synthesis, red blood cell AChE only returns to normal as new blood cells are produced. Hence, AChE may be used to monitor exposure even at a

time when all of the organophosphate insecticide has been metabolized and excreted. This was illustrated in a monitoring study of 8 human subjects who had been accidentally poisoned with the organophosphate dichlorvos; AChE reverted towards baseline as a linear function of time, and the estimated time to normalization was 82 days,[83] despite the fact that dichlorvos has a short half-life in humans. In a human volunteer who ingested a single dose of 5 mg dichlorvos, urinary excretion had practically stopped after 9 days.[84] Hence, AChE can be used to monitor exposure to carbamates in the short term, and to organophosphates in the short to medium term. It cannot be used to monitor long-term exposure (years).

The interpretation of acetylcholinesterase activity analyses among pesticide-exposed persons is complicated by a considerable interindividual variability. In a study of 40 blood donors from the USA, AChE/Hb measured with the Test-Mate device (the same type of device as used in the PEXADU study) had a mean of 27.1 U/g, an SD of 2.9 U/g and a range of 21.9-37.3 U/g.[60] In a recent study among 242 healthy volunteers in Germany, the ratio between the 97.5th percentile and the 2.5th percentile of AChE/Hb was 1.5; the assay was similar to the one used by the Test-Mate.[85] When AChE/Hb is used for occupational safety monitoring of pesticide-exposed workers, it is therefore recommended to compare results from each person to their own pre-exposure AChE/Hb instead of relying on population reference values.[86]

While detailed subjective information on pesticide exposure was available in the PEXADU project, the subjective exposure information did not correlate with AChE/Hb, as described in Paper IV and Paper V. This might indicate that AChE/Hb was primarily influenced by some other factor than insecticide exposure,[11] so that AChE/Hb would be a poor exposure metric. However, it might also be because AChE/Hb reflects total exposure to organophosphate and carbamate insecticides (e.g., spraying, bystander exposure, re-entry work in sprayed fields, consumption of fruit and vegetables with insecticide residues), and we only have subjective information on spraying. AChE/Hb is a well-established biomarker of exposure to cholinesterase inhibitor insecticides.[11 86] We therefore decided to use AChE/Hb as our only exposure metric, and keep the potential for confounding in mind when interpreting statistical analyses. The subjective exposure information will only later be used for statistical analyses of exposure vs. health if we can validate it against other biomarkers, such as urine metabolites or pesticide residues in the passive samplers worn by participants (see page 24). In the following sections, I will describe the extensive quality checks conducted to ensure the validity of our AChE/Hb data.

6.4.2.2.2 Results from negative control

The manufacturer of the Test-Mate analyzer recommends that quality control is performed on each day of testing by analyzing a sample from a person with no known exposure to organophosphate or carbamate insecticides,[60] and I volunteered. Quality controls were performed on my capillary blood on each day of testing, as well as on some training days before data collection started. Results are plotted in Figure 6-4. To enable analysis of within-day variance for the negative control results, on the last 8 days of the project two analyses were carried out within 10 minutes of each other. Both results are plotted in Figure 6-5. Analyses of variance for the AChE analyses done on my blood are presented in Table 6-3 (all phases, limited to the first measurement of the day) and Table 6-4 (repeated measurements on the last 8 days of phase 3).

Results in Table 6-3 are split into total variance (σ^2_{TOT}), between-phase variance (σ^2_{BPh}) and within-phase/between-day variance (σ^2_{WPh}). It is clear that almost all the variance in both AChE, hemoglobin and AChE is due to day-to-day variability in the results. This can be confirmed visually in Figure 6-4, where there is no clear trend in results between phases. This suggests that AChE reagents were not damaged by temperature or other factors during storage, as described on page 41.

The results in Table 6-3 cannot tell us about the source of the within-day variance, but it must be the sum of true biological variance in the measured quantities, and the variance due to measurement errors. To determine the relative importance of these two sources of within-day variability, we turn to Table 6-4. Results are split into between-day variance (σ^2_{BD}) and within-day variance (σ^2_{WD}). We find that the vast majority of the variance over these 8 days is due to within-day variability. As it is highly unlikely that the (unobserved) true levels of hemoglobin and AChE changed in my blood in the few minutes passing between each analysis, the within-day variance in Table 6-4 must be due to measurement errors. It should be noted that my mean AChE/Hb was ~30 U/g, which is close to the reference value of 31.4 U/g reported by the manufacturer of the Test-Mate ChE device.[60]

Table 6-3: Analysis of variance for negative control AChE (first result of the day, all phases)

Variable	Arithmetic mean	σ^2_{TOT}	σ^2_{BPh}	σ^2_{WPh}	$\sigma^2_{WPh}/\sigma^2_{TOT}$
AChE (U/ml)	4.20	0.045	0.000	0.045	1.000
Hemoglobin (g/dL)	13.83	0.193	0.001	0.192	0.995
AChE/Hb (U/g)	30.36	1.370	0.000	1.370	1.000

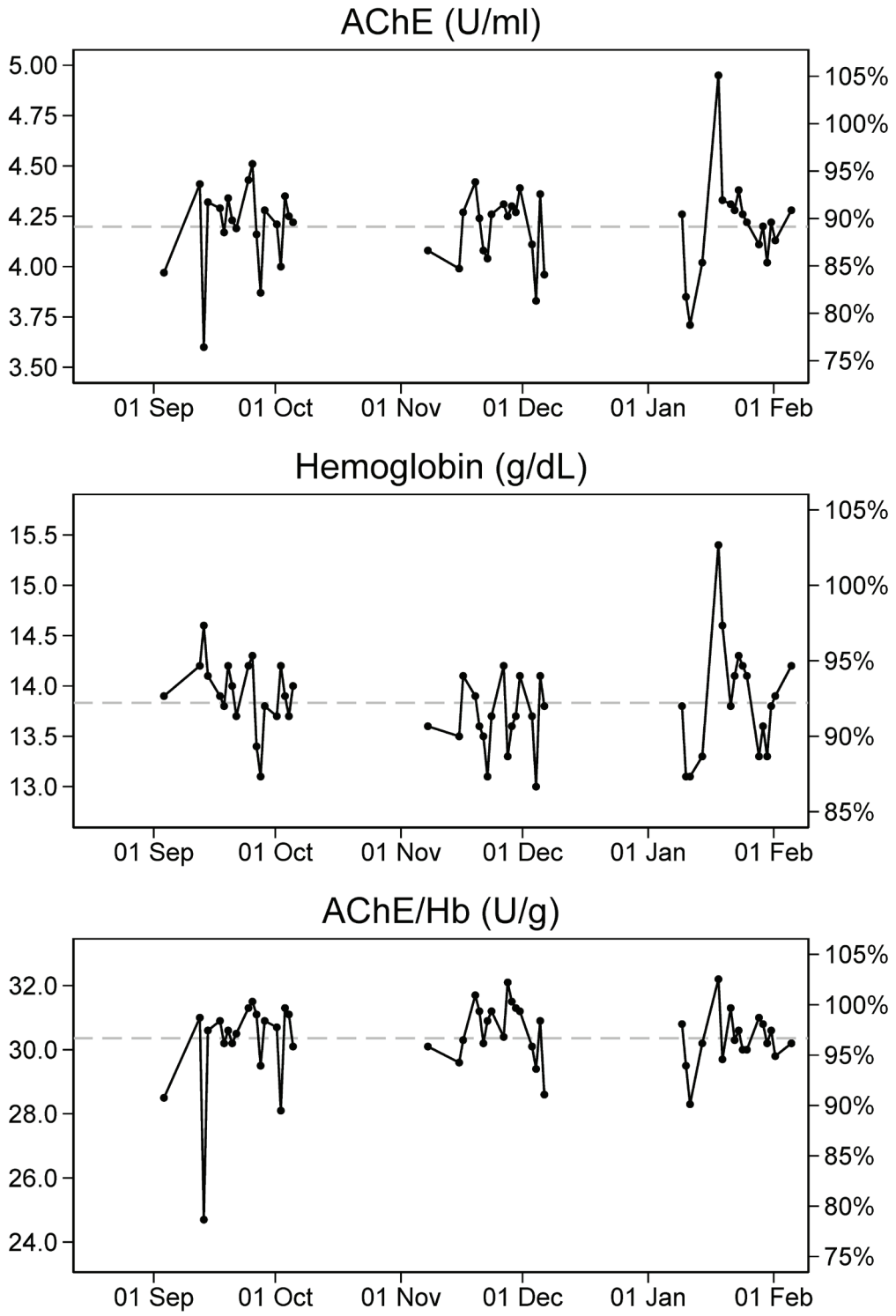
σ^2_{TOT} = total variance. σ^2_{BPh} = between-phase variance. σ^2_{WPh} = within-phase variance = between-day variance. The data corresponds to the plots in Figure 6-4. The very first measurement done by the nurse after training was excluded, as it contained an obvious error ($Hb = 4.0\ g/dL$).

Table 6-4: Analysis of variance for repeated negative control AChE (last 8 days of phase 3)

Variable	Arithmetic mean	σ^2_{TOT}	σ^2_{BD}	σ^2_{WD}	$\sigma^2_{WD}/\sigma^2_{TOT}$
AChE (U/ml)	4.10	0.025	0.000	0.025	1.000
Hemoglobin (g/dL)	13.66	0.140	0.007	0.133	0.951
AChE/Hb (U/g)	30.03	0.502	0.000	0.502	1.000

σ^2_{TOT} = total variance. σ^2_{BD} = between-day variance. σ^2_{WD} = within-day variance = between-analysis variance.

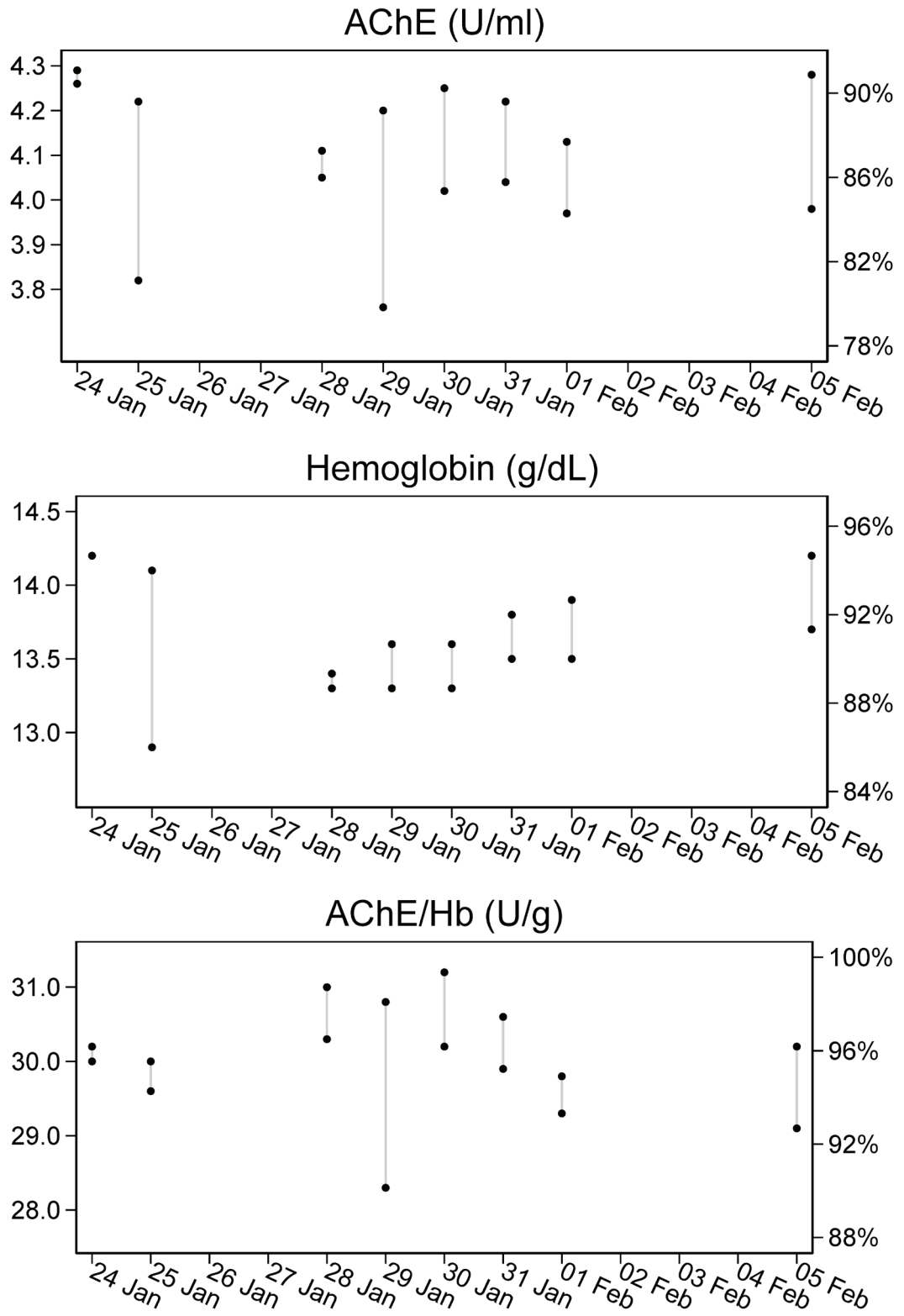
Figure 6-4: AChE quality control data from negative control



Hb = hemoglobin. AChE/Hb = hemoglobin-adjusted AChE.

Left-hand y axis shows absolute values, right-hand y axis shows percent normal as defined by manufacturer of AChE analyzer. Dashed gray lines are means across all phases (see Table 6-3, page 46). All measurements made with the same device. In the cases where more than one measurement was made on a specific day, only the first value is included. The very first measurement done by the nurse after training was excluded, as it contained an obvious error ($Hb = 4.0\ g/dL$).

Figure 6-5: Repeated AChE quality control data from negative control



Hb = hemoglobin. AChE/Hb = hemoglobin-adjusted AChE.

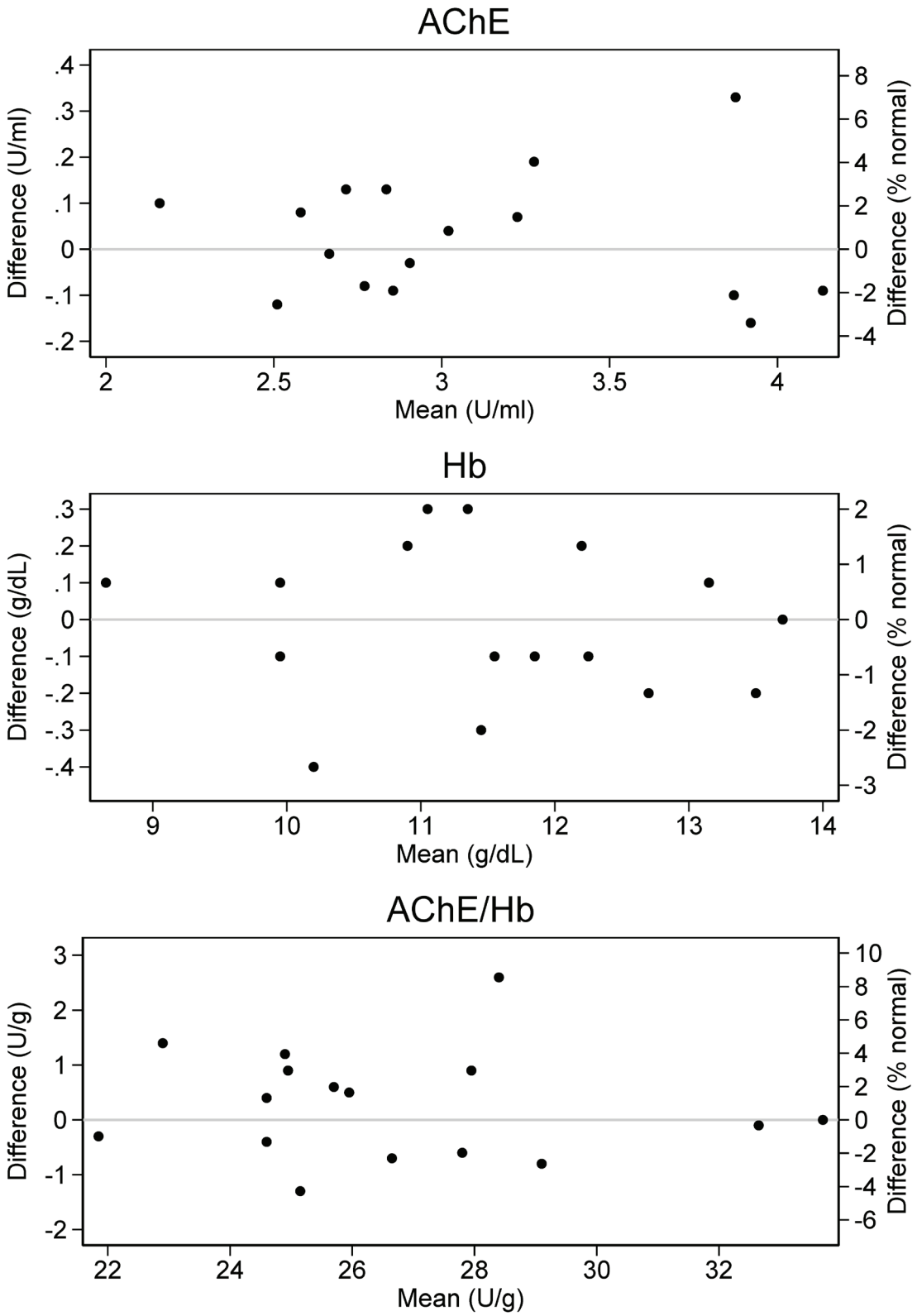
Left-hand y axis shows absolute values, right-hand y axis shows percent normal as defined by manufacturer of AChE analyzer.

6.4.2.2.3 Influence of operating procedures

During the second phase of the PEXADU project, I discovered that the nurse responsible for analyzing AChE had slightly changed the test procedure. In phase 1 and the beginning of phase 2, she vigorously shook the test tube after adding reagent for the analysis. After reading the manufacturer's instructions[60] carefully, she had switched to gently turning the test tube back and forth after adding reagents. If this change in procedures could influence the results, it could make it difficult to compare AChE results across phases.

On two separate days, I randomly selected a total of 16 participants whose venous blood samples were analyzed twice for AChE - once where the test tube was shaken vigorously after adding reagent, and once where the tube was turned over gently. Results from this quality control are presented in Bland-Altman plots in Figure 6-6. There is no systematic difference in results from the two mixing procedures, only random errors. The change in procedure is therefore unlikely to have biased our results.

Figure 6-6: Bland-Altman plots of the influence of operating procedures on AChE results



Difference = (result from vigorous mixing) – (result from gentle mixing)

Right-hand y-axes show differences in percent of the normal level of the variable (e.g., AChE = 4.71 U/ml). See text for details.

6.4.2.2.4 Analysis of variance for study participants

Table 6-1 presents an analysis of variance for AChE, hemoglobin and AChE/Hb for study participants, based on linear mixed effect models that only include random terms for family and participant. The variance is split into between-family variance (σ^2_{BF}), between-person variance (σ^2_{BP}) and within-person/between-phase variance (σ^2_{WP}). We notice that family relations (genetics and shared environment) account for only 6% of the variance in AChE, 23% of the variance in hemoglobin, and 0% of the variance in AChE/Hb. Differences between individuals seems to matter the most, with between-person variance accounting for 71%, 45% and 82% of the total variance in AChE, hemoglobin and AChE/Hb, respectively. But there is also considerable within-person variance (23% for AChE, 33% for hemoglobin and 18% for AChE/Hb). In addition, we note that the mean AChE/Hb for participants (25.8 U/g) was considerably lower than the mean result from myself (negative control, mean 30.4 U/g; see page 45). This difference in mean AChE/Hb is expected due to the presumed insecticide exposure of the participants.

Table 6-5: Analysis of variance for participant's AChE analyses

Variable	$\bar{x}$	σ^2_{TOT}	σ^2_{BF}	σ^2_{BP}	σ^2_{WP}	$\sigma^2_{BF}/\sigma^2_{TOT}$	$\sigma^2_{BP}/\sigma^2_{TOT}$	$\sigma^2_{WP}/\sigma^2_{TOT}$
AChE (U/ml)	3.00	0.307	0.018	0.219	0.070	0.058	0.713	0.228
Hemoglobin (g/dL)	11.66	1.917	0.433	0.853	0.631	0.226	0.445	0.329
AChE/Hb (U/g)	25.79	15.818	0.000	12.923	2.894	0.000	0.817	0.183

$\bar{x}$ = arithmetic mean. σ^2_{TOT} = total variance. σ^2_{BF} = between-family variance. σ^2_{BP} = between-person variance. σ^2_{WP} = within-person variance = between-phase variance. Number of persons = 364, number of measurements = 1071.

As mentioned above (see page 45), the within-day variance in negative control results (Table 6-4) are likely due to measurement errors. Participant results shown in Figure 6-6 indicate that the size of measurement errors from the Test-Mate are independent of the size of the measured quantity. Hence, participants' within-person variance must be the sum of the variance attributable to biological changes and the variance attributable to measurement errors:[87]

Equation 6-2

$$\sigma^2{}_{WP,biology+error} = \sigma^2{}_{WP,biology} + \sigma^2{}_{error}$$

If the size of the measurement error depended on the biological quantity, the situation would be more complex.[87] Equation 6-2 can be rearranged to obtain

Equation 6-3

$$\sigma^2{}_{WP,biology} = \sigma^2{}_{WP,biology+error} - \sigma^2{}_{error}$$

According to this equation, we can estimate the proportion of the within-person variance attributable to biological changes by subtracting the variance due to errors (listed in Table 6-4) from the overall within-person variance. I applied this formula to results from Table 6-5; results are shown in Table 6-6.

Table 6-6: Estimated proportion of variance in participants' AChE results attributable to measurement errors

Variable	σ^2_{TOT}	σ^2_{WP}	σ^2_{error}	$\sigma^2_{WP,biology}$	$\sigma^2_{error}/\sigma^2_{TOT}$	$\sigma^2_{error}/\sigma^2_{WP}$	$\sigma^2_{WP,biology}/\sigma^2_{WP}$
AChE (U/ml)	0.307	0.070	0.045	0.025	0.145	0.638	0.362
Hemoglobin (g/dL)	1.917	0.631	0.193	0.437	0.101	0.306	0.694
AChE/Hb (U/g)	15.818	2.894	1.370	1.525	0.087	0.473	0.527

σ^2_{TOT} = total variance (copied from Table 6-5). σ^2_{WP} = within-person variance = (copied from Table 6-5). σ^2_{error} = variance due to errors (copied from Table 6-4). $\sigma^2_{WP,biology}$ = within-person variance due to biological changes.

Table 6-6 shows that measurement errors contributed an estimated 15%, 10% and 9% of the total variance in AChE, hemoglobin and AChE/Hb, respectively. For within-person variance, the numbers are 64% for AChE, 31% for hemoglobin and 47% for AChE/Hb. In other words, while variance due to measurement errors is a relatively small proportion of total variance, it is responsible for about half of the within-person variance. Hence, it seems that our measurements of AChE/Hb primarily reflect biological differences between individuals.

The manual for the Test-Mate analyzer states that "intraindividual variability of (…) erythrocyte (…) cholinesterase is less than 5% per week and less than 10% per month".[60] The within-person coefficients of variation (including both measurement error and biological changes) among participants can be estimated to 9% for AChE, 7% for hemoglobin and 7% for AChE/Hb. The demonstrated intraindividual variability was thus roughly within the expected range.

6.4.2.2.5 Quality control of results from the Test-Mate ChE device

After each participant's examination in each phase, I reviewed the results and informed the participant before they left the examination center. Towards the end of phase 1, I was puzzled by the results from the Test-Mate ChE analyzer: I had the impression that approximately half the study population was anemic (defined as hemoglobin ≤12.9 g/dL for men and ≤11.9 g/dL for women).[88] While anemia is a public health problem in Uganda,[89] I deemed such a high prevalence of anemia unlikely.

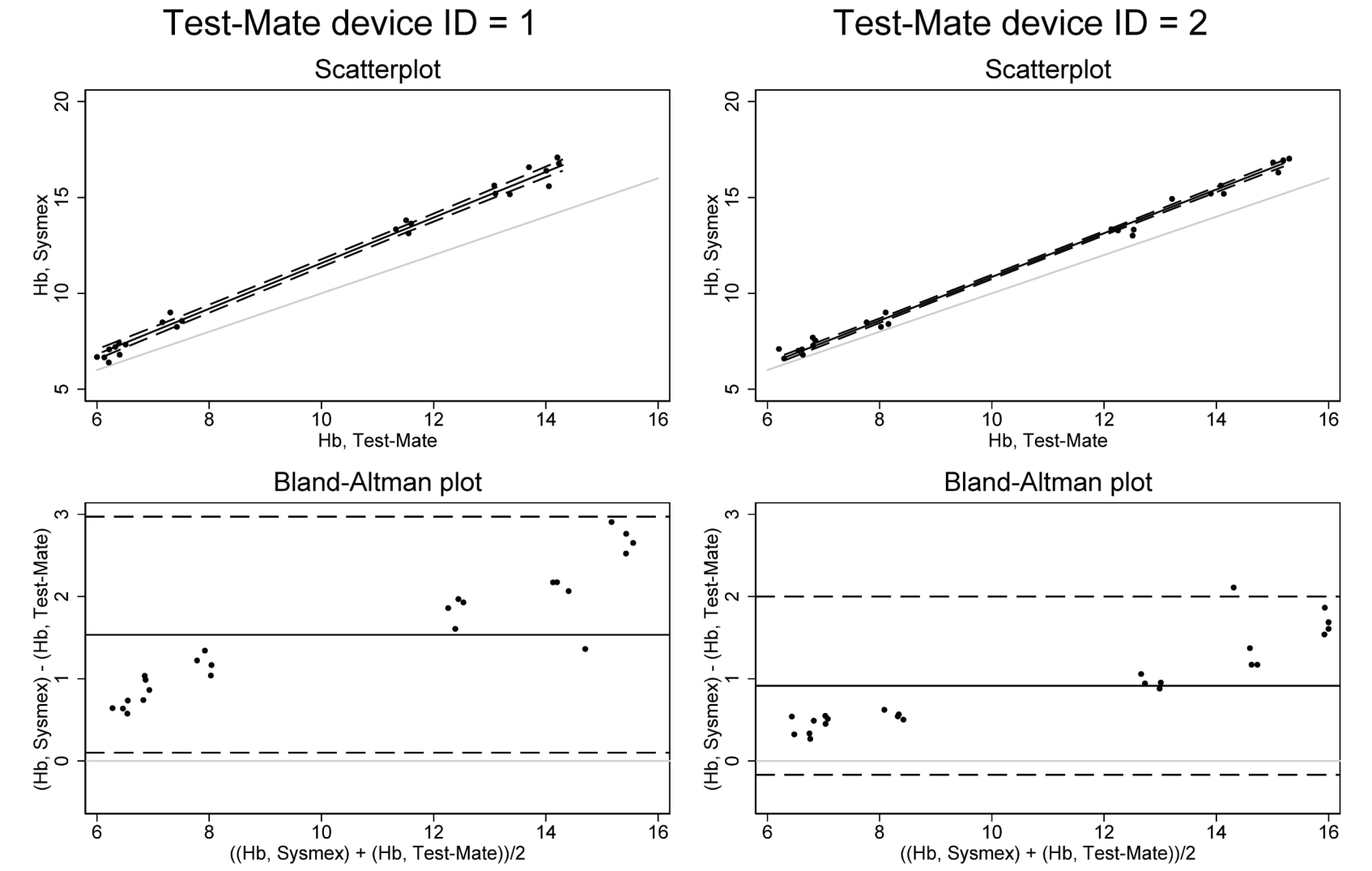
If measurements of hemoglobin were biased, our main exposure metric (AChE/Hb) might also be biased, which could potentially lead to problems during our statistical analyses of exposure vs. health outcomes. To investigate this issue, quality control analyses were conducted in Denmark in the end of November 2019. 6 anonymized K-EDTA whole blood patient samples were provided by the Department of Clinical Biochemistry, Aarhus University Hospital (courtesy of project coordinator Uffe Lund Lystbæk). Along with the samples, I received information on their hemoglobin concentration, as determined by the analyzer Sysmex XN-9000 (Sysmex Corporation, Kobe, Japan). The samples had been selected by the staff at the Department of Biochemistry to represent a wide span of hemoglobin values.

I analyzed each sample four times with each of two different Test-Mate devices: Device number 1 (the device used for all analyses during data collection in Uganda) and device 2 (a spare device that had not been used during data collection). In each round of measurements (1-4), the order of the samples was randomized using a pseudo-random number generator, and so was the order of the devices used. All samples were analyzed using the same batch of reagents that been left over from the PEXADU project. I wrote results down on paper at the time of analysis and later double-entered them into a database.

Figure 6-7 presents results from a comparison of results from the two Test-Mate devices with the results from the Sysmex device (considered the "gold standard"). Hemoglobin values from both of the Test-Mate devices are negatively biased, and this bias is larger for device 1 than for device 2. The influence of this bias on AChE/Hb was investigated by plotting results from the two Test-Mate devices against each other, as presented in Figure 6-8. The figure confirms that compared to device 2, device 1 has negative bias in the hemoglobin values presented. However, the refigure also shows that device 1 has a similar negative bias in AChE. These two biases almost cancel each other out so that AChE/Hb is approximately the same from the two devices.

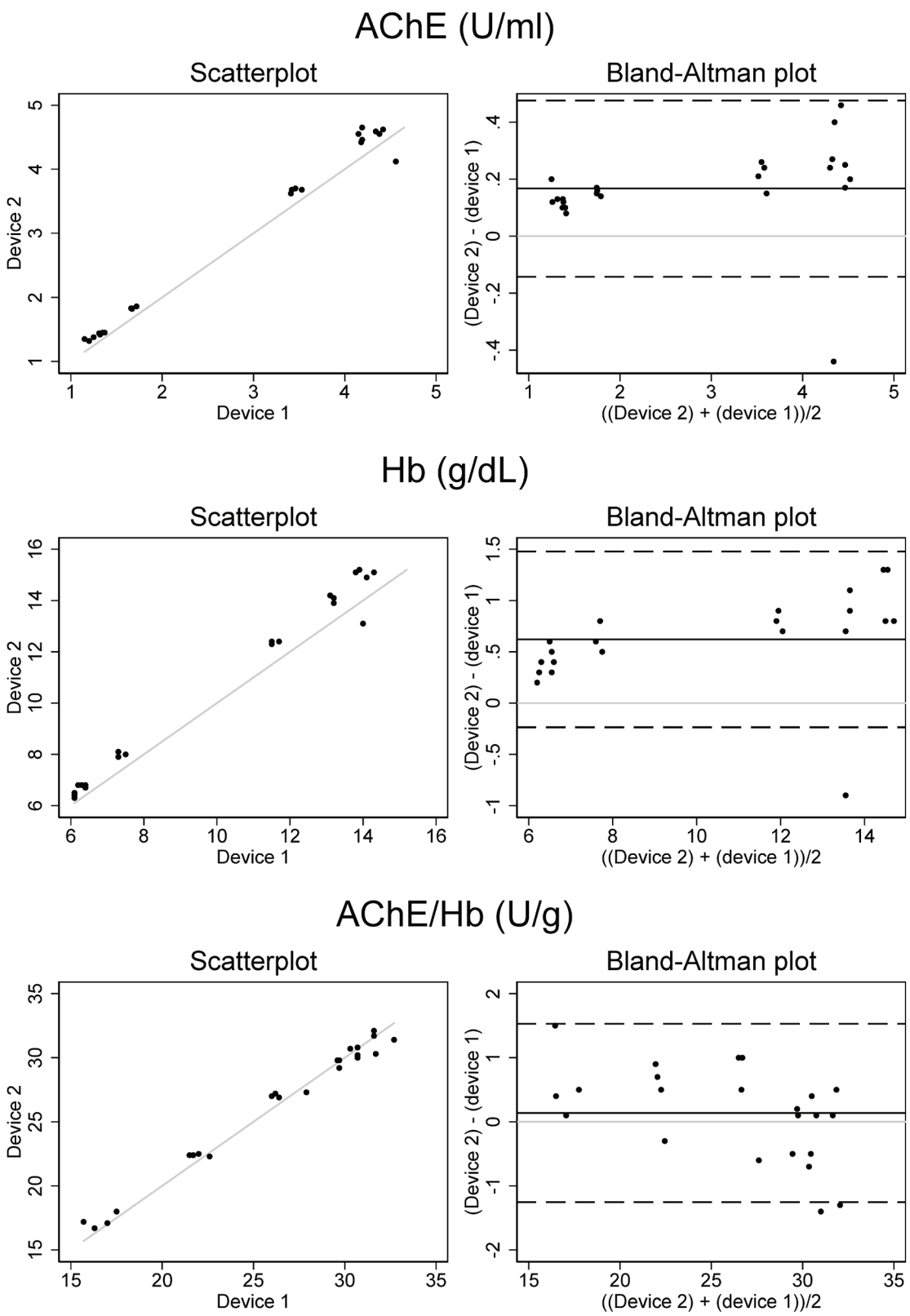
These analyses do not tell whether AChE results from Test-Mate device 1 or 2 are most accurate. To answer that question, we would have to compare AChE from both devices to results from a "gold standard" device. Unfortunately, AChE is not one of the biochemical analyses offered at Aarhus University Hospital. However, since AChE/Hb was almost the same for the two devices, I do not believe that this exposure metric was biased to a considerable degree. Figure 6-8 does suggest that for device 1 compared to device 2, AChE/Hb is slightly biased upwards for high values of AChE/Hb, and slightly biased downwards for low values of AChE/Hb. If results from device 2 were more accurate than results from device 1, such bias in results from device 1 would lead to a flattening of exposure-response relationships, as outlined in Figure 6-9. That means that any bias in AChE/Hb is more likely to lead to under-estimation than over-estimation of exposure-response relationships in our analyses of health effects.

Figure 6-7: Venous hemoglobin measured by Sysmex vs. Test-Mate



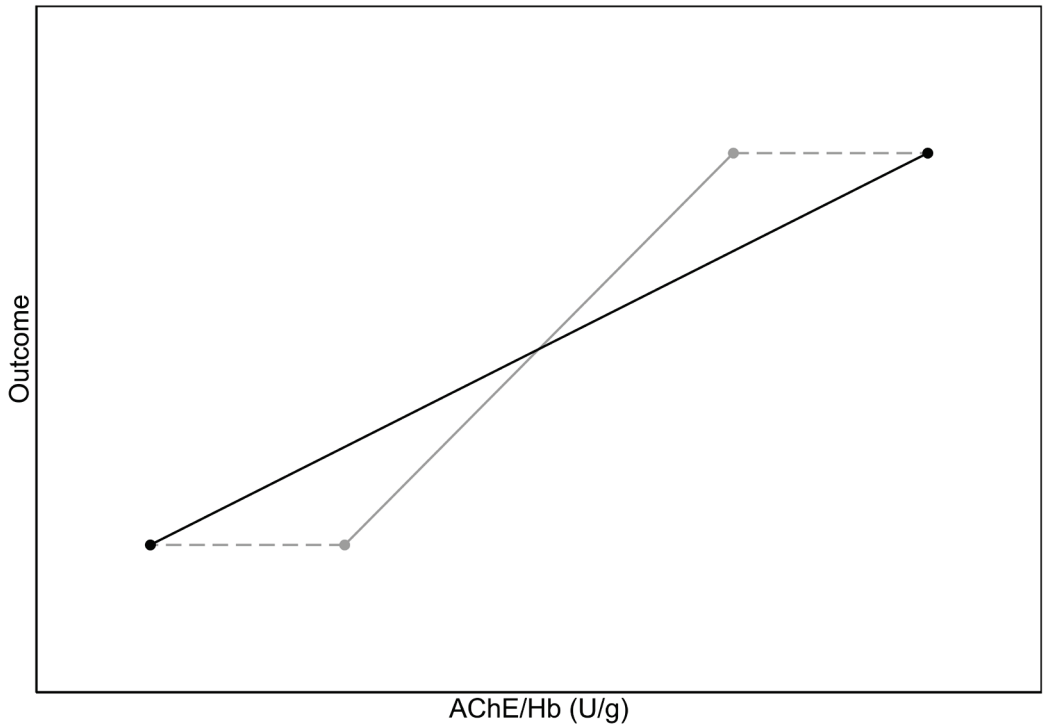
In the scatterplots, markers have been slightly jittered to avoid overlap. The gray line is the line of equivalence (y = x). The black lines represent trend with 95% confidence interval, derived using a linear mixed effect model with sample ID as a random effect. In the Bland-Altman plots, the gray line is located at zero. The solid black line represents the mean difference between devices, and the dashed black lines represent the upper and lower boundary of the 95% prediction interval, respectively.

Figure 6-8: Comparison of AChE, Hb and AChE/Hb between two Test-Mate devices



In the scatterplots, the gray line is the line of equivalence (y = x). In the Bland-Altman plots, the gray line is located at zero. The solid black line represents the mean difference between devices, and the dashed black lines represent the upper and lower boundary of the 95% prediction interval, respectively.

Figure 6-9: Schematic representation of potential effect of measurement error in AChE/Hb on exposure-response relationships



Gray markers represent two theoretical "true" pairs of AChE/Hb and outcome, and the line connecting them represents the "true" exposure-response relationship. Black markers represent the same two points, but parallel shifted under the assumptions that outcome is perfectly measured, and that AChE/Hb measurements are biased downwards for low values and upwards for high values. The black line represents the resulting, observed exposure-response relationship.

6.4.2.3 Pulmonary function parameters

6.4.2.3.1 Standardization and quality assurance of participant results

Three nurses were employed as data collectors in the PEXADU project. Before data collection started, I provided a two-day course to the nurses on the theory of obstructive airway diseases and pulmonary function testing with a focus on quality assessment and participant coaching. Spirometry was carried out in a standardized manner according to ATS criteria[46] to ensure maximum data quality.

Before statistical analyses, data quality for all spirometries were assessed according to modified ATS criteria.[46] Across all phases of the PEXADU project, participants had performed 4,931 individual blows with the MicroDL spirometer. I created an electronic form in ODK[90] collect. The form showed the flow-volume and volume-time curve from each blow in turn and prompted me to state whether the curves showed any of the individual issues in Table 6-7 (modified from the online appendix of Paper V). Once all blows had been processed, the database was imported in Stata 15. Based on the presence

or absence of individual signs, each blow was categorized as acceptable/unacceptable or usable/unusable according to the criteria in Table 6-7. I dropped results from all examinations where there was < 2 acceptable blows, and results from non-repeatable examinations (defined as a difference between best and second-best usable value ≥ 0.25 L for either FEV_1 or FVC).

Table 6-7: Spirometry quality assessment criteria for individual blows

	Disqualifies blow from being…		
	Acceptable	Usable for FEV_1	Usable for FVC
Cough in 1st second	x	x	x
Volume-time curve without plateau	x		
Obstruction of mouthpiece	x		
Sub-maximal blowing effort	x		
Leak around mouthpiece	x		
Slow start of exhalation	x	x	x
Extra inhalation (within 1st second)	x	x	
Extra inhalation (after 1st second)	x		x

Due to the magnitude of the task of assessing all 4,931 blows for quality, complete assessment in duplicate was infeasible. To evaluate the validity of my assessments, a second physician with experience in pulmonary function testing (Wajd Abbas Hassan Hansen, WAHH) also assessed all blows from phase 1 (1,723 blows total from 304 individuals) using the same system. Table 6-8 shows the level of agreement between the assessments made by WAHH and myself; we agreed on the quality of the examination in 94.7% of the 304 cases.

Table 6-8: Interrater agreement for quality control of 304 spirometries from phase 1

		MRHH	
		OK	Problem
WAHH	OK	276	2
	Problem	14	12

A "problem" is defined as a spirometric examination with < 2 acceptable blows, or non-repeatable results. MRHH = Martin Rune Hassan Hansen. WAHH = Wajd Abbas Hassan Hansen.

When one outcome is much more common than the alternatives, a high level of agreement might be due to chance. This could be the case here, as most examinations were classified as "OK" by both assessors. Hence, the kappa statistic[74] might be a better measure of agreement. The kappa statistic[74] compares the level of agreement observed (A_{obs}) to the level expected by random chance (A_{exp}) and is defined by Equation 6-4:

Equation 6-4

$$\varkappa = \frac{A_{obs} - A_{exp}}{1 - A_{exp}}$$

In case of perfect agreement between two assessors, then $A_{obs} = 1$ and $\varkappa = 1$. On the other hand, if any agreement is solely due to random chance, then $A_{obs} = A_{exp}$ and $\varkappa = 0$. Using Stata's kap command, I calculated $\varkappa = 0.57$, indicating fair to good interrater agreement.[74]

For the phase 1 examinations classified as "OK" by both assessors, I calculated the difference in best FEV_1 and FVC values from each assessment. Summarized results are shown in Table 6-9. While a few discrepant values were seen, overall there was excellent agreement between results from the two assessors.

Table 6-9: Difference in FEV_1 and FVC based on quality control by two different assessors

	Difference = MRHH - WAHH			
Parameter	Mean	Median [IQR]	Minimum	Maximum
FEV_1 (L)	0.002	0.000 [0.000; 0.000]	0.000	0.110
FVC (L)	0.005	0.000 [0.000; 0.000]	-0.110	0.180

MRHH = Martin Rune Hassan Hansen. WAHH = Wajd Abbas Hassan Hansen.

Based on these results, we deemed it justifiable to rely only on my quality assessment for all curves. The quality assessments for phase 1 examinations made by WAHH were not used further.

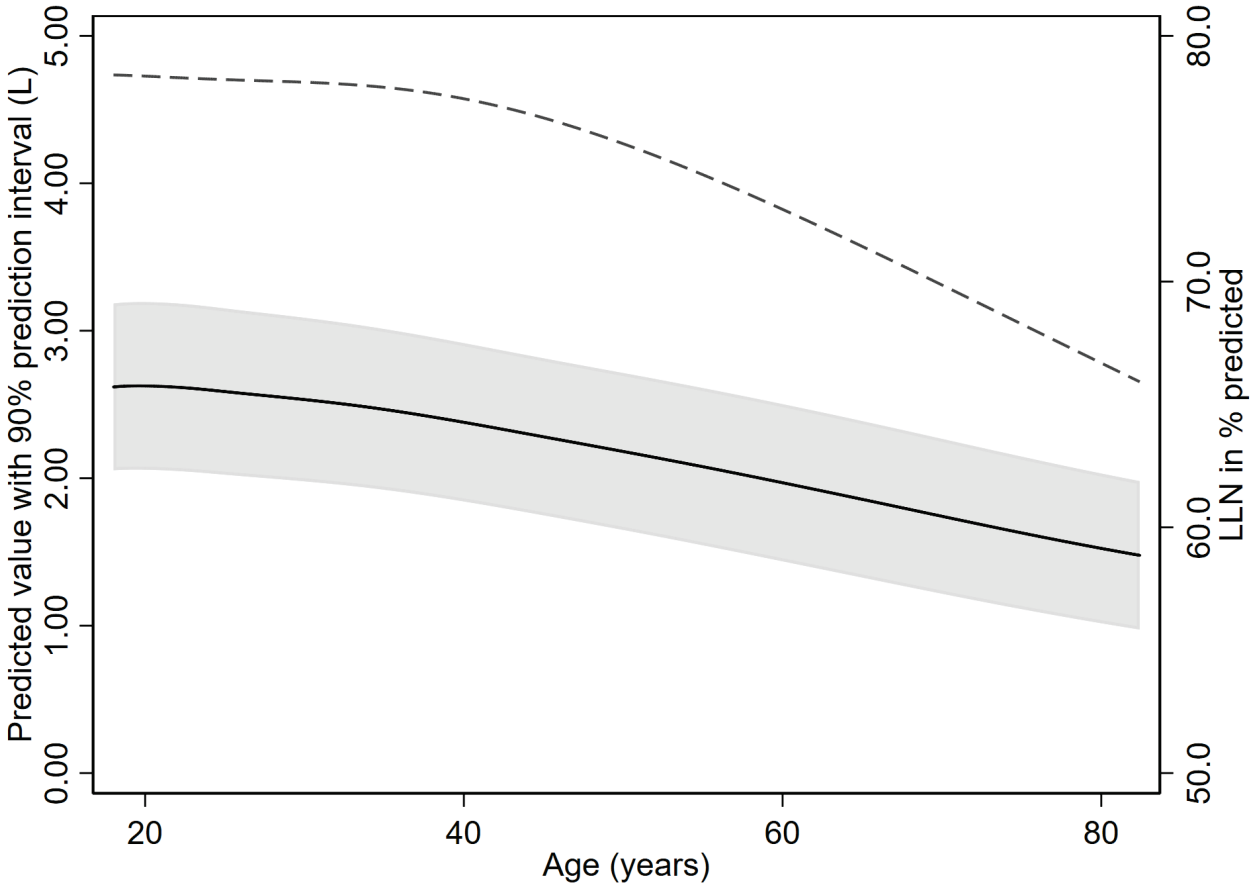
6.4.2.3.2 Choice of reference values

For each person who performed spirometry, we converted results to Z-scores using the Global Lung Function Initiative equations (GLI-2012), to account for the well-known correlation between lung function and ethnicity, age, sex, and height.[47 91] Each Z-score is equal to the difference between a person's measurement and predicted value, divided by the standard deviation of the predicted value.

Unfortunately, spirometry reference values for Ugandans are unavailable. While a suboptimal solution, we decided to compare participant's results to reference values for African-Americans.

The Global Lung Function Initiative taskforce recommends expressing spirometry results as Z-scores instead of percent predicted and state that Z-scores *"are free from bias due to age, height, sex and ethnic group"*.[47] This is illustrated in Figure 6-10 that shows GLI-2012 predicted values for FEV_1 as a function for age for African-American females with height 155.9 cm (the median height of females in the PEXADU project), as well as lower limit of normal (5th percentile of predicted) for FEV_1 in percent of predicted FEV_1. LLN in percent of predicted decreases from 78.4% for 20-year-olds to 66.7% for 80-year-olds. Hence, if we had expressed spirometry results as percent predicted instead of Z-scores, we would have failed to take into account that e.g. an FEV_1 of 70% predicted is abnormal for a 20-year-old, but within the normal range for an 80-year-old.

Figure 6-10: FEV_1 predicted value and LLN as a function of age



Solid line shows predicted value for FEV_1. Shaded gray area shows the corresponding 90% prediction interval. Lower bound of the gray area is the 5th percentile = lower limit of normal (LLN). Dashed line shows the LLN in percent of the predicted value. All calculations based on the GLI 2012 equations,[47 91] with ethnicity set to African-American, sex set to female, and height set to the median age for females in the PEXADU population (155.9 cm).

6.4.2.3.3 Reversibility of obstruction

When spirometry is used for the clinical diagnosis of airway obstruction in clinical practice, bronchodilator medicine is used to distinguish between asthma and COPD. Obstruction in asthma is reversible, while obstruction in COPD is not (or reversible to a much lesser degree).[41 42] We could not get ethical clearance to administer bronchodilator medication in the PEXADU study population. Therefore, we could not distinguish between reversible and non-reversible obstruction in the study population.

6.4.2.3.4 Calibration checks

On each day of testing, I checked the calibration of the MicroDL spirometers before use. A 3-liter calibration syringe (MIR 919000, Medical International Research Inc., Rome, Italy) was emptied three times under each of three different conditions: Slowly (as slowly as possible while keeping the movement smooth), medium, and fast (as fast as possible). Several different spirometers and turbines was used during the project. The device and/or turbine was switched if the calibration checks showed unacceptable imprecision or bias. Calibration check results are presented and discussed on page 72.

6.4.2.4 Glycemic regulation

In the PEXADU project, HbA_{1c} was used as the main metric of glycemic regulation, and FPG was a secondary outcome. The choice was made to simplify the logistics of data collections, as HbA_{1c} does not vary diurnally and its measurement does not require fasting.[64] We analyzed both HbA_{1c} and FPG as continuous measures, since hyperglycemia is a cause of excess morbidity and mortality, even below the cut-offs that define diabetes mellitus.[92] Furthermore, our study population was too small to use outright diabetes mellitus as the main outcome.

6.4.2.4.1 Validity of HbA_{1c} and FPG measurements

Quantification of glycemic status by Hba_{1c} is not without problems. The relationship between average plasma glucose levels and HbA_{1c} can be modified by a number of factors including age, ethnicity, sickle cell trait, sickle cell anemia, glucose-6-phosphate dehydrogenase (G6PD) deficiency, and some forms of HIV medication.[19]

Quality control data for glucose and HbA_{1c} were unfortunately not collected, making it difficult to assess the precision and accuracy of our measurements directly. However, indirect information can be gleaned by comparing FPG and HbA_{1c} from the same persons at the same time. If our devices are precise and accurate, we would expect results to be relatively concordant, though it is known that FPG and HbA_{1c} may not classify the exact same individuals as diabetic.[19] Compared to a combination of FPG, HbA_{1c} and OGTT, HbA_{1c} alone has a sensitivity of 30% for the diagnosis of diabetes mellitus.[19]

Across all phases of the PEXADU project, we performed 591 simultaneous tests of FPG and HbA_{1c} in 307 individual participants. The results can be categorized as normal, raised or diabetes according to standard ADA criteria[19] shown in Table 2-1 on page 7. In Table 6-10, I have cross-tabulated the classification of the 591 samples according to FPG and HbA_{1c}. 68% of tests have concordant FPG and HbA_{1c} classifications. 28% of tests are slightly discordant (normal/raised or raised/diabetes), while 4% of tests are highly discordant (normal/diabetes).

Table 6-10: Cross-tabulation of categorization of glycemic status assessed by HbA_{1c} and FPG

		Classification of HbA_{1c}		
		Normal	**Raised**	**Diabetes**
Classification of FPG	**Normal**	370 62.6%	90 15.2%	22 3.7%
	Raised	64 10.8%	25 4.2%	6 1.0%
	Diabetes	3 0.5%	4 0.7%	7 1.2%

Top numbers are counts. Percentages are relative to the total number of tests (591).

During data collection, I noticed some of these discrepancies in HbA_{1c} and FPG, and considered the following possible explanations:

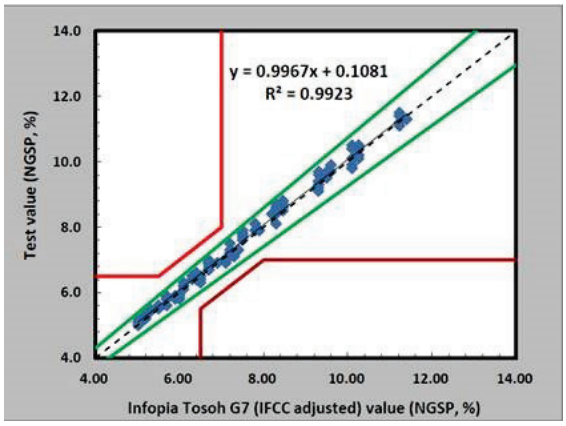
- Non-fasting participants: When FPG is elevated and HbA_{1c} is not, the participant might not truly be fasting. When I asked directly, some participants with normal HbA_{1c} and FPG apparently in the diabetic range confirmed that they had in fact eaten before the test. Such FPG results were deleted. Other participants might also have eaten without admitting it.
- Temperature damage to reagents: As described on page 41, temperatures in the examination center were generally within the range recommended by the manufacturer of the glucose and HbA_{1c} devices.
- Operator error: All tests were performed by either a nurse or a laboratory technician according to the manufacturers' instructions.[81 82] When I supervised the staff, I did not note any deviations from protocol.
- Device malfunction or bad reagents: I shipped the HemoCue HbA1c 501 device to the manufacturer for quality control when the project was over. There, it was tested with reagent packs from both of the lots that had been used in the PEXADU project. Results are shown in Figure 6-11; no clear problems were detected.

Sickle cell trait/anemia and G6PD deficiency are both relatively prevalent in Uganda.[93-96] Some participants may have one of these conditions. Sickle cell anemia is a hemolytic anemia seen in

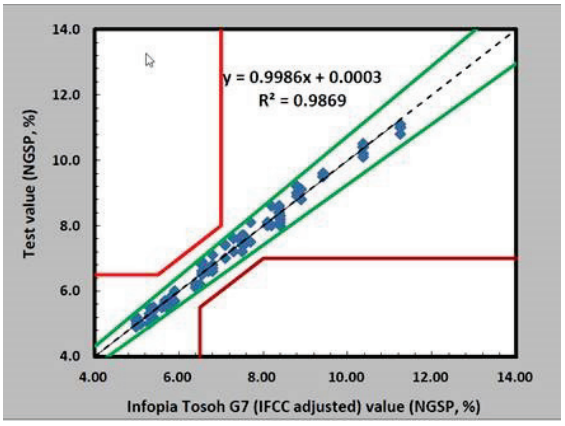
individuals homozygous for the variant hemoglobin HbS, while individuals with sickle cell trait are heterozygous carriers and rarely have any symptoms.[97] G6PD deficiency is an X-linked recessive disorder that can lead to hemolysis during episodes of oxidative stress.[98] Anemia can bias HbA_{1c} due to changed erythrocyte lifespan[64], and for the same reason it may also bias AChE/Hb, as old erythrocytes have lower AChE activity than young erythrocytes.[99] To rule out confounding from anemia, when I analyzed HbA_{1c} vs. AChE/Hb I included a sensitivity analysis with hemoglobin concentration as an independent variable; results were unchanged. Sickle cell trait could have biased our HbA_{1c} results, as HbS interferes with the assay used by the HemoCue HbA1c 501.[100] However, sickle cell trait does not influence AChE,[101] so it does not have the potential to confound relationships between HbA_{1c} and AChE/Hb.

Figure 6-11: Quality assurance results for HemoCue HbA1c 501 device

Reagent lot F17G26H17EL



Reagent lot F18F21F27EL



"Infopia Tosoh G7" is the name of another HbA_{1c} analyzer. Results from the HemoCue HbA1c 501 are plotted on the y-axis. Quality testing performed by Osang Healthcare Co., Ltd. (South Korea) with samples that were not from the PEXADU population. Figures reproduced with permission from HemoCue (personal communication, Maria Hagbjärn, HemoCue AB, November 14, 2019).

6.4.2.4.2 Handling of HbA1c < 4% NGSP

The HemoCue HbA1c 501 device has a lower limit of quantitation (LOQ) of 4% NGSP (= 20 mmol/L). Approximately 3% of our analyses were < LOQ and reported simply as "<4%" by the device. Before analyzing HbA_{1c} as a continuous metric, we had to decide how to handle observations < LOQ. We considered the following three strategies:[102]

1. Exclude the observations with HbA_{1c} < LOQ from all calculations
2. Assign a fixed value to all observations
3. Impute values using more advanced methods such as Maximum Likelihood Estimation (MLE)

While we decided to use strategy 1 in some sensitivity analyses, the loss of information and statistical power was deemed unacceptable for the main analyses. Strategy 2 is easy to implement, but has the disadvantage that it does not take the variance of values < LOQ into account.[102] Strategy 3 was theoretically the best, as it could take into account that values below the LOQ have a certain variability.[102] However, the Stata regression command implementing MLE (`truncreg`) could not account for the interdependence of the data (multiple rounds of examinations, family relationships). Hence, we settled for strategy 2, which was deemed acceptable in light of the relatively low number of measurements < LOQ.[102 103] As described in detail in the online appendix of Paper IV, we assumed that HbA_{1c} values < LOQ follow a triangular distribution between 3% NGSP and LOQ, resulting in an assigned value of 3.71% NGSP = 16.8 mmol/mol.

6.4.3 Selection problems

Study results can be affected by selection bias if the likelihood of participating depends on both the exposure and the outcome under investigation.[104] E.g., if farmers in ill health and with high exposure are more interested in the study findings and therefore have a higher likelihood of participating, this can introduce a spurious association between high exposure and ill health. As the PEXADU project was a follow-up study, selection problems could potentially arise both at the time of recruitment and during follow-up. It is unfortunately difficult to assess the potential for selection problems before the baseline examination, as I have little data available regarding non-participants. Loss to follow-up between phases is highly unlikely to influence our results, as we were able to follow up among 354 participants in phase 3, out of the 364 participants at baseline (< 3% lost).

Almost all participants gave blood samples for analysis of HbA_{1c} and AChE/Hb at each visit. Therefore, selection problems between project phases are unlikely to bias the results of HbA_{1c} vs. AChE/Hb. On

the other hand, FPG was measured in a convenience-based subsample of the study population, and this could be a potential source of bias. Persons who were tested for FPG had a mean AChE/Hb that was 0.68 [0.42; 0.94] U/g lower than participants who were not tested. While it is impossible to investigate directly if persons who were tested also had different FPG than those who were not tested, the issue can be examined indirectly, as we would expect similar patterns in FPG and HbA_{1c}. Among persons tested for FPG, mean HbA_{1c} was 0.57 [-0.34; 1.47] mmol/mol higher than in those who were not tested. While the difference in HbA_{1c} is not statistically significant, analyses of FPG vs. AChE/Hb might therefore be biased by selection problems, but such bias would introduce an association between high AChE/Hb and high FPG, which is the opposite of the observed association (see page 31). Hence, neither our results for HbA_{1c} nor FPG can be explained by selection bias after participant inclusion.

In each phase of the project, we excluded a number of persons from spirometry, some refused to undergo spirometry, and some performed spirometry that did not fulfill quality criteria (page 57). Hence, spirometry results are only available from 74-80% participants in each phase. Across all project phases, participants with spirometry available had mean AChE/Hb that was 0.31 [-0.02; 0.65] U/g higher than participants without. Furthermore, mean FEV_1 Z-score in phase 1 was 0.19 [-0.12; 0.50] higher among persons for whom data are also available in phase 2, compared to those without data in phase 2. When comparing phases 1/2 and 2/3 in the same manner, the mean difference was 0.30 [0.02; 0.59] and 0.26 [-0.02; 0.53], respectively. This indicates that participants with spirometry data available might both have had higher lung functions and slightly higher AChE/Hb compared to those participants who did not. This could cause a spurious association between low AChE/Hb and low pulmonary function, which is in fact the direction of the association we observed (see page 31). To estimate exactly how much of the observed association could be due to selection bias, a formal quantitative bias analysis would be needed, and we plan to conduct such an analysis in the future. Until then, the risk of selection bias must be kept in mind when interpreting results.

6.4.4 Confounding

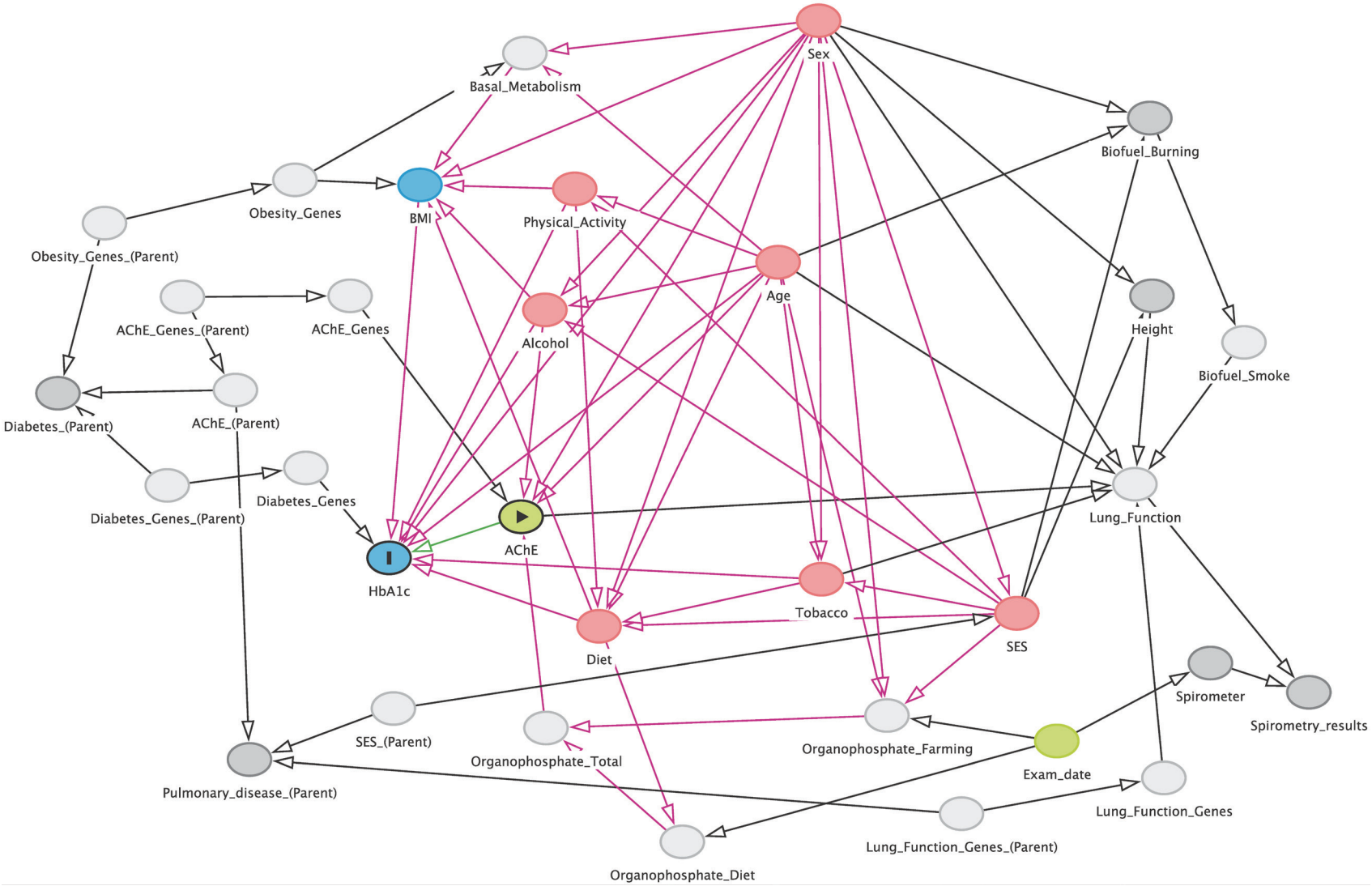
6.4.4.1 Confounder selection

Confounders for Paper IV and Paper V were selected a priori, based on analysis of Directed Acyclic Graphs (DAGs) containing information on assumed causal relationships between study variables. The DAGs for glycemic regulation and pulmonary function are shown in Figure 6-12 and Figure 6-13, respectively.

When drawing the DAGs to decide on which confounders to adjust for in my analyses of AChE vs. pulmonary function and glycemic regulation, I assumed that the only factors inheritable from parent to child were genes and socioeconomic status. In other words, I excluded any direct heritability of behavioral factors such as dietary pattern and attitude towards smoking, and I disregarded any causal link from parents' health status to their children's behavior. I believe that this is justified, as several studies in rural Uganda have shown a poor level of knowledge of the causes of diabetes mellitus,[105-107] asthma[108 109] and COPD.[110] A person who is unaware of the link between specific behaviors and disease (e.g., physical inactivity as a risk factor for diabetes mellitus) is unlikely to change behaviors in response to parents' illness. Even if the person has knowledge of the link between behavior and illness, this may not lead to a change in behavior. This was illustrated by a 2015 study among residents of the rural Kasese District in Western Uganda[111]; knowledge of diabetes mellitus was not associated with cardiometabolic risk factors (e.g., BMI, physical activity level, diet).[111]

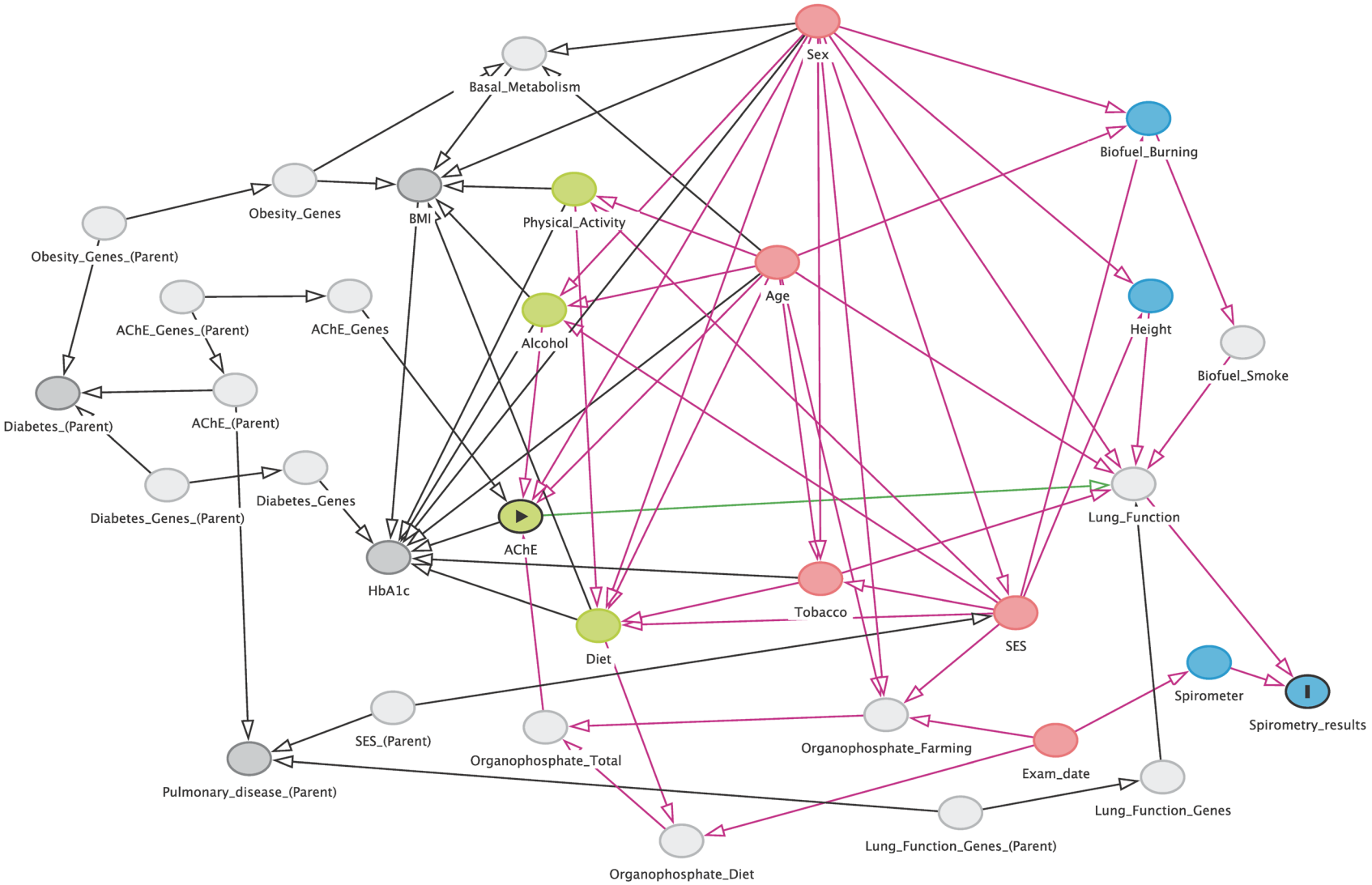
In our pre-published analysis protocol, we had stated that all analyses would be adjusted for project phase as an independent variable account for unknown time-variant confounders. However, in the final models we decided to leave out project phase. In the two following sections, I will explain why we did so. I will also explain why we left out the device ID of the spirometer used to test participants' pulmonary functions, even though Figure 6-13 indicates that the specific spirometer could be a confounder if spirometers are not calibrated to the same standards.

Figure 6-12: Directed Acyclic Graph for glycemic regulation in the PEXADU project



Green variable marked ▶ is the exposure variable. Remaining green variables are parents of the exposure. Blue variable marked "I" is the outcome. Remaining blue variables are parents of the outcome. Red variables are parents of both exposure and outcome. Light gray variables are unmeasured. Dark gray variables "other" variables. Green paths are causal. Red paths are biasing or "back-door" paths between exposure and outcome. Black paths are "other" paths.

Figure 6-13: Directed Acyclic Graph for spirometry in the PEXADU project



Green variable marked ▶ is the exposure variable. Remaining green variables are parents of the exposure. Blue variable marked "I" is the outcome. Remaining blue variables are parents of the outcome. Red variables are parents of both exposure and outcome. Light gray variables are unmeasured. Dark gray variables "other" variables. Green paths are causal. Red paths are biasing or "back-door" paths between exposure and outcome. Black paths are "other" paths.

6.4.4.2 Ambient air pollution as a possible confounder

$PM_{2.5}$ (airborne particulate matter with an aerodynamic diameter < 2.5 µm) and PM_{10} (airborne particulate matter with an aerodynamic diameter < 10 µm) are two important components of ambient air pollution. Exposure to such pollutants can impair lung function, also among otherwise healthy individuals. A recent systematic review and metaanalysis showed that each 10 µg/m^3 increase in acute exposure to $PM_{2.5}$ was associated with a -7.0 [-11.8; 2.3] ml change in FEV_1 among healthy adults, while each 10 µg/m^3 increase in long-term exposure to PM_{10} was associated with an annual -8.7 ml [-15.4; -2.1] change in FEV_1.[112] Epidemiological studies also show that ambient air pollution is associated with increased risk of diabetes mellitus.[113] Therefore, we considered whether concurrent temporal variation in both ambient air pollution and insecticide exposure had the potential to confound our results, and should be adjusted for in analyses, by using project phase as a proxy for exposure levels that were likely to change over time.

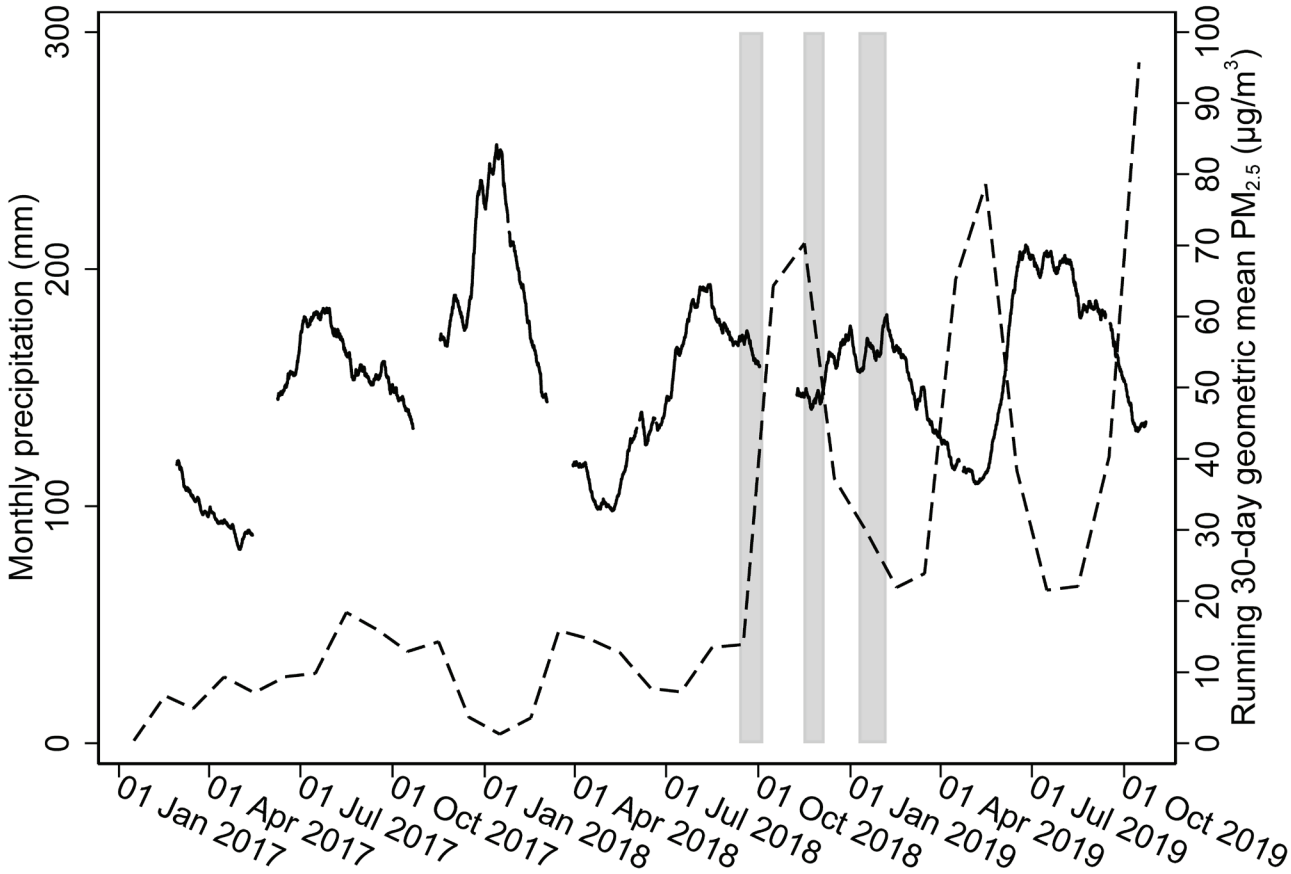
Publically available data on $PM_{2.5}$ from an air quality monitoring station located at the U.S. Embassy in Kampala were downloaded from an online repository (https://airnow.gov). Of course, care should be taken before extrapolating measurements from urban Kampala to semi-urban Wakiso approximately 17 kilometers away, but one would expect the overall temporal trends in air pollution across the year to be similar in the two locations. To emphasize overall trends in $PM_{2.5}$ over day-to-day fluctuations, I calculated a running 30-day geometric mean of $PM_{2.5}$. Historical data on monthly precipitation in Kampala was also obtained online (https://worldweatheronline.com).

Figure 6-14 shows plots of $PM_{2.5}$ and precipitation as a function of time. Large fluctuations in both precipitation and PM2.5 are seen in 2017-2019, and $PM_{2.5}$ is negatively correlated with precipitation. $PM_{2.5}$ ranges from 27 µg/m^3 in May 2017 to 84 µg/m^3 in January 2018. In the PEXADU project phases, highlighted in gray in the figure, differences in $PM_{2.5}$ are smaller but still noticeable. $PM_{2.5}$ seems higher in phase 1 and 3 compared with phase 2. As described further on page 31, there was a clear downwards trend in pulmonary function metrics across study phases, while we observed no temporal trends in HbA_{1c} and FPG. The lack of clear temporal covariance between ambient air pollution, glycemic regulation and pulmonary function makes it unlikely that ambient air pollution can considerably confound our results.

Due to the distance between the air monitoring station in Kampala and the homes of study participants in the Wakiso District, day-to-day fluctuations in ambient air pollution might differ between the two locations, even if the overall annual trends are the same. We therefore found that

if we wanted to adjust for ambient air pollution in our statistical models, the most reasonable way to do so would be to include project phase as an independent dummy variable, and our analysis protocol said that we would do exactly this.[114] However, adjustment for project phase in this way could pose a problem. Descriptive statistics showed that AChE/Hb decreased across study phases (see page 31), indicating increasing exposure to cholinesterase inhibiting pesticides. Hence, adjusting for project could lead to bias towards the null, potentially masking a real effect of AChE/Hb. On this background, we decided to remove project phase as an independent variable in our main models, and only re-included it in sensitivity analyses.

Figure 6-14: Temporal pattern of precipitation and ambient air pollution, Kampala 2017-2019



Thick black line = running 30-day geometric mean $PM_{2.5}$ concentration. Data is only shown for periods where data were available for at least 80% of the last 30 days, and 80% of the data available were valid. Dashed line = monthly precipitation in mm. Gray areas = phases of the PEXADU project.
$PM_{2.5}$ data provided by the U.S. embassy in Kampala (https://airnow.gov/index.cfm?action=airnow.global_summary#Uganda$Kampala, accessed 2019-11-27).
Precipitation data provided by WorldWeatherOnline.com(https://www.worldweatheronline.com/kampala-weather-history/kampala/ug.aspx, accessed 2019-11-17).

6.4.4.3 Spirometer ID as a possible confounder of pulmonary function

As described on page 61, several different spirometers were used during the project. Any systematic differences in the performance of the devices would have the potential to bias our analysis, with the direction of the bias depending on device calibration. To investigate whether spirometer ID should be included as a covariate in our analyses of pulmonary function, I analyzed the spirometer calibration check data (collection described on page 61).

Figure 6-15 (page 74) shows results from the calibration checks, stratified by the device and turbine used. The figure only includes data from calibration checks done with equipment that was also used to test at least one participant on the same date as the check. The gray lines are located at 3.00 L ±3% (the acceptable range of results, according to the ATS[46]). The black lines show trend in results, based on a mixed effect model of the following structure:

Equation 6-5

$$FVC = \beta_0 + \beta_{FEF} \times FEF_{25-75} + \alpha + \varepsilon$$

where FVC is the FVC reported by the device in the calibration check (should be 3.00 liters, if there was zero imprecision or bias), β_0 is the intercept, β_{FEF} is a regression coefficient, FEF_{25-75} is the FEF_{25-75} reported in the calibration check (a measure of how fast the piston was pushed), α is a random effect for date of testing, and ε is an error term. To take non-linearity in the trend into account, FEF_{25-75} was generally modeled using restricted cubic splines with four knots. One combination of spirometer and turbine was used only on a few participants on a single day, so these results were instead analyzed under the assumption of linearity in a simple linear regression model.

Table 6-11 provides summary statistics for the calibration data, in the form of the mean FVC reported during calibration for each device, weighted by the number of participants tested with that specific device on the same date.

Figure 6-15 and Table 6-11 show that the imprecision of the MicroDL spirometers was generally a little higher than the ± 3% recommended by the ATS.[46] Statistically significant biases are seen, e.g. weighted mean FVC reported by spirometer 1284 and turbine 1284 = 3.020 [3.015; 3.025] L. However, the biases are numerically small, and largely independent of the speed of pushing the piston during calibration (Figure 6-15). There is a clear link between the project phase and the spirometer used. E.g., all phase 3 examinations were done with equipment that had not been used on a single participant in phase 1. As described below, most spirometric indices for participants

decreased from phase 1 to 2, and from 2 to 3 (see page 31). This temporal pattern does not match the pattern of which spirometers were used in each phase, and the size and direction of their biases. Hence, I do not believe that differences in accuracy between spirometers were responsible for the observed trends in pulmonary function among participants. Imprecision leads to bias toward the null hypothesis and cannot explain any correlations demonstrated.

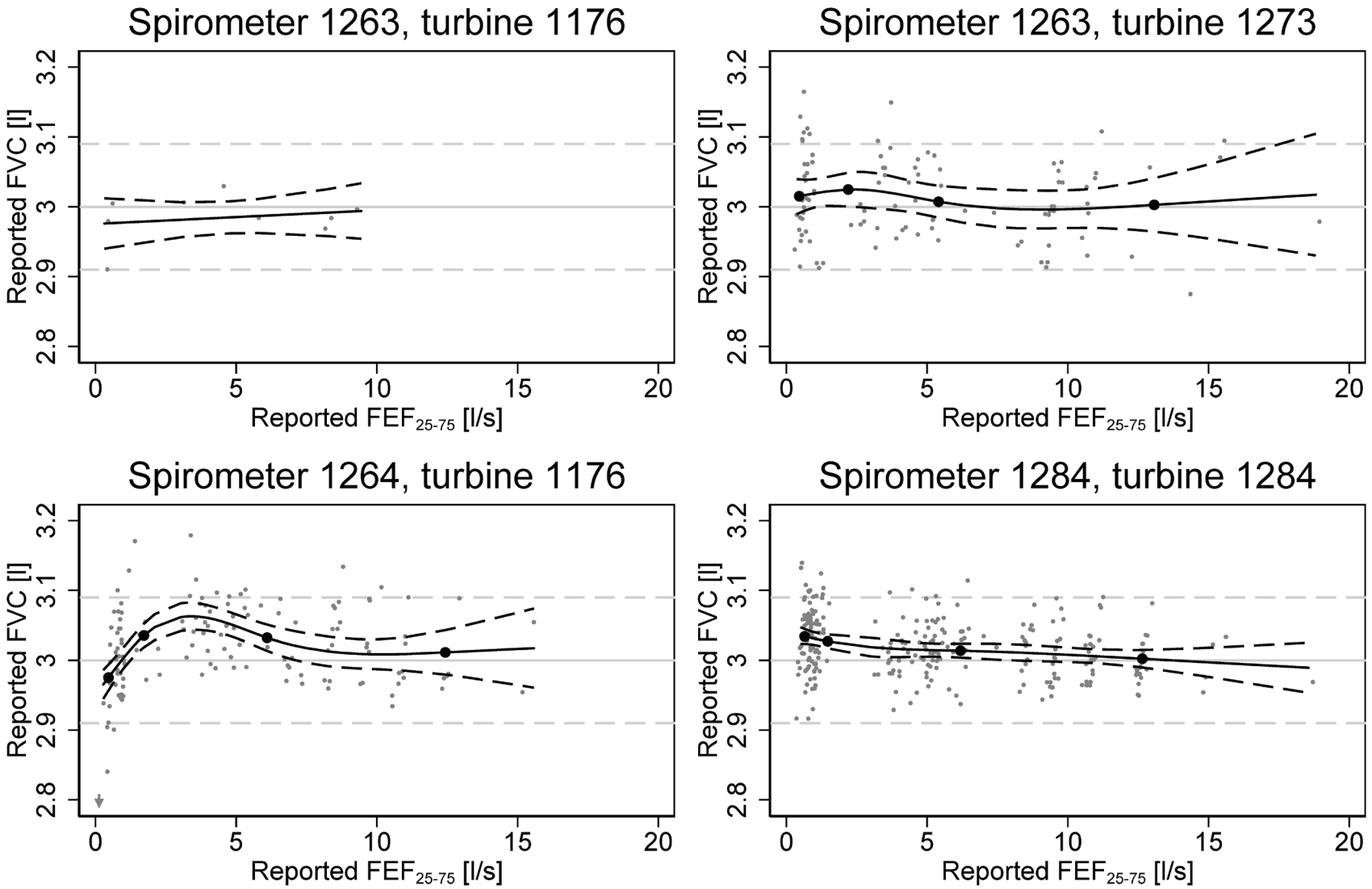
Table 6-11: Summary statistic for MicroDL calibration results

MicroDL		**Participant spirometries, n**				**Mean FVC**	**95% CI**	**95% PI**
Spirometer	**Turbine**	Ph. 1	Ph. 2	Ph. 3	Total			
1263	1176	14	0	0	14	2.984	[2.963; 3.006]	[2.919; 3.050]
1263	1273	114	18	0	132	3.013	[3.002; 3.025]	[2.901; 3.126]
1264	1176	176	39	0	215	3.029	[3.019; 3.038]	[2.922; 3.135]
1284	1284	0	238	271	509	3.020	[3.015; 3.025]	[2.934 ; 3.106]

The "FCV" is the volume of air reported by the spirometer during calibration, and should theoretically be 3.00 liters. When calculating the mean reported FVC, the calibration data were weighted by the number of persons tested with the device on the same date as the calibration check.

As different spirometers were used at different times in the project, adjusting the analyses for spirometer ID would be tantamount to adjusting for project phase. As described on page 70, adjusting for project phase might just as well introduce bias as remove bias. Because of this risk of over-adjustment, and because analysis of the calibration check data showed that differences between spirometers were unlikely to explain the trends in spirometry results between phases, we decided to leave out spirometer ID from our final regression models. In a sensitivity analysis, we included project phase as a covariate. Due to the strong correlation between project phase and spirometer ID (see Table 6-11), we did not include spirometer ID as a separate variable in the models.

Figure 6-15: Calibration data for spirometers, stratified by device ID and turbine ID



Gray dots represent individual plunges of the calibration syringe (plunges with FVC < 2.8 shown as arrows pointing downward). Gray lines located at 3.00 L ±3%. Black lines show trend from mixed effect model (see text for details); solid line shows estimate, dashed lines 95% CI. Black dots show the location of knots for restricted cubic splines. Data for spirometer 1263 + turbine 1176 were modelled under the assumption of linearity due to the low number of data points.

6.5 Evaluation of methods in Paper VI

Readers might find it unfair that I criticize Gouge *et al* for using a suboptimal analysis strategy in their original paper,[54] and then I use a mixed effect model for my analyses of the same data in Paper VI. Mixed effect models were likely not implemented in the statistical software available to Gouge *et al* in 1994. However, I also performed a sensitivity analysis (not described in Paper VI), where I compared each subject's FVC to his/her baseline FVC using a paired *t*-test, which *was* available in 1994. Results showed the same pattern as my main analysis, with FVC decreasing by 5.8 [1.9; 9.9] percentage-points of normal for asthmatics two hours after pyridostigmine administration, while no effect was seen for healthy subjects.

7 Discussion of results

7.1 Summary of findings and methodological considerations

Our study in the COBIN-D population (Paper II) showed lower prevalence of diabetes mellitus among persons who had ever used pesticides, compared to persons why had never done so, with adjusted OR 0.68 [0.52; 0.90]. However, large demographic differences between the exposed and unexposed groups mean that residual confounding is likely, even though we did adjust for a number of well-known determinants of diabetes risk, such as Body Mass Index and physical activity level. Paper II can be criticized for its cross-sectional study, and for investigating the effect of "pesticide" exposure in a broad sense, as different classes of pesticides have very different modes of action (see page 2). However, Paper II should be considered a pilot study, and we originally planned to conduct a follow-up study with better exposure characterization in the same population. We cancelled the follow-up study because we discovered that pesticide exposure levels in the population were lower than expected. Paper II fulfilled the aim of adjusting analyses of pesticide exposure vs. diabetes for known risk factors (see page 20), but because of the risk of residual confounding, I have low confidence in the interval validity of the results. That does not mean that the study is without merit, as it provides clues for how future studies on health effects of pesticides should be conducted. While a relatively large random sample of the general population might seem like an optimal study population, demographic differences between exposed and non-exposed persons could be too large to overcome by statistical adjustment. Therefore, health effects of pesticides might be better investigated in a study design with careful matching of exposed and non-exposed subjects, e.g. by comparing organic and conventional farmers. The studies described in Paper IV and Paper V were planned after we gained this important insight.

In Paper IV, we found an association between low AChE/Hb and decreased HbA_{1c} in the PEXADU population, while Paper V demonstrated an association between low AChE/Hb and decreased pulmonary function metrics in the same population. This does not support a causal link between exposure to cholinesterase inhibitor insecticides and diabetes, but it does support a link between exposure and impaired pulmonary function. Compared to Paper II and many previous studies on health effects of pesticides, the PEXADU project had a stronger study design using repeated measures of both exposure and outcome variables, which will theoretically increase both precision and accuracy of exposure-response relationships, though associations might still be due to reverse causality. Participants were recruited from two farmer's organizations, which minimized

demographic differences between high- and low-exposed subjects, thus decreasing the risk of residual confounding after we adjusted for well-known confounders selected *a priori*. We had objective measures for both exposure and outcome, and we performed extensive quality control on both. Repeated analyses of red blood cell acetylcholinesterase in blood samples from an unexposed control gave stable results across the entire project, giving me confidence that the observed temporal trends in AChE/Hb among participants is not due to degradation of reagents for the test or equipment failure. We did not see any correlation between levels of AChE/Hb and coarse subjective exposure information at baseline, which might indicate that other factors than insecticide exposure were causing the variance in AChE/Hb in the study population. On the other hand, across all project phases participants' mean AChE/Hb was 25.8 U/g, while the mean AChE/Hb of the unexposed control was 30.4 U/g. This is what we would expect to see if AChE/Hb was being inhibited by widespread exposure to insecticides. Spirometry quality was assessed using standardized criteria; phase 1 spirometries were assessed in duplicate, and inter-rater agreement was fair to good, indicating that the assessments from the main assessor are reliable. As discussed on page 70 and page 72, findings in Paper IV and Paper V are unlikely to be explained by temporal trends in air pollution in the study area, or by which spirometers were used to test participants in each project phase. Results from Paper VI provides strong evidence than acute cholinesterase inhibition can lead to pulmonary function impairment in humans, which supports the hypothesis that the statistical associations demonstrated in Paper V represent causal effects.

The main common limitation of Paper IV and Paper V is the convenience-based recruitment strategy, leading to a risk of selection bias. I have very little data available on individuals who were invited for the PEXADU study, but who were not among the 380 individuals who showed up at the examination center. This makes it difficult to assess the potential for selection bias before the baseline examination. Selection processes from baseline onwards cannot explain our findings on glycemic regulation, but they may explain an unknown proportion of the findings regarding lung function. Future work on the PEXADU data will include quantitative bias analysis to account for selection problems. For Paper IV, another important limitation is the possibility of reverse causality; the demonstrated association between low AChE/Hb and decreased HbA_{1c} may be due to blood glucose levels affecting AChE/Hb,[115] rather than the other way around.

While confidence in the internal validity of the PEXADU study findings must be somewhat tempered by the risk of reverse causality in Paper IV, and the possibility of selection bias in Paper V, I still find

that the PEXADU study is methodologically stronger and has higher internal validity than most of the previous studies on the relationship between cholinesterase inhibitor insecticides, diabetes mellitus and pulmonary function. Regarding the external validity of the findings, the results are probably generalizable to other smallholder farmers in Eastern Africa and similar settings, but caution is warranted before extrapolating results to populations with presumed lower exposure levels, such as consumers eating fruits and vegetables with pesticide residues.

7.2 Comparison with previous literature

A scoping review on exposure to cholinesterase inhibiting insecticides and perturbed glycemic regulation showed that there was only suggestive evidence for a causal link between the two. E.g., Ranjbar *et al* found no association between urinary levels of organophosphate insecticide metabolites and FPG, HbA_{1c} or HOMA-IR (a measure of insulin resistance) in a large cross-sectional sample of the general US population,[25] while Nascimento *et al* examined 54 children from an agricultural area of Brazil who were environmentally exposed to pesticides, and found that BChE was negatively correlated with FPG (indicating that exposure to cholinesterase inhibitor insecticides leads to increased FPG).[28] On the other hand, Shapiro *et al* examined pregnant women from Canada and found a negative correlation between urinary levels of organophosphate insecticide metabolites early in pregnancy, and the risk of being diagnosed with gestational diabetes mellitus or gestational glucose intolerance later in pregnancy.[26] However, there was a high risk of bias due to confounding from diet, as the major route for the included women was likely through diet, and estimates were not adjusted for the intake of fruit and vegetables.[26] A number of other studies have found increased blood glucose levels or increased risk of diabetes among exposed persons, but I deemed that all of these studies had "probably high" risk of bias. There was no clear pattern in the exposure metric used (organophosphate metabolites, cholinesterase enzyme activity) and the findings of the study (no association, positive/negative association) when comparing the previous studies between themselves, or when comparing Paper V with the previous studies (Table 2-2 and Table 2-3). Overall, I have low confidence in the evidence for an association between cholinesterase inhibitor insecticides and diabetes mellitus.

Previous studies on pulmonary function among subject exposed to cholinesterase inhibitor insecticides are more consistent. As described on page 17, a recent systematic review by Ratanachina *et al* found "tentative evidence" for a negative effect of exposure on FEV_1/FVC, but most studies were cross-sectional and had limited confounder control.[5] Ratanachina *et al* recommended "further

studies with better and more comprehensive adjustments for potential confounders and co-exposures, particularly the effects of other occupational factors in each working environment".[5] While Paper V has some weaknesses, it fits the evidence gap identified by Ratanachina *et al*: We adjusted for well-known confounders and co-exposure, and we used a stronger study design. Because of the agreement between Paper V and previous studies on the same subject, I am moderately confident that a causal link exists between exposure to cholinesterase inhibitor insecticides and lung function impairment.

7.3 Implications for policy, practice and future research

Paper IV and Paper V focus specifically on cholinesterase inhibitor insecticides, and we have not yet investigated effects of other classes of pesticides on pulmonary health and glycemic regulation. In the PEXADU project, we collected urine samples for the analysis of pesticide metabolites, and participants wore passive pesticide samplers for the analysis of pesticide residues. Due to difficulties in obtaining permission to export the samples from Uganda, they have not yet been analyzed at the time of writing (January 2020). Once we get permission for exportation, we should be able to analyze these samples and investigate effects of other classes of pesticides that were commonly used in the PEXADU study population (pyrethroid insecticides and dithiocarbamate fungicides).

Because of the burden of morbidity and mortality caused by diabetes mellitus[6] and obstructive airway disease,[6] and because of the increasing use of pesticides[3] in the intensifying agricultural production system, more knowledge is needed on which specific compounds are related to which specific health outcomes. Without this knowledge, rational cost-benefit analyses on the use of pesticides are impossible. We do not need more studies investigating health effects of exposure to "pesticides" as a broad class; such studies are unlikely to produce actionable evidence. If possible, studies should include objective measures of exposure to specific compounds or classes of compounds, though this may not be financially feasible for studies conducted in low-income settings. While collection of detailed subjective information on exposure could be an alternative to objective measures, self-reported exposure may not correlate well with actual exposure, as indicated in Paper IV and Paper V. Studies primarily relying on self-reported exposure metrics should therefore validate these metrics against objective measures in a subset of the study population. Farmers are often exposed to a multitude of different agrochemicals, and the effects of the chemicals may interact. While studies on mixtures of chemicals might better represent the real-life exposure and effects of pesticides compared to single-exposure studies, it is important that future studies still collect

information on individual compounds contained in the mixtures. Furthermore, future studies should collect information on and account for important confounders of the relationships pesticide exposure and health outcomes. The relevance of specific factors as confounders will depend on the health effect under investigation. For example, there is no need for further studies on pulmonary effects of pesticides that do not account for sex, age, height, ethnicity and tobacco smoking.

While it is true than an observational study cannot prove a causal link between two factors, I think that it would be a mistake to describe the observed correlation between low acetylcholinesterase and decreased pulmonary function in the PEXADU population as merely a "statistical association". Mechanistically, one would expect exposure to cholinesterase inhibitor insecticides to lead to bronchoconstriction, and human experimental studies with the less toxic cholinesterase inhibitor pyridostigmine shows that it can impair lung function in the short term. It is therefore not unreasonable to think that the demonstrated correlations between acetylcholinesterase inhibition and decreased pulmonary function may represent a causal effect. Exposure to cholinesterase inhibitor insecticides should therefore be minimized. This does not mean that the compounds should never be used, as they are e.g. important for the control of insect-borne diseases,[10] but it does mean that farmers should take basic precautions such as not spraying against the wind, and should wear personal protective equipment during spraying.

8 Conclusions

The studies included in this PhD dissertation have investigated possible health effects of pesticides in present-day use by a combination of literature review and epidemiological studies. The main findings in relation to the PhD project aims are listed below.

- A scoping review of previous studies on diabetes in relation to exposure to cholinesterase inhibitor insecticides showed that the evidence for a causal link was only suggestive. Most previous studies were cross-sectional, had inadequate confounder control, and gave somewhat conflicting results. A more comprehensive systematic review is underway and will include evidence on all neuroactive non-organochlorine insecticides.
- In a cross-sectional study of a general-population sample in Nepal, we found no indication of a link between self-reported pesticide use and diabetes. Subjects who had ever used pesticides had lower risk of diabetes, but this was probably due to residual confounding.
- A follow-up study among smallholder farmers in Uganda showed that low acetylcholinesterase activity was associated with decreased blood sugar levels. While we cannot rule out that the association was due to reverse causality, the result does not support a causal link between exposure to cholinesterase inhibitor insecticides and diabetes.
- In the same follow-up study in Uganda, low acetylcholinesterase activity was associated with decreased lung function, also after confounder adjustment. Mechanistic evidence from previous human exposure studies with the cholinesterase inhibitor drug pyridostigmine indicates that the association may due to a causal link between exposure to cholinesterase inhibitor insecticides and lung function impairment.

To support data management in the epidemiological study in Uganda, I developed a piece of software that allows data to be exported from the HemoCue HbA1c 501 device in digital format. The software will be published as open source to simplify data collection in future epidemiological studies on diabetes.

Further studies on exposure to cholinesterase inhibitor insecticides are needed, and should include lower-exposed populations, and measurements of cholinesterase enzyme activity should be supplemented with other objective measures of exposure. In addition, we need studies on other currently used classes of pesticides, also with objective measures of exposure. Efforts to protect farmers and pesticide applicators from harmful effects of pesticides should be strengthened.

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Appendices

Previous studies on cholinesterase inhibitor pesticides and diabetes

Table A-1: Study characteristics (occupational exposure)

First author	Year	Country	Study design	Study population	Exposure biomarker(s)	Outcome	n	% female	% exposed	Ethnicity
Bhatnagar[116]	1980	India	Cross-sectional	Pesticide factory workers and "healthy subjects"	BChE	FPG	57	NR	74	NR
Bhatnagar[117]	1982	India	Cross-sectional	Pesticide factory workers and unexposed controls	BChE	FPG	90	NR	83	NR
Owczarzy[118]	1982	Poland	Cross-sectional	Pesticide factory workers and unexposed controls	AChE (not Hb-adjusted) and BChE	FPG and OGTT	66	0	48	NR
Berberian[119]	1987	Egypt	Cross-sectional	Pesticide applicators and unexposed controls	BChE	FPG	NR	NR	NR	NR
Parron[120]	1996	Spain	Cross-sectional	Greenhouse workers	AChE (not Hb-adjusted) and BChE	FPG	105	0	24% high-exposed	NR
Rojas[27]	1996	Venezuela	Combination of cross-sectional and follow-up	Pesticide mixers, pesticide applicator pilots, unexposed pilots	AChE (not Hb-adjusted)	FPG	73	NR	45	NR
Jintana[121]	2009	Thailand	Cross-sectional	Organophosphate applicators and healthy controls	AChE/Hb and BChE	FPG	120	45	75	Thai
Patil[29]	2009	India	Cross-sectional	Healthy pesticide applicators and healthy unexposed controls	BChE	FPG	90	0	67	NR
Elhalwagy[122]	2010	Egypt	Cross-sectional	Pesticide applicators (cotton fields)	AChE (not Hb-adjusted)	FPG	210	0	71	NR
Tsatsakis[32]	2011	Greece	Cross-sectional	Farmers, farmworkers and rural residents	Hair OP metabolites: DEDTP, DEP, DETP, DMP	DM	220	28	77	NR
Bayrami[33]	2012	Iran	Cross-sectional	Farmers and unexposed workers from same village	BChE	FPG	80	0	50	NR
Raafat[123]	2012	Egypt	Cross-sectional	Farmers and unexposed controls (university employees)	Blood OP: Malathion	HOMA-IR	188	0	52	NR

Abbassy[30]	2014	Egypt	Cross-sectional	Farmers	BChE	FPG	NR	0	NR	NR
Sudjaroen[124]	2015	Thailand	Cross-sectional	Conventional farmers and organic farmers	BChE	FPG	71	NR	42	NR
Garcia-Garcia[39]	2016	Spain	Combination of cross-sectional and follow-up	Greenhouse workers and healthy unexposed controls	AChE/Hb and BChE	FPG	280	53	68	NR
Marrero[31]	2017	Venezuela	Cross-sectional	Organophosphate applicators and unexposed controls (lab technicians)	BChE	FPG	30	43	57	NR
Sudjaroen[125]	2017	Thailand	Cross-sectional	Conventional farmers and organic farmers	BChE	FPG	80	NR	56	NR
Velmurugan[24]	2017	India	Cross-sectional	Farmers and non-farming villagers	BChE Plasma OPs: Chlorpyrifos, malathion, methyl-parathion, monocrotophos	DM	802	NR	N/A	NR
Ahmadi[126]	2018	Iran	Cross-sectional	Pesticide applicators and unexposed controls from same area	BChE	FPG	204	0	49	NR
Cotton[127]	2018	Australia	AChE/Hb monitored in follow-up design (4 measurements in 12 weeks). FPG analyzed in cross-sectional design.	Farmers and unexposed controls (non-farmers)	AChE/Hb	FPG	55	15	75	NR
Arevalo-Jaramillo[34]	2019	Ecuador	Cross-sectional	Exposed rural women (from high- and low-exposure areas) and unexposed controls (women from city)	BChE	FPG	115	100	54	NR
Rathish[128]	2019	Sri Lanka	Cross-sectional	Farmers	AChE/Hb	FPG and OGTT	46	0	50	Sinhalese

See legend on page 91.

Table A-2: Study characteristics (environmental or unknown mode of exposure)

First author	Year	Country	Study design	Type of exposure	Study population	Exposure biomarker(s)	Outcome	n	% female	% exposed (CC: % cases)	Ethnicity
Cecchi[35]	2012	Argentina	Cross-sectional	Unclear	Pregnant women from area with intensive agriculture	AChE/Hb BChE	FPG	97	100	40	NR
El-Morsi[36]	2012	Egypt	Case-control	Environmental	Children with DM type 1 and healthy controls (= siblings of other pediatric patients)	Serum OPs: Malathion, profenofos, chlorpyrifos-methyl	DM type 1	110	62	68	NR
Ranjbar[25]	2015	USA	Cross-sectional	Environmental	General non-institutionalized US population	Urine OP metabolites: DEDTP, DEP, DETP, DMP, DMDTP, DMTP	FPG HbA_{1c} HOMA-IR	2227	48	N/A	NR
Shapiro[26]	2016	Canada	Follow-up	Environmental	Pregnant women	Urine OP metabolites: DEDTP, DEP, DETP, DMDTP, DMP, DMTP	GDM or G-IGT	1195	100	N/A	~ 5/6 white, 1/6 non-white
Nascimento[28]	2018	Brazil	Combination of cross-sectional and follow-up	Environmental	Children from agricultural area	AChE (not Hb-adjusted)	FPG	54	52	N/A	NR

Legend for Table A-1 and Table A-2:

- N/A = not applicable
- NR = not reported
- CC = case-control

Table A-3: Confounder adjustment (occupational exposure)

First author	Sex	Age	Physical activity	Tobacco	Alcohol	Family history of DM	HLA genotype	Weight status	Other factors	Risk of confounding
Bhatnagar[116]	0	0	0	0	0	0	0	0		High
Bhatnagar[117]	0	0	0	0	0	0	0	0		High
Owczarzy[118]	+	0	0	0	0	+	0	0		High
Berberian[119]	0	0	0	0	0	0	0	0		High
Parron[120]	+	0	0	0	0	0	0	0		High
Rojas[27]	+	+	0	+	+	0	0	0		Probably high
Jintana[121]	(+)	0	0	0	0	0	0	0		High
Patil[29]	+	+	0	+	+	0	0	0		Probably high
Elhalwagy[122]	+	0	0	0	0	0	0	0		High
Tsatsakis[32]	+	+	0	0	0	0	0	0	Occupation	Probably high
Bayrami[33]	+	+	0	0	0	0	0	0	Education	Probably high
Raafat[123]	+	0	0	0	0	+	0	+		High
Abbassy[30]	+	+	0	+	0	0	0	0		Probably high
Sudjaroen[124]	0	0	0	0	0	0	0	(+)		High
Garcia-Garcia[39]	+	+	0	+	+	0	0	+		Probably high
Marrero[31]	(+)	(+)	0	0	0	0	0	0		Probably high
Sudjaroen[125]	0	(+)	0	0	0	0	0	0		High
Velmurugan[24]	+	+	0	0	0	+	0	+		Probably high
Ahmadi[126]	+	0	0	0	0	0	0	0		High
Cotton[127]	0	(+)	0	0	0	0	0	0		High
Arevalo-Jaramillo[34]	+	+	0	0	0	0	0	0	"Habits" and previous jobs	Probably high
Rathish[128]	+	0	0	+	0	0	0	0	Ethnicity	High

For legend, see page 93.

Table A-4: Confounder adjustment (environmental or unclear mode of exposure)

First author	Sex	Age	Physical activity	Tobacco	Alcohol	Family history of DM	HLA genotype	Weight status	Other factors	Risk of confounding
Cecchi[35]	+	(+)	0	+	+	0	0	(+)		Probably high
El-Morsi[36]	(+)	(+)	0	0	0	+	0	0	Previous diagnosis of atopic or autoimmune disease	Low
Ranjbar[25]	+	+	0	+	0	0	0	+	Ethnicity, socio-economic status, diet, fasting duration, urinary creatinine	Probably low
Shapiro[26]	+	+	0	0	0	0	0	+	Ethnicity, education, urine-specific gravity	Probably high
Nascimento[28]	+	+	0	0	0	0	0	+	Pubertal stage	Probably low

Legend for Table A-3 and Table A-4:

- + = accounted for by matching, adjustment in multivariate model or other appropriate method
- (+) = not formally accounted for. However, distributions are similar in the exposure groups, so bias is unlikely.
- 0 = not accounted for

Table A-5: Study results (occupational exposure)

First author	Exposure metric	Effect of exposure on glucose		Effect of exposure on biomarker	
		Overall	Effect estimates	Overall	Effect estimates
Bhatnagar[116]	Group-based	-	FPG (mmol/L): Workers 4.40 [4.17; 4.63], controls 5.61 [5.29; 5.93], diff. -1.21 [-1.60; -0.81]	-	BChE (IU/ml): Workers 1.98 [1.69; 2.27], controls 3.70 [3.23; 4.17], diff. -1.72 [-2.27; -1.17]
Bhatnagar[117]	Group-based	-	FPG (mmol/L): Workers 4.61 [4.44; 4.77], controls 5.61 [5.47; 5.75], diff. -1.00 [-1.22; -0.79]	-	BChE (IU/ml): Workers 1.96 [1.81; 2.11], controls 3.70 [3.23; 4.17], diff. -1.74 [-2.24; -1.25]
Owczarzy[118]	AChE modeled as continuous variable AChE dichotomized into < or ≥ 2 µmol/L	+	Negative correlation between FPG, AChE ($r = -0.49$, $p < 0.01$) and BChE ($r = -0.51$, $p < 0.01$) Persons with AChE < 2 µmol/ml had sign. higher FPG and post-load OGTT results than persons with AChE ≥ 2 µmol/ml.	N/A	N/A
Berberian[119]	Group-based	+	FPG±SD (mmol/L): Exposed 7.05±0.64, controls 4.27±0.27, diff. 2.78, $p < 0.05$	-	BChE 64.7% in exposed group compared to controls
Parron[120]	Group-based	(+)	FPG (mmol(L): High-exposed 5.10 [4.64; 5.56], low-exposed 4.98 [4.67; 5.30], diff. 0.12 [-0.44; 0.68]	(-)	AChE (U/ml): High-exposed 14.23 [13.59; 14.87], low-exposed 14.42 [13.94; 14.89], diff. -0.19 [-0.99; 0.61] Mean BChE (U/ml): High-exposed 3.96 [3.60; 4.32], low-exposed 4.03 [3.86; 4.21], diff. -0.07 [-0.48; 0.33]
Rojas[27]	Group-based	+	FPG (mmol/L): Exposed group before spraying season 4.92 [4.27; 5.56], exposed group in spraying season 5.91 [4.88; 6.94], controls 4.65 [4.21; 5.08]. Diff. 0.27 [-1.02; 1.56] before spraying season, 1.26 [0.14; 2.37] in spraying season.	-	AChE (unit NR): Exposed group before spraying season 2.90 [2.80; 3.00], exposed group in spraying season 2.35 [2.17; 2.53], controls 2.61 [2.51; 2.71]. Diff. 0.29 [0.06; 0.52] before spraying season, -0.26 [-0.47; -0.05] in spraying season.
Jintana[121]	Group-based	(-)	FPG (mmol/L): Exposed group 5.07 [4.95; 5.19], controls 5.21 [4.99; 5.43], diff. -0.14 [-0.39; 0.11]	-	AChE (U/g): Exposed group in low-exposure season 29.8, exposed group in high-exposure season 20.73, controls 38.98, $p < 0.01$ for diff. between groups. BChE (U/mL): Exposed group in low-exposure season 4.91, exposed group in high-exposure season 3.73, controls 5.96, $p < 0.05$ for diff. between groups.

Patil[29]	Group-based	+	FPG (mmol/L): Exposed group 5.77 [5.75; 5.80], controls 5.22 [5.17; 5.27], diff. 0.56 [0.50; 0.61]	-	BChE (U/mL): Exposed group 4.83 [4.78; 4.87], controls 6.99 [6.92; 7.06], diff. -2.16 [-2.25; -2.08]
Elhalwagy[122]	Group-based	(-)	FPG±SD (mmol/L): Conventional motor sprayers 5.11±0.44, knapsack motor sprayers 4.36±0.34, controls 5.91±0.52, p = 0.187	-	AChE±SD (mol/mL/min): Conventional motor sprayers 1.68±0.06, knapsack motor sprayers 1.55±0.07, controls 2.07±0.12, p = 0.002
Tsatsakis[32]	Metabolites modelled as continuous variables	(+)	Non-significant positive relationship between DMP, DEP and odds of DM.	N/A	N/A
Bayrami[33]	Group-based	(-)	FPG (mmol/L): Exposed 4.33 [3.73; 3.93], controls 4.86 [4.52; 5.19], diff. -0.52 [-1.21; 0.17]	-	BChE (U/mL): Exposed 31.24 [29.04; 33.44], controls 35.73 [34.21; 37.25], diff. -4.49 [-7.16; -1.86]
Raafat[123]	Malathion modeled as continuous variable	+	Positive correlation between HOMA-IR and malathion level. R^2 = 0.316, p = 0.0097	N/A	N/A
Abbassy[30]	Group-based	+	FPG (mmol/L), non-smokers: Pesticide applicators 5.48, farmworkers 4.68, controls 4.83. p < 0.05 for applicators vs. controls, p > 0.05 for farmworkers vs. controls. FPG (mmol/L), smokers: Pesticide applicators 6.11, farmworkers 5.77, controls 4.51. p < 0.05 for pesticide applicators and farmworkers vs. controls.	-	BChE (U/mL), non-smokers: Pesticide applicators 2.00 [1.92; 2.07], farmworkers 2.21 [2.14; 2.29], controls 2.28 [2.20; 2.35], diff. For PA -0.28 [-0.39; -0.17], diff. for FW -0.06 [-0.17; 0.04] BChE (U/ml), smokers: PA 1.84 [1.78; 1.89], FW 1.99 [1.95; 2.04], controls 2.19 [2.14; 2.23], diff. for PA -0.35 [-0.42; -0.28], diff for FW -0.19 [-0.26; -0.13]
Sudjaroen[124]	Group-based	(-)	FPG (mmol/L): Conventional farmers 5.24 [5.01; 5.47], organic farmers 5.63 [5.25; 6.02], diff. -0.39 [-0.84; 0.05]	(+)	BChE (U/mL): Conventional formers 8.09 [7.64; 8.54], organic farmers 7.96 [7.56; 8.36], diff. 0.13 [-0.47; 0.73]
Garcia-Garcia[39]	Group-based	+/-	FPG coefficients from mixed effect model (mmol/L): High-exposure vs. low-exposure period 0.16 [0.01; 0.31], exposed persons vs. controls -0.91 [-1.21; -0.62]	+/-	AChE/Hb (µmol/min/g) coefficients from mixed effect model: High-exposure vs. low-exposure period -1.13 [-2.66; 0.39], exposed vs. controls -10.39 [-11.69; -9.09] BChE (unit unknown) coefficients: High-exposure vs. low-exposure period -143.3 [-186.7; -99.9], exposed vs. controls 148.5 [89.2; 207.8]
Marrero[31]	Group-based	+	FPG (mmol/L): Exposed persons 5.39 [5.02; 5.76], controls 4.72 [4.45; 5.00], diff. 0.67 [0.21; 1.12]	-	BChE (U/L): Exposed persons 6.75 [6.26; 7.24], controls 8.65 [7.78; 9.53], diff. -1.91 [-2.91, -0.91]
Sudjaroen[125]	Group-based	+	FPG (mmol/L): Conventional farmers 6.02 [5.77; 6.28], organic farmers 5.18 [4.95; 5.41], diff. 0.84 [0.50; 1.18]	-	BChE (U/mL): Conventional farmers 8.41 [7.96; 8.86], organic farmers 9.17 [8.80; 9.54], diff. -0.76 [-1.35; -0.17]

Velmurugan[24]	BChE summarized by diabetes status OP metabolites modeled as continuous variables	+	No significant difference in BChE between diabetics and non-diabetics, $p < 0.40$ OR for DM (based on combination of self-report and HbA_{1c}): MCP: Q1 [ref.], Q4 1.70 [0.86; 1.37], p_{trend} 0.032 CHL: Q1 [ref.], Q4 1.82 [0.31; 1.25], p_{trend} 0.044 MAL: Q1 [ref.], Q4 1.08 [0.54; 2.16], p_{trend} 0.654 MPAR: Q1 [ref.], Q4 2.67 [1.23; 2.80], p_{trend} 0.048	N/A	N/A
Ahmadi[126]	Group-based	+	FPG mean±SD (mmol/L): Exposed group 5.38±0.89, controls 5.21±1.62, p = 0.356 OR for FPG > 109 mmol/L, exposed compared to controls: 4.08 [1.57; 10.65]	-	BChE mean±SD (U/mL): Exposed group 0.23±0.10, controls 0.30±0.07, p < 0.001
Cotton[127]	Group-based	(-)	OR for IFG among farmers: 0.44 [0.13; 1.55]	-	Point estimates of AChE/Hb (U/g) lower for farmers than controls. 27.5 vs. 31.6 at baseline.
Arevalo-Jaramillo[34]	Group-based	(-)	FPG mean±SD (mmol/L): High-exposed 4.77±2.22 (p = 0.10), low-exposed 4.78±2.20 (p = 0.10), controls 4.85±1.39	(+)/-	BChE mean±SD (U/mL): High-exposed 4.96±1.03 (p = 0.16), low-exposed 3.30±1.73 (p < 0.01), controls 4.57±1.28
Rathish[128]	AChE dichotomized by the median	(+)	FPG mean (IQR): High-exposed farmers 5.05 (4.61; 5.38), low-exposed farmers 4.94 (4.55; 5.55), p = 0.8948 OGTT $AUC_{0\text{-}120}$ mean (IQR) (mmol×min/L): High-exposed 809 (654; 898), low-exposed 744 (659; 894), p = 0.55	N/A	N/A

For legend, see page 97.

Table A-6: Study results (environmental or unclear mode of exposure)

First author	Exposure metric	Effect of exposure on glucose		Effect of exposure on biomarker	
		Overall	Effect estimates	Overall	Effect estimates
Cecchi[35]	Group-based	0	No difference in FPG between different women examined in spraying and non-spraying seasons	-	AChE and BChE significantly lower for women examined in spraying season ($p < 0.01$)
El-Morsi[36]	Group-based	+/-	OR 4.11 [1.74; 9.69] for malathion > LOD for cases, OR 0.13 [0.02; 0.69] for profenofos, OR 0.16 [0.05; 0.49] for chlorpyrifos-methyl. All concentrations significantly higher among cases.	N/A	N/A
Ranjbar[25]	Metabolites dichotomized into < LOD and ≥ LOD	0	No significant or clear numerical differences in FPG, HOMA-IR or HbA_{1c} for any of the metabolites.	N/A	N/A
Shapiro[26]	First-trimester urine metabolites modelled as continuous variables	-	OR for GDM or G-IGT across quartiles of metabolites: DEP: Q1 1 [ref.], Q2 0.7 [0.4; 1.4], Q3 0.4 [0.2; 0.9], Q4 0.9 [0.4; 1.9], $p_{trend} = 0.58$ Sum(DMP + DMTP): Q1 1 [ref.], Q2 0.9 [0.5; 1.6], Q3 0.5 [0.3; 0.9], Q4 0.5 [0.2; 0.9], $p_{trend} < 0.01$	N/A	N/A
Nascimento[28]	Comparison of high-exposure and low-exposure period. BChE as continuous variable in high-exposure period.	+	FPG (mmol/mol): Low-exposure period 4.85 [4.76; 4.94], high-exposure period 5.33 [5.24; 5.43], p for diff. < 0.001. Correlation between FPG and BChE in high-exposure period: r = -0.509, p < 0.001 (adjusted).	-	AChE (U/mL): Low-exposure period 12.05 [11.55; 12.55], high-exposure period 12.01 [11.39; 12.63], diff. not significant. BChE (U/mL): Low-exposure period 8.58 [8.21; 8.96], high-exposure period 5.67 [4.87; 6.45], p for diff. < 0.001.

Symbology for overall effects in Table A-5 and Table A-6:

- + = statistically significant positive relationship
- (+) = positive relationship, not statistically significant
- 0 = no association
- (-) = negative association, not statistically significant
- - = statistically significant negative association

Note that when the biomarker is ChE, the expected relationship between exposure and biomarker is negative. As organophosphate and carbamate insecticides inhibit ChE, low ChE enzyme activity indicates high exposure.

Anthropometry in the PEXADU project

Measurement of height

Height was measured without shoes or socks in accordance with WHO standard procedures,[66] using a stadiometer (SM-SZ-300, Sumbow Medical Instruments Co. Ltd., Ningbo, China) purchased from a local distributor. Ugandan women often have elaborate hairstyles (braiding and extensions) that may make it difficult to measure height accurately. We assumed the body height was constant across the phases, and used each participant's mean height across all phases where he/she participated.

Measurement of weight

Weight was measured using a medical weighing scale (seca® robusta 813, seca gmbh & co., Hamburg, Germany), purchased from a local distributor. Measurement was done in accordance with WHO standard procedures[66], with participant wearing light clothes, with empty pockets and bare feet. The resolution of the weighing scale was 0.1 kg.

The gravitational acceleration is slightly larger at the poles compared to at the equator. This means that a scale calibrated for use at a specific latitude may have a slight bias if it is used at a different latitude without recalibration. A seca® representative informed me that scales sold in Uganda are calibrated for Central Europe (Klaus Rosenbøl, personal communication, December 10, 2018). The International Gravity Formula[129] can be used to calculate the gravitational acceleration *g* at a specific latitude φ:

$$g_{\varphi} = g_0 \times (1 + \alpha \times (\sin \varphi)^2 + \beta \times (\sin \varphi)^2)$$

where $g_0 = 9.780318\, N/kg$, $\alpha = 0.0053024$ and $\beta = -0.0000058$.

Wakiso is located at a latitude of approximately 0.4 decimal degrees, while seca® headquarters in Hamburg are located at a latitude of approximately 53.6 decimal degrees. Using the International Gravity Formula, we calculate that

$$\gamma = \frac{g_{Hamburg}}{g_{Wakiso}} = \frac{9.8139\, {}^{N}/_{kg}}{9.7803\, {}^{N}/_{kg}} = 1.003$$

To obtain an unbiased estimate of mass, measurements from Wakiso were multiplied by $\gamma = 1.003$. Failing to correct the weights would most likely not affect our results considerably, but as the correction was trivial to implement, I saw no reason not to. After correcting weights for the gravity in Uganda, I subtracted 1 kg to account for the wearing of clothes.

Co-authorship declarations

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Declaration of co-authorship concerning article for PhD dissertations

Full name of the PhD student: Martin Rune Hassan Hansen

This declaration concerns the following article/manuscript:

Title:	Exposure to neuroactive non-organochlorine insecticides, and diabetes mellitus and related metabolic disturbances: Protocol for a systematic review and meta-analysis
Authors:	Martin Rune Hassan Hansen, Erik Jørs, Annelli Sandbæk, Henrik Albert Kolstad, Jörg Schullehner, Vivi Schlünssen

The article/manuscript is: Published ☒ Accepted ☐ Submitted ☐ In preparation ☐

If published, state full reference:
Hansen, Martin Rune Hassan, et al. "Exposure to neuroactive non-organochlorine insecticides, and diabetes mellitus and related metabolic disturbances: Protocol for a systematic review and meta-analysis." Environment international 127 (2019): 664-670.
https://doi.org/10.1016/j.envint.2019.02.074

If accepted or submitted, state journal:

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B. Has done most of the work (67-90 %)
C. Has contributed considerably (34-66 %)
D. Has contributed (10-33 %)
E. No or little contribution (<10%)
F. N/A

Page **1** of **2**

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Category of contribution	Extent (A-F)
The conception or design of the work:	B
Free text description of PhD students contribution ***(mandatory)*** MRHH conceived the need for a systematic review on insecticide exposure and glycemic regulation. The study was designed in collaboration especially with VS, but all authors contributed.	
The acquisition, analysis, or interpretation of data:	F
Free text description of PhD students contribution ***(mandatory)*** This is a protocol paper; it does not contain original data.	
Drafting the manuscript:	A
Free text description of PhD students contribution ***(mandatory)*** MRHH drafted the first version of the manuscript.	
Submission process including revisions:	A
Free text description of PhD students contribution ***(mandatory)*** MRHH is the corresponding author of the paper and took care of submission to the journal, communication with editor and reviewers, and implemented revisions (as agreed upon with the remaining authors).	

Signatures of first- and last author, and main supervisor

Date	Name	Signature
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23/01-20	Vivi Schlünssen	V. H

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Signature of the PhD student

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Declaration of co-authorship concerning article for PhD dissertations

Full name of the PhD student: Martin Rune Hassan Hansen

This declaration concerns the following article/manuscript:

Title:	Pesticide exposure and diabetes mellitus in a semi-urban Nepali population: a cross-sectional study
Authors:	Martin Rune Hassan Hansen, Bishal Gyawali, Dinesh Neupane, Erik Jørs, Annelli Sandbæk, Per Kallestrup, Vivi Schlünssen

The article/manuscript is: Published ☒ Accepted ☐ Submitted ☐ In preparation ☐

If published, state full reference:
Hansen, Martin Rune Hassan, et al. "Pesticide exposure and diabetes mellitus in a semi-urban Nepali population: a cross-sectional study." International Archives of Occupational and Environmental Health [published online ahead of print December 14, 2019].
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Category of contribution	Extent (A-F)
The conception or design of the work:	C
Free text description of PhD students contribution ***(mandatory)*** This study was conducted as part of the COBIN project, of which BG is the primary investigator. MRHH prepared the data collection instruments related to pesticide exposure in collaboration with EJ and VS.	
The acquisition, analysis, or interpretation of data:	B
Free text description of PhD students contribution ***(mandatory)*** BG collected all the data. MRHH carried out extensive data clean-up, and all analyses presented in the paper. MRHH interpreted the findings primarily in collaboration with VS.	
Drafting the manuscript:	A
Free text description of PhD students contribution ***(mandatory)*** MRHH drafted the first version of the manuscript.	
Submission process including revisions:	A
Free text description of PhD students contribution ***(mandatory)*** MRHH is the corresponding author of the paper and took care of submission to the journal, communication with editor and reviewers, and implemented revisions (as agreed upon with the remaining authors).	

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Title:	HemoDownloader: A utility for downloading data from HemoCue HbA1c 501 devices using a graphical user interface
Authors:	Martin Rune Hassan Hansen, Vivi Schlünssen, Annelli Sandbæk

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Category of contribution	Extent (A-F)
The conception or design of the work:	A
Free text description of PhD students contribution ***(mandatory)*** MRHH conceived the need for the program and wrote the source code without inputs from VS or AS.	
The acquisition, analysis, or interpretation of data:	F
Free text description of PhD students contribution ***(mandatory)*** This paper does not contain original data.	
Drafting the manuscript:	A
Free text description of PhD students contribution ***(mandatory)*** MRHH drafted the first version of the manuscript.	
Submission process including revisions:	A
Free text description of PhD students contribution ***(mandatory)*** MRHH is the corresponding author of the paper and took care of submission to the journal.	

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Authors:	Martin Rune Hassan Hansen, Erik Jørs, Annelli Sandbæk, Daniel Sekabojja, John C. Ssempebwa, Ruth Mubeezi, Philipp Staudacher, Samuel Fuhrimann, Alex Burdorf, Bo Martin Bibby, Vivi Schlünssen

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Occupational and Environmental Medicine (https://oem.bmj.com/)

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Category of contribution	Extent (A-F)
The conception or design of the work:	B
Free text description of PhD students contribution ***(mandatory)*** MRHH, EJ, AS and VS contrived the study. MRHH designed the study in collaboration with the remaining authors.	
The acquisition, analysis, or interpretation of data:	B
Free text description of PhD students contribution ***(mandatory)*** MRHH was the primary investigator of the study and lead the data collection team in Uganda. MRHH, VS and BMB designed the analysis plan. MRHH cleaned up data and did all statistical analyses. MRHH did the initial interpretation of analysis results, aided by VS.	
Drafting the manuscript:	A
Free text description of PhD students contribution ***(mandatory)*** MRHH drafted the first version of the manuscript.	
Submission process including revisions:	A
Free text description of PhD students contribution ***(mandatory)*** MRHH is the corresponding author of the paper and took care of submission to the journal.	

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Signature of the PhD student

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Full name of the PhD student: Martin Rune Hassan Hansen

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Title:	Organophosphate and carbamate insecticide exposure is related to decreased pulmonary function among smallholder farmers in Uganda: A short-term follow-up study
Authors:	Martin Rune Hassan Hansen, Erik Jørs, Annelli Sandbæk, Daniel Sekabojja, John C. Ssempebwa, Ruth Mubeezi, Philipp Staudacher, Samuel Fuhrimann, Torben Sigsgaard, Alex Burdorf, Bo Martin Bibby, Vivi Schlünssen

The article/manuscript is: Published ☐ Accepted ☐ Submitted ☒ In preparation ☐

If published, state full reference:

If accepted or submitted, state journal: Thorax (https://thorax.bmj.com)

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23/01-20	Vivi Schlünssen	Vivi S

Date: 23/01-20

Martin Rune Hassan Hansen

Signature of the PhD student

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Declaration of co-authorship concerning article for PhD dissertations

Full name of the PhD student: Martin Rune Hassan Hansen

This declaration concerns the following article/manuscript:

Title:	Pyridostigmine impairs pulmonary function in asthmatic subjects: Re-analysis of results from an observational study
Authors:	Martin Rune Hassan Hansen, Vivi Schlünssen

The article/manuscript is: Published ☐ Accepted ☐ Submitted ☒ In preparation ☐

If published, state full reference:

If accepted or submitted, state journal: Military Medicine (https://academic.oup.com/milmed)

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F. N/A

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The conception or design of the work:	A
Free text description of PhD students contribution ***(mandatory)*** MRHH conceived the need for the study, and planned all analyses without any inputs from VS.	
The acquisition, analysis, or interpretation of data:	A
Free text description of PhD students contribution ***(mandatory)*** MRHH carried out all analyses and interpreted results.	
Drafting the manuscript:	A
Free text description of PhD students contribution ***(mandatory)*** MRHH drafted the first version of the manuscript. VS critically reviewed the draft.	
Submission process including revisions:	A
Free text description of PhD students contribution ***(mandatory)*** MRHH is the corresponding author of the paper and took care of submission to the journal and communication with editor.	

Signatures of first- and last author, and main supervisor

Date	Name	Signature
23/01-20	Martin Rune Hassan Hansen	Martin Rune Hassan Hansen
23/01-20	Vivi Schlünssen	Vivi Schlünssen

Date: 23/01-20

Martin Rune Hassan Hansen

Signature of the PhD student

Ethical approval for the COBIN-D project

Government of Nepal

Nepal Health Research Council (NHRC)

Estd. 1991

Ref. No.: 766

10 November 2016

Mr. Bishal Gyawali
Principal Investigator
Aarhus University
Denmark

Subject: Approval of research proposal entitled **Community based management of Diabetes in Nepal: Study protocol for a cluster-randomized trial**

Dear Mr. Gyawali,

It is my pleasure to inform you that the above-mentioned proposal submitted on **09 September 2016** (**Reg.no. 263/2016** please use this Reg. No. during further correspondence) has been approved by NHRC Ethical Review Board on **09 November 2016**.

As per NHRC rules and regulations, the investigator has to strictly follow the protocol stipulated in the proposal. Any change in objective(s), problem statement, research question or hypothesis, methodology, implementation procedure, data management and budget that may be necessary in course of the implementation of the research proposal can only be made so and implemented after prior approval from this council. Thus, it is compulsory to submit the detail of such changes intended or desired with justification prior to actual change in the protocol before the expiration date of this approval. Expiration date of this study is **August 2018.**

If the researcher requires transfer of the bio samples to other countries, the investigator should apply to the NHRC for the permission. The researchers will not be allowed to ship any raw/crude human biomaterial outside the country; only extracted and amplified samples can be taken to labs outside of Nepal for further study, as per the protocol submitted and approved by the NHRC. The remaining samples of the lab should be destroyed as per standard operating procedure, the process documented, and the NHRC informed.

Further, the researchers are directed to strictly abide by the National Ethical Guidelines published by NHRC during the implementation of their research proposal and submit progress report and full or summary report upon completion.

As per your research proposal, the total research amount is **USD. 4,200.00** and accordingly the processing fee amount to **NRs. 10,719.00** . It is acknowledged that the above-mentioned processing fee has been received at NHRC.

If you have any questions, please contact the Ethical Review M & E section of NHRC.

Thanking you,

.....
Dr. Khem Bahadur Karki
Member Secretary

Tel: +977 1 4254220, Fax: +977 1 4262469, Ramshah Path, PO Box: 7626, Kathmandu, Nepal
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Ethical approvals for the PEXADU project

MAKERERE UNIVERSITY

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COLLEGE OF HEALTH SCIENCES
SCHOOL OF PUBLIC HEALTH
HIGHER DEGREES, RESEARCH AND ETHICS COMMITTEE

July 03rd, 2018

MARTIN RUNE HASSAN HANSEN
Principal Investigator, Protocol (577)
Section for Environment, Work & Health, Department of Public Health
Aarhus University, Denmark

Re: Approval of a Project proposal for the study entitled: PESTICIDE EXPOSURE, ASTHMA AND DIABETES IN UGANDA (PEXADU)

This is to inform you that, the Higher Degrees, Research and Ethics Committee (HDREC) has granted approval to the above referenced study, the HDREC reviewed the proposal during an ad-hoc HDREC meeting held on 22 March, 2018 and made some suggestions and comments which you have adequately incorporated:

Please note that your study protocol number with HDREC is **577**. Please be sure to reference this number in any correspondence with HDREC. Note that the initial approval date for your proposal by HDREC is 03rd/07/2018, and therefore approval expires at every annual anniversary of this approval date. The current approval is therefore valid until: **02nd/07/2019**.

Continued approval is conditional upon your compliance with the following requirements:

1) No other consent form(s), questionnaire and/or advertisement documents should be used. The consent form(s) must be signed by each subject prior to initiation of any protocol procedures. In addition, each subject must be given a copy of the signed consent form.

2) All protocol amendments and changes to other approved documents must be submitted to HDREC and not be implemented until approved by HDREC except where necessary to eliminate apparent immediate hazards to the study subjects.
3) Significant changes to the study site and significant deviations from the research protocol and all unanticipated problems that may involve risks or affect the safety or welfare of subjects or others, or that may affect the integrity of the research must be promptly reported to HDREC.
4) All deaths, life threatening problems or serious or unexpected adverse events, *whether related to the study or not,* must be reported to HDREC in a timely manner as specified in the National Guidelines for Research Involving Humans as Research Participants.

- Please complete and submit reports to HDREC as follows:

Makerere University School of Public Health
Valid Thru:
02 JUL 2019
Higher Degrees, Research and Ethics Committee

a) For renewal of the study approval – complete and return the continuing Review Report – Renewal Request (Form 404A) at least 60 days prior to the expiration of the approval period. The study cannot continue until re-approved by HDREC.

b) Completion, termination, or if not renewing the project – send a final report within 90 days upon completion of the study.

- Finally, the legal requirement in Uganda is that all research activities must be registered with the National Council of Science and Technology. The forms for this registration can be obtained from their website www.uncst.go.ug. Please contact the Administrative Assistant of the Higher Degrees, Research and Ethics Committee at wtusiime@musph.ac.ug or telephone number (256)-393 291 397 if you encounter any problems.

Yours sincerely

Dr. Suzanne Kiwanuka
Chairperson: Higher Degrees, Research and Ethics Committee

Enclosures:

a) A stamped, approved study documents (informed consent documents):

Martin Rune Hassan Hansen

From: Research Management - UNCST <research@uncst.go.ug>
Sent: 31. august 2018 09:06
To: Martin Rune Hassan Hansen
Cc: Hansen
Subject: Study Approval - (HS234ES)

Uganda National Council for Science and Technology

(Established by Act of Parliament of the Republic of Uganda)

Dear Martin Hansen,

I am pleased to inform you that on **31/08/2018**, the Uganda National Council for Science and Technology (UNCST) approved your study titled, **Pesticide Exposure, Asthma and Diabetes in Uganda**. The Approval is valid for the period of **31/08/2018** to **31/08/2019**.

Your study reference number is **HS234ES**. Please, cite this number in all your future correspondences with UNCST in respect of the above study.

Please, note that as Principal Investigator, you are responsible for:

1. Keeping all co-investigators informed about the status of the study.
2. Submitting any changes, amendments, and addenda to the study protocol or the consent form, where applicable, to the designated local Research Ethics Committee (REC) or Lead Agency, where applicable, for re-review and approval prior to the activation of the changes.
3. Notifying UNCST about the REC or lead agency approved changes, where applicable, within five working days.
4. For clinical trials, reporting all serious adverse events promptly to the designated local REC for review with copies to the National Drug Authority.
5. Promptly reporting any unanticipated problems involving risks to study subjects/participants to the UNCST.
6. Providing any new information which could change the risk/benefit ratio of the study to the UNCST for review.
7. Submitting annual progress reports electronically to UNCST. Failure to do so may result in termination of the research project.

Please, note that this approval includes all study related tools submitted as part of the application.

Yours sincerely,

Winfred Badanga Nazziwa
For: Executive Secretary
UGANDA NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

Paper I

Exposure to neuroactive non-organochlorine insecticides, and diabetes mellitus and related metabolic disturbances: Protocol for a systematic review and meta-analysis

Hansen MRH, Jørs E, Sandbæk A, Kolstad HA, Schullehner J, Schlünssen V.

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Review article

Exposure to neuroactive non-organochlorine insecticides, and diabetes mellitus and related metabolic disturbances: Protocol for a systematic review and meta-analysis☆

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Pesticide
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ABSTRACT

Objectives: To assess whether exposure to specific classes of neuroactive non-organochlorine insecticides is associated with diabetes mellitus or related metabolic traits.

Methods: Eligibility criteria: Any type of epidemiological and human exposure studies providing an exposure contrast to neuroactive non-organochlorine insecticides and a measure of association to diabetes mellitus or related metabolic traits. We will include published peer-reviewed studies in both English and non-English language.

Information sources: Articles will be located in the NCBI PubMed, Embase, Scopus, Web of Science and LILACS databases, supplemented with manual searching of reference lists and articles citing the included studies.

Risk of bias assessment: Risk of bias in individual studies will be assessed using tools from the Navigation Guide systematic review methodology, while the risk of bias at the outcome level will be assessed according to the Grading of Recommendations Assessment, Development, and Evaluation (GRADE) guidelines.

Data synthesis and analysis: When studies are sufficiently similar in population, exposure, comparator and effect estimate to meaningfully allow quantitative synthesis, we will perform meta-analysis. Otherwise, results will be summarized qualitatively.

Funding: The authors are paid employees of their respective institutions. MRHH is a Ph.D. student working under grants from Aarhus University and the National Research Centre for the Working Environment.

Registration: PROSPERO CRD42017068861.

1. Background

The global prevalence of diabetes mellitus has been rapidly increasing over the last decades, reaching 8.5% in 2014 (World Health Organization, 2016), and it is estimated that 3.7 million people die annually as a consequence of diabetes mellitus or higher-than-optimal

☆ Protocol registration and changes: This publication is an update of a protocol first registered in the International Prospective Register of Systematic Reviews (PROSPERO) on June 9, 2017 and updated on March 25, 2019 (registration number CRD42017068861, http://www.crd.york.ac.uk/PROSPERO/display_record.php?ID=CRD42017068861). All elements of the protocol have been adjusted to adhere with best practices for systematic reviews. Apart from preliminary searches in scientific databases to assist in the design of search strings, no work on the systematic review was performed before the protocol was changed.

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blood glucose (World Health Organization, 2016). Epidemiological studies have suggested a link between exposure to some pesticides and development of diabetes mellitus (Velmurugan et al., 2017; Wang et al., 2011; Saldana et al., 2007; Montgomery et al., 2008), which is worrying in the light of the widespread use of pesticides – the annual global consumption of pesticides is estimated at 2.6 billion kg (Atwood and Paisley-Jones, 2017). A recent systematic review and meta-analysis found a summary OR of 1.58 (95% confidence interval 1.32–1.90) for diabetes mellitus when comparing top vs. bottom tertiles of any pesticide exposure (Evangelou et al., 2016).

1.1. Description of the outcome

Diabetes mellitus is a heterogeneous group of diseases that can be classified into four major categories (American Diabetes Association, 2018) based on patients' clinical features and the underlying pathology:

- Type 1 diabetes: Autoimmune destruction of the insulin-producing cells of the pancreas.
- Type 2 diabetes: Relative insulin deficiency due to decreased insulin production and/or insulin resistance.
- Gestational diabetes: Diabetes mellitus diagnosed in the second or third semester of pregnancy that was not clearly overt diabetes prior to gestation.
- Specific types of diabetes due to other causes: E.g., monogenic, drug- or chemical-induced diabetes mellitus.

Overt diabetes mellitus represents the end of a continuous spectrum of hyperglycaemia, insulin resistance, and decreased insulin production. This is reflected in the changes of diagnostic criteria made by the World Health Organization (WHO) based on studies showing negative health effects of hyperglycaemia: The 1980 cut-off for fasting plasma glucose was 8.0 mmol/l (WHO Expert Committee on Diabetes Mellitus, 1980), changed to 7.8 mmol/l (World Health Organization, 1985) in 1985 and to the current value of 7.0 mmol/l in 1998 (Alberti and Zimmet, 1998). Even with the current definition, higher-than-optimal blood glucose values below the diagnostic cut-off are associated with significant morbidity and mortality (World Health Organization, 2016).

The risk of type 1 diabetes mellitus is influenced by HLA (Human Leukocyte Antigen) genotype (American Diabetes Association, 2018). The risk of type 2 diabetes mellitus increases with old age, physical inactivity, overweight, family history, use of some medications such as glucocorticoids, and among some ethnic groups (American Diabetes Association, 2018). In agreement with the ability of some therapeutic drugs to increase the risk of diabetes mellitus among predisposed individuals (American Diabetes Association, 2018), exposure to non-therapeutic chemicals can also play a role in the aetiology of some cases (American Diabetes Association, 2014). E.g., the rodenticide Vacor was toxic to beta cells and lead to a phenotype similar to type 1 diabetes in humans who attempted suicide with the compound (Karam et al., 1980).

1.2. Description of the exposure

Pesticides are compounds used for killing unwanted organisms, and based on their target organisms they can be broadly categorized as insecticides, nematicides, rodenticides, herbicides or fungicides (Casida, 2009). These broad classes can be further subdivided based on the specific mode of action of individual compounds (Casida, 2009). Because of the broad definition of the term “pesticide”, it encompasses a huge number of chemical substances: in May 2018 the European Union pesticide database alone contained 1367 different active substances (*EU Pesticides Database*, n.d.).

1.3. Rationale for a systematic review

While the putative association between pesticide exposure and diabetes mellitus has been assessed in several reviews (Evangelou et al., 2016; Lasram et al., 2014; Mostafalou and Abdollahi, 2016; Jaacks and Staimez, 2015; Song et al., 2016; Kim et al., 2017; Leso et al., 2017; Xiao et al., 2017), previous reviews likely missed relevant evidence due to search strategies that were not sufficiently sensitive. A new review with a more comprehensive search strategy is warranted.

Higher-than-optimal blood glucose and diabetes mellitus should be considered part of a pathophysiological spectrum rather than separate entities. A comprehensive review should therefore include not only studies on outright diabetes, but also associated metabolic traits such as impaired fasting glucose, glucose intolerance, insulin resistance, decreased insulin production and hyperglycaemia below the diagnostic limits for diabetes mellitus. Out of eight previous reviews that we have identified, the authors of two (Evangelou et al., 2016; Mostafalou and Abdollahi, 2016; Song et al., 2016) searched only for studies on “diabetes”, two reviews did not list their search strategy (Lasram et al., 2014; Kim et al., 2017) and four (Jaacks and Staimez, 2015; Song et al., 2016; Leso et al., 2017; Xiao et al., 2017) also searched for diabetes-associated metabolic traits – but used few synonyms.

Pesticides are a heterogeneous group of chemical compounds (Casida, 2009), and articles on specific compounds may not include terms such as “pesticide” or “insecticide”, meaning they may not turn up when searching a database using the generic term “pesticide”. In our view, this has not been taken properly into account by authors of previous reviews. E.g., neonicotinoid insecticides have an estimated 27% share of the insecticide market in monetary value (Sparks and Nauen, 2015), yet none of the previous reviews specified specific search terms for neonicotinoids (two reviews (Lasram et al., 2014; Song et al., 2016) focused on other classes of pesticides, and one (Kim et al., 2017) of the remaining five did not list the search strategy).

Because of the continuous nature of the disease process, our systematic review will not only focus on diabetes mellitus as defined by the diagnostic criteria at the time of the individual studies; we will also include studies on hyperglycaemia, insulin resistance and decreased insulin production. As it is not practically feasible to perform a sensitive search for literature about all pesticidal compounds nor biologically meaningful to assess them as one, we choose to focus on the neuroactive non-organochlorine insecticides that comprise 85% of the global market for insecticides in monetary value (Sparks and Nauen, 2015). A list of the classes of insecticides to be included can be seen in the section “Types of exposures” below, while individual compounds are listed in Online appendix A.

2. Objectives

Through a systematic review and meta-analysis of human epidemiological studies, we aim to assess whether exposure to specific classes of neuroactive non-organochlorine insecticides is associated with diabetes mellitus, or related metabolic traits such as impaired fasting glucose, glucose intolerance, or insulin resistance.

3. Methods

The systematic review will be carried out according to the Navigation Guide methodology for systematic reviews in environmental health (Woodruff and Sutton, 2014; Woodruff et al., 2011), and will be reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher et al., 2009). This protocol conforms to the PRISMA Protocols (PRISMA-P) guidelines, and its abstract conforms to the PRISMA for Abstracts (PRISMA-A) guidelines (Beller et al., 2013).

The reviewer team has members trained in systematic review methodology, as well as expertise in occupational and environmental

Table 1
Included outcomes.

Outcome	Definition	Current ADA diagnostic criteria (American Diabetes Association, 2018)
Diabetes mellitus[a]	Clinical disease characterized by a varying degrees of hyperglycaemia, insulin resistance and decreased insulin production.	Fasting plasma glucose ≥ 7.0 mmol/l OR 2-hour plasma glucose ≥ 11.1 mmol/l during standardized oral glucose tolerance test OR Glycated haemoglobin A ≥ 48 mmol/mol OR Random plasma glucose ≥ 11.1 mmol/l
Impaired fasting glucose	Higher-than-normal fasting glucose that does not meet diagnostic criteria for diabetes mellitus	5.6 mmol/l ≤ fasting plasma glucose ≤ 6.9 mmol/l
Impaired glucose tolerance	Higher-than-normal 2-hour plasma glucose during standardized oral glucose tolerance test that does not meet diagnostic criteria for diabetes mellitus	7.8 mmol/l ≤ 2-hour plasma glucose ≤ 11.0 mmol/l
Prediabetes	Higher-than-normal glucose levels that do not meet diagnostic criteria for diabetes mellitus	Impaired fasting glucose OR Impaired glucose tolerance OR 39 mmol/mol ≤ glycated haemoglobin A ≤ 47 mmol/mol
Hyperglycaemia	Increased blood sugar level, expressed either as a continuous measure or categorized.	–
Insulin resistance	Decreased sensitivity to insulin in peripheral tissues, determined by simultaneous measurement of insulin and plasma glucose levels. Expressed as continuous or categorical measure.	–
Decreased insulin production	Decreased endogenous insulin levels, expressed either as continuous measure or categorized.	–

ADA = American Diabetes Association.
[a] The clinical diagnosis of diabetes mellitus normally requires confirmation with a second test, unless the patient has classic hyperglycaemic symptoms or a hyperglycaemic crisis (American Diabetes Association, 2018). However, in epidemiological studies, repeat testing is often not performed.

medicine, chemical risk assessment and clinical endocrinology with a focus on diabetes mellitus. The review process will be managed using the DistillerSR (*DistillerSR*, n.d.) web application for systematic reviews to ensure transparency.

In case any methodological changes from this protocol are made during the review, they will be listed under the heading "Differences between protocol and review" in the final article.

3.1. Eligibility criteria

3.1.1. Types of populations

Any human population.

3.1.2. Types of exposures

Occupational or environmental exposure to neuroactive non-organochlorine insecticides, defined as non-organochlorine insecticidal compounds whose main toxico-dynamic target is the nervous system according to the classification system by the Insecticide Resistance Action Committee (IRAC) (IRAC International MoA Working Group, 2017). To make the task manageable, we will not include DDT, its analogs or other organochlorine insecticides (IRAC subgroups 2A and 3B), since most of these compounds are already severely restricted due to health risk concerns (Stockholm Convention, 2010), and currently have a small market share (Sparks and Nauen, 2015). Nor will we include nicotine, even though it can be categorized as a neuroactive insecticide, because we consider it obsolete as a pesticide, and it is estimated to have no significant market share (Sparks and Nauen, 2015).

Specifically, the included classes of insecticides (with IRAC group or subgroup numbers in parentheses) are:

- Carbamates (1A)
- Organophosphates (1B)
- Fiproles (2A)
- Pyrethroids and pyrethrins (3A)
- Neonicotinoids (4A)
- Sulfoximines (4C)
- Butenolides (4D)
- Spinosyns (5)
- Avermectins and milbemycins (6)
- Pymetrozine (9B)
- Flonicamid (9C)
- Nereistoxin analogs (14)
- Amitraz (19)
- Oxadiazines (22A)
- Semicarbazones (22B)
- Diamides (28).

The individual insecticides in these classes can be seen in Appendix A.

We will include studies with objective measurements of exposure, as well as more crude exposure measures, as long as the insecticide used is stated and falls within one of the above-mentioned categories. The more crude exposure measures may include professions, industries, work sites, questionnaire information, job-exposure matrices etc., no matter if dichotomous (yes/no) or (semi)quantitative.

3.1.3. Types of comparators

Humans exposed to no or lower levels of neuroactive non-organochlorine insecticides than the more highly exposed population. The difference in exposure level in included studies does not need to be formally tested. E.g., we will include studies comparing conventional farmers with the background population, even if no more sophisticated exposure metrics are used (such as biomarkers or subjective information on pesticide usage duration, frequency and intensity). The validity of the exposure assessment and its implications for the confidence in the study findings will instead be discussed as part of the risk of bias assessment (see below).

3.1.4. Types of outcomes

Table 1 lists included outcomes with the definitions that will be used in this systematic review, as well as current diagnostic criteria from the American Diabetes Association (American Diabetes

Association, 2018), where applicable. Authors of original studies may have used other diagnostic criteria. E.g., as previously mentioned the WHO has used three different diagnostic cut-offs for fasting plasma glucose since 1980 (WHO Expert Committee on Diabetes Mellitus, 1980; World Health Organization, 1985; Alberti and Zimmet, 1998). For this reason, during data extract we will record the exact diagnostic criteria used by original study authors (see Section 3.4).

Because of the heterogeneity of the pathophysiology of different types of diabetes (American Diabetes Association, 2018), throughout the review we will manage results for the following types separately: Type 1, type 2, gestational and unspecified. We will not consider studies whose outcome is random plasma glucose or glucosuria, nor studies on monogenic forms of diabetes. When the outcome in a study is diabetes mellitus, it must be defined by register data, fasting plasma glucose, oral glucose tolerance test, glycated haemoglobin A (HbA_{1c}), or a combination of these measures, optionally in combination with patient self-report. We will not include studies where diabetes mellitus is defined only by patient self-report or by measurement of random plasma glucose or glycosuria. The reason for including studies relying on a combination of patient self-report and objective measures is that patients receiving treatment for diabetes may be normoglycaemic, yet they should still be considered diabetic.

3.1.5. Types of studies

3.1.5.1. Inclusion criteria

Epidemiological (e.g., cross-sectional, case-control, cohort, but *not* ecological) and human exposure studies providing an exposure contrast to the neuroactive non-organochlorine insecticides listed in Appendix A and a measure of association to the outcomes of interest. Studies without a measure of association will be included if they provide enough information to calculate such a measure.

3.1.5.2. Exclusion criteria.

- Case reports, case series, ecological studies, animal studies, ex vivo studies (including studies on human cells and tissues) and in silico studies.
- Studies only considering exposure to “insecticides” or “pesticides” as broad categories.
- Studies on exposure to a mixture of pesticides that includes compounds that cannot be classified as neuroactive non-organochlorine insecticides (unless authors present separate results on the effect of neuroactive non-organochlorine insecticides, adjusted for the effect of other pesticides).
- Studies only reporting prevalence or incidence of diabetes in an insecticide-exposed group, but without an exposure contrast.
- Studies on insecticide poisoning requiring acute medical treatment.

Animal, ex vivo and in silico studies are excluded for pragmatic reasons. We consider it infeasible to review both epidemiological and mechanistic evidence on all non-organochlorine neuroactive insecticides in the same systematic review. We consider evidence from epidemiological studies most relevant, but it will be highly relevant in the future to follow-up with a systematic review on mechanistic evidence.

Studies on patients requiring acute medical treatment for insecticide poisoning are excluded because hyperglycaemia is a part of the acute stress response (Marik and Bellomo, 2013), and the hyperglycaemia seen among such patients may be an unspecific marker of physiological stress. We are aware that hyperglycaemia has been reported among patients acutely intoxicated with pyrethroids (Kim et al., 2015), organophosphates (Moon et al., 2016), carbamates (Satar et al., 2005) and neonicotinoids (Todani et al., 2008).

If we identify studies with overlapping populations, we will abstract data and perform risk of bias assessment for them all (see below). When synthesizing results, we will only include results from one article per study population per exposure-outcome pair (e.g., any occupational organophosphate insecticide exposure vs. fasting plasma glucose as continuous measure). We will use the results from the study that has the lowest risk of bias across the evaluated domains (see below). If studies have the same risk of bias, we will include the largest study (number of participants or amount of follow-up time, depending on study design).

3.1.5.3. Years considered. Any year of publication. All searches will be re-run within 12 months of publication of the systematic review. New hits will be screened in the same manner as hits in the original search (see below). When deciding on whether to include new eligible studies in the systematic review, their potential influence on the conclusions of the systematic review will be weighed against the resulting delay in publication. If we choose not to include such new studies, we will provide a comprehensive list of the studies in an appendix.

3.1.5.4. Publication language. We will not exclude any articles based on language, but we will only actively search for articles using English terms (except for the LILACS database, where Spanish, French and Portuguese terms will also be used). When screening studies for inclusion, articles in languages other than the ones spoken by the reviewers (Danish, English, German, Norwegian and Spanish) will be translated into English using Google Translate (https://translate.google.com/). All included studies (and all screened studies where we are in doubt about inclusion after automatic translation) will be translated to English by a human translator.

3.1.5.5. Publication status. Peer-reviewed publications.

3.1.6. Types of effect measures

While included studies have to provide an effect measure as described in the inclusion criteria, the type of effect measure will not influence the decision to in- or exclude studies.

Because of the known and strong correlations between e.g. family history, age and diabetes mellitus, we will not be able to convert adjusted odds ratios for diabetes mellitus into relative risks, since the conversion requires an a common “assumed control risk” for all the non-exposed persons (O'Connor, 2013). We will only combine adjusted odds ratios and adjusted relative risks in meta-analysis if the prevalence of diabetes mellitus in the studies reporting odds ratios is $\leq 10\%$, since the odds ratio will then be numerically very similar to the relative risk (Sterne and Kirkwood, 2010).

3.2. Information sources and search

Searches will be performed in the following scientific databases:

- NCBI PubMed (https://www.ncbi.nlm.nih.gov/pubmed/)
- Embase (https://www.embase.com)
- Scopus (https://www.scopus.com)
- Web of Science (https://apps.webofknowledge.com)
- LILACS (http://lilacs.bvsalud.org).

The search terms for each of these 5 databases are included in Online appendices B–F.

The search strategy was primarily developed by MRHH, but with inputs from the remaining authors. For each outcome of interest, a list of synonyms was compiled. MeSH (Medical Subject Heading) and Emtree keywords were identified in the NCBI PubMed and Embase databases, respectively. The MeSH terms and keywords were exploded and the related words included in free text. Spanish, French and Portuguese terms were identified in the European Union's terminology database (IATE) (IATE (Interactive Terminology for Europe), n.d.). For exposure terms, we also identified and exploded MeSH terms and keywords from PubMed/Embase. Names of compounds belonging to each included class of insecticides were compiled from the IRAC

classification (IRAC International MoA Working Group, 2017), supplemented with exploded MeSH terms, Emtree keywords and the World Health Organization guide to classification of pesticides by hazard (World Health Organization, 2009). Non-English compound names were identified in IATE, an online database of pesticide common names (Wood, n.d.) and lists of pesticides approved or banned in Ecuador, Mexico and Spain. Chemical Abstracts Service (CAS) numbers for individual compounds were identified in NCBI PubChem (https://pubchem.ncbi.nlm.nih.gov/) and aforementioned database of pesticide common names (Wood, n.d.).

A hand search for potentially eligible articles will be performed in the reference list of included articles, among the articles that cite any of the included articles, and in the reference lists of existing reviews on the subject. Experts on negative health effects of pesticides will be presented with the list of included studies and will be asked to identify any further eligible studies.

We have decided not to perform a grey literature search as part of the systematic review. We know that we might inadvertently exclude some studies, but we judge it to be more important to perform a very sensitive search strategy for published papers.

The removal of in vitro, in silico and animal studies (see eligibility criteria above) will be done manually rather than by the use of search filters because studies might have been erroneously indexed in the article databases.

3.3. Study selection

Records and data will be managed using the DistillerSR (*DistillerSR*, n.d.) web application for systematic reviews. Search results will be directly imported, followed by de-duplication and study selection within DistillerSR. Two independent reviewers (MRHH and JS) will screen search hits for eligibility first at the title and abstract level, then at the full text level. Conflicts will be solved by consulting a third review author. In the systematic review, a flow-chart of article in- and exclusion will be provided in accordance with the PRISMA (Moher et al., 2009) guidelines.

3.4. Data extraction and data items

Two independent investigators (MRHH and JS) will design and pilot data extraction forms until agreement is achieved, with mediation by a third investigator if agreement cannot be achieved. Data will also be extracted by two separate investigators (MRHH and JS), with mediation by a third investigator in case of disagreements.

We will prepare three separate data extraction forms – one for extracting study characteristics, one for assessing risk of bias (see included parameters in the section "Risk of bias assessment"), and one for extracting effect estimates. The forms will be filled out in the order listed (i.e. risk of bias assessment will be done before extraction of effect estimates). The form for extracting effect estimates will have separate fields for each identified exposure-outcome pair. Data items to be extracted can be seen in draft versions of the forms for recording study characteristics and effect estimates in Online appendix G; the final forms will be implemented in DistillerSR.

The study design will be assessed by the systematic review authors instead of relying on labels used by investigators of primary studies. If the outcome is diabetes mellitus, we will assess and record whether it was type 1, type 2, gestational or unspecified, also without relying on labels in the primary study.

Effect estimates will be extracted with their 95% confidence intervals. If no confidence interval is available, but an exact p-value is, we will extract the p-value and calculate a 95% confidence interval. If insufficient data are available to calculate a confidence interval, the corresponding author of the study in question will be contacted to obtain the data necessary for the calculation. When extracting data for binary outcomes, priority will be given to relative effects measures such as risk ratio, odds ratio and hazard ratio depending on the study design. Absolute effect measures such as risk differences will be included if no relative effect measures are available. For continuous outcomes such a fasting plasma glucose, we will give equal priority to relative and absolute effect measures.

If results from both unadjusted and adjusted analyses are presented in a study, we will normally include results from the most adjusted one. Analyses including both pesticide exposure and being a farmer/pesticide applicator as independent variables at the same time will be considered over-adjusted, in which case we will usually select a less adjusted model. If the only other model available is completely unadjusted, we will instead choose the over-adjusted model and take this into account when assessing the risk of "other bias" in the study (see below).

3.5. Risk of bias assessment

Risk of bias will be considered both at the outcome and the individual study level. The risk of bias at the outcome level will be assessed as part of the assessment of the quality of evidence (see below). All assessment of risk of bias will be done by two separate investigators (MRHH and JS) with mediation by a third investigator (VS) in case of disagreements. The risk of bias assessment will be managed in DistillerSR.

In accordance with the Navigation Guide systematic review methodology, risk of bias in individual studies will be assessed across a number of domains (Woodruff and Sutton, 2014). We will use the same domains as a previous systematic review on the reproductive toxicity of the biocide triclosan (Johnson et al., 2016):

- Sequence generation
- Allocation concealment
- Baseline differences
- Blinding
- Exposure assessment
- Outcome assessment
- Confounding
- Incomplete outcome data
- Selective outcome reporting
- Conflict of interest
- Other bias.

For each domain in the risk of bias, studies will be rated as "low risk", "probably low risk", "probably high risk", "high risk" or "not applicable" according to the instructions listed in Online appendix H. Support for judgement (in the form of verbatim quotes from the original articles and/or comments from the systematic review authors) will be provided in an online appendix as suggested by the Cochrane Handbook (Deeks et al., 2011).

When assessing the risk of bias due to confounding, the factors considered important confounders will depend on the type of diabetes mellitus. For type 1 diabetes mellitus, important confounders will be ethnicity and HLA (Human Leukocyte Antigen) genotype or family history of diabetes mellitus. For type 2, gestational and non-specified diabetes mellitus, important confounders will be ethnicity, age, sex, family history of diabetes mellitus, diet, weight status and physical activity level.

To assess the risk of bias due to selective reporting, we will attempt to locate published protocols of included studies.

In the domain "Conflict of interest", we will include both financial and non-financial conflicts of interest as laid out in the standard form (*Editors ICoMJ. ICMJE Conflict of Interest form*) for reporting conflicts of interest published by the International Committee of Medical Journal Editors. We will search for undisclosed conflicts using a modification of the method used in a previous systematic review (Mandrioli et al., 2016). In short, if study authors did not provide a disclosure or

disclosed no conflict of interest, we will identify papers written by the same authors indexed in the five scientific databases listed in the section "Information sources and search". We will then review disclosures in these other papers. We will also search for authors' curricula vitae on Google (https://www.google.com/). In case a relevant conflict of interest is uncovered during the search, the search will end and the study be classified as if the conflict of interest had been declared in the study paper.

3.6. Assessing quality of evidence

The quality of evidence at the level of each exposure-outcome pair will be graded according to the Grading of Recommendations Assessment, Development, and Evaluation (GRADE) guidelines (Guyatt et al., 2008), adapted to the setting of occupational and environmental medicine as described in the Navigation Guide (Woodruff and Sutton, 2014). We will establish an initial level of certainty in the evidence based on the predominating study design that can then be up- or downgraded based on a priori defined factors, leading to an overall certainty rating (high, moderate, low or very low). While the GRADE guidelines by default consider randomized controlled trials high quality and observational studies low quality (Guyatt et al., 2008), we will by default consider observational studies moderate quality in keeping with the Navigation Guide (Woodruff and Sutton, 2014). The standard GRADE guidelines will be used when deciding to lower (study limitations, inconsistency of results, indirectness of evidence, imprecision, publication bias) or increase the certainty in the evidence (large magnitude of effect, plausible confounding would reduce a demonstrated effect, dose-response gradient) (Guyatt et al., 2008).

Risk of publication bias will be assessed qualitatively if we identify 9 studies or less for an exposure-outcome pair, while we will use funnel plots if we identify at least 10 studies.

3.7. Summary measures and synthesis of results

For each exposure-outcome pair where we include two or more studies, we will qualitatively assess whether the studies are sufficiently similar in exposed population, comparator and type of effect estimate to allow quantitative synthesis in a meaningful manner. E.g., studies on environmentally and occupationally exposed persons will not be combined quantitatively, unless biomarkers show that exposure levels are comparable. Results on categorical outcomes (e.g., prediabetes) will not be quantitatively synthesized unless definitions of categories are the same across studies. This assessment will be done by two separate investigators, with mediation by a third investigator in case of disagreements. If quantitative synthesis is deemed inappropriate, we will synthesize the results qualitatively based on a discussion of study homo-/heterogeneity. If we deem that at least two studies are sufficiently similar for quantitative synthesis, we will quantify the heterogeneity of their results using the I^2 statistic (Sterne and Kirkwood, 2010) and combine them in a meta-analysis using a random effects model (Sterne and Kirkwood, 2010) (to account for any cross-study heterogeneity) with inverse variance weighting. Adjusted and unadjusted effect estimates will not be combined quantitatively. The summary measures will depend on the effect measures reported in the individual studies. E.g., if articles report odds ratios, we will calculate meta-odds ratios, and if articles report regression coefficients from linear regression models on blood glucose values, we will calculate meta-coefficients.

3.8. Additional analyses

To assess the impact (direction and size) of the risk of bias in individual studies, we will repeat any quantitative synthesis for each exposure-outcome pair, stratified by each risk of bias domain and by overall risk of bias (low risk or probably low risk vs. probably high risk, high risk or unclear risk), provided that each stratum contains at least two studies. If the strata contain less than two studies each, we will qualitatively assess the impact of the risk of bias.

Since many insecticides are lipophilic, weight status may influence the relationship between absorbed dose and measured biomarkers. Therefore, quantitative analyses will be repeated, stratified by weight status (population overweight vs. not overweight), provided each stratum contains at least two studies. If strata are smaller, results will be assessed qualitatively.

Since in silico, in vitro and animal studies will not be included in the systematic review, we will refrain from speculating on the possible mechanistic links between exposure to neuroactive non-organochlorine insecticides and disruption of glucose homeostasis. Also, since we will not review the benefits of using these compounds (e.g., in agriculture and public health programs targeted against malaria) in this systematic review, we will give no recommendations regarding their use.

3.9. Data sharing

The DistillerSR database and audit log for the systematic review will be exported and included in the final publication as an online supplement to ensure complete transparency.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.02.074.

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Conflicts of interest

AS reports personal fees from Novo Nordisk Denmark, outside the submitted work. The remaining authors declare that they have no conflicts of interest (financial or otherwise) in relation to the subject of the systematic review.

Author contributions

MRHH and VS contrived the systematic review. MRHH and VS gathered the review team. MRHH and VS lead and all authors contributed to the development of the protocol. MRHH and JS are the lead reviewers of this systematic review. MRHH wrote the first draft of this protocol and EJ, AS, HAK, JS and VS made substantial contributions to the designs of the systematic review and to revisions of the manuscript. MRHH is the guarantor of the systematic review.

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The opinions expressed in this article are the responsibility of the authors alone and do not necessarily reflect the policies of their respective institutions.

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Paper II

Pesticide exposure and diabetes mellitus in a semi-urban Nepali population: a cross-sectional study

Hansen MRH, Gyawali B, Neupane D, Jørs E, Sandbæk A, Kallestrup P, Schlünssen V.

International Archives of Occupational and Environmental Health.

Published online ahead of print December 14, 2019.

For copyright reasons, paper II has been removed from this version of the document.

The paper is available at https://doi.org/10.1007/s00420-019-01508-2

Paper III

HemoDownloader: A utility for downloading data from HemoCue HbA1c 501 devices using a graphical user interface

Hansen MRH, Schlünssen V, Sandbæk A.

Submitted to SoftwareX.

Paper III has not been published yet and has therefore been removed from this version of the document.

Program source code for paper III

Submitted to SoftwareX.

```
##  HemoDownloader 1.1
##  A GUI UTILITY FOR DOWNLOADING DATA FROM HEMOCUE® HBA1C 501 DEVICES
##  Copyright © 2018-2019 Martin Rune Hassan Hansen <martinrunehassanhansen@ph.au.dk>

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##  VERSION 1.0.1, 2018-09-22
##  Change compared with version 1.0: The program now gracefully handles HbA1c values listed as "< 4 %".

##  VERSION 1.0.2b, 2018-09-24
##  Change compared with version 1.0.1: The program now gracefully handles HbA1c values listed as "> 14 %".

##  VERSION 1.1, 2019-07-02
##  Changes compared with version 1.0.2b:
##      * Added legal notice
##      * Added help
##      * Added license information
##      * Added 'About' information
##      * Added debug mode
##      * Changed default timeout
##      * Changed headings in exported files
##      * Added more tests of data integrity

__version__ = 1.1


##  IMPORT NECESSARY MODULES
import tkinter as tk
from tkinter import ttk
from tkinter import simpledialog
from tkinter import filedialog
from os import path
import re, csv, datetime

modulesNotFound = False
try:
    import serial, serial.tools.list_ports
except ModuleNotFoundError:
    modulesNotFound = True
try:
    import xlsxwriter
except ModuleNotFoundError:
    modulesNotFound = True
try:
    import xlwt
except ModuleNotFoundError:
    modulesNotFound = True


##  MAIN WINDOW
class settingsWindow(ttk.Frame):

```

```
66      ##  INITIALIZE THE WINDOW.
67      def __init__(self, master):
68          #   Define geometry of window.
69          ttk.Frame.__init__(self, master=master)
70          self.master.title('HemoDownloader 1.1 - Transfer data from HemoCue® HbA1c 501')
71          self.master.resizable(False, False)
72
73          #   Create menubar
74          self.menubar = tk.Menu(root)
75          self.filemenu = tk.Menu(self.menubar, tearoff=0)
76          self.filemenu.add_command(label="Exit", command=root.destroy)
77          self.menubar.add_cascade(label="File", menu=self.filemenu)
78          self.editmenu = tk.Menu(self.menubar, tearoff=0)
79          self.helpmenu = tk.Menu(self.menubar, tearoff=0)
80          self.helpmenu.add_command(label="Instructions for use", command=lambda:
            self.showHelpBox("Instructions for use"))
81          self.helpmenu.add_command(label="About HemoDownloader", command=lambda:
            self.showHelpBox("About HemoDownloader"))
82          self.helpmenu.add_command(label="License information", command=lambda:
            self.showHelpBox("License information"))
83          self.menubar.add_cascade(label="Help", menu=self.helpmenu)
84          root.config(menu=self.menubar)
85
86          #   Create widgets.
87          self.firstTimeExecuted = True
88          self.initialDir = r'\\'
89          self.outputFilePathLabel = ttk.Label(root, text="File to save:")
90          self.outputFilePathLabel.grid(row = 2, column=0, sticky='W', pady=15, padx=10)
91
92          self.outputFilename = ''
93          self.outputFilenameLabel = ttk.Entry(root,textvariable=self.outputFilename,
            width=50, state='readonly')
94          self.outputFilenameLabel.grid(row=2, column=1, sticky='W', padx=10)
95
96          #   Define list of filetypes for save-as menu.
97          self.tabularFiletypes = [('CSV','*.csv'),('Tab Separated
            Values','*.tsv'),('Excel Workbook','*.xlsx'),('Excel 97-2003
            Workbook','*.xls'),('All files','*.*')]
98
99          #   More widgets
100         self.saveAsButton = ttk.Button(root,text='Save As...',
            command=self.save_as_filename)
101         self.saveAsButton.grid(row=2, column=2, sticky='W', padx=10)
102
103         self.comportLabel = ttk.Label(root, text="Serial port connection to use:")
104         self.comportLabel.grid(row = 3, column=0, sticky='W', padx=10)
105
106         self.comportLongName = tk.StringVar()
107         self.comportMenu = ttk.OptionMenu(root, self.comportLongName, *[],
            command=self.select_comport)
108         self.comportMenu.grid(row=3, column=1, sticky='W', padx=10)
109         self.update_serial_port_list()
110         self.registerComportConnectionErrorboxState(False)
111
112         self.timeoutLabel = ttk.Label(root, text="Connection timeout:")
113         self.timeoutLabel.grid(row = 4, column=0, sticky='W', padx=10, pady=15)
114
115         self.timeoutString = tk.StringVar()
116         self.timeoutStringChoices = ['dummy','30 seconds','1 minute','2 minutes','3
            minutes','5 minutes','10 minutes']
117         self.timeoutMenu = ttk.OptionMenu(root, self.timeoutString,
            *self.timeoutStringChoices, command=self.set_timeoutSeconds)
118         self.timeoutMenu.grid(row=4, column=1, sticky='W', padx=10)
119         self.timeoutString.set(self.timeoutStringChoices[5])
120         self.set_timeoutSeconds()
121
122         #  Display warning text stating that the software is not approved for medical
```

```
            use.
123         self.warningText = """This software is intended for use in epidemiological
            studies only.
124         It is *not* approved as a medical device and must not be used as such.
125         That means the software must not be used on human beings for diagnosis,
            prevention,
126         monitoring, prediction, prognosis, treatment or alleviation of disease or any
            other medical purposes.\n
127         By clicking 'RECEIVE DATA' below you are agreeing not to use the software as a
            medical device.
128         """
129         self.warningContent = tk.StringVar()
130         self.warningContent.set(self.warningText)
131         self.warningTextBox = ttk.Label(root, justify='center',
            textvariable=self.warningContent)
132         self.warningTextBox.grid(row=6, column=0,columnspan=3, pady=0)
133
134         #  Button that has to be clicked to start transmission.
135         self.startButton = ttk.Button(self.master, text='RECEIVE DATA',
            command=self.recordData)
136         self.startButton.grid(row=7, column=0,columnspan=3, pady=(5,15))
137
138     ##  REGISTER WHETHER THERE IS AN OPEN ERROR BOX REPORTING THAT THE SERIAL
        CONNECTION COULD NOT BE OPENED.
139     def registerComportConnectionErrorboxState(self, state):
140         self.comportConnectionErrorboxOpen = state
141
142     ##  SELECT WHICH COMPORT TO USE
143     def select_comport(self, longComportName):
144         try:
145             self.comportLongName.set(longComportName)
146             self.comportShortName = self.comportsDict[self.comportLongName.get()]
147         except KeyError:
148             self.after(2500, self.update_serial_port_list)
149
150     ##  GET AN ALPHABETICALLY SORTED LIST OF AVAILABLE SERIAL PORTS.
151     def list_serial_ports(self):
152         try:
153             comports = list(serial.tools.list_ports.comports(include_links=False))
154             if len(comports) != 0:
155                 self.comportsDict = {}
156                 for comport in comports:
157                     self.comportsDict.update({comport[1]:comport[0]})
158             else:
159                 self.comportsDict = {'[NO SERIAL PORTS AVAILABLE]':'-'}
160         except:
161             self.comportsDict = {'[NO SERIAL PORTS AVAILABLE]':'-'}
162         self.comportsLongNames = sorted(self.comportsDict)
163
164     ##  UPDATE THE LIST OF SERIAL PORTS EVERY 2.5 SECONDS AND SHOW AN ERROR BOX IF THE
        SELECTED SERIAL PORT BECOMES UNAVAILABLE.
165     def update_serial_port_list(self):
166         self.list_serial_ports()
167         previouslySelectedComport = self.comportLongName.get()
168         menu = self.comportMenu["menu"]
169         menu.delete(0,"end")
170         for string in self.comportsLongNames:
171             menu.add_command(label=string, command=lambda value=string:
            self.select_comport(value))
172         if previouslySelectedComport != '' and previouslySelectedComport != '[NO SERIAL
            PORTS AVAILABLE]':
173             previousComportAmongCurrentPorts =
            self.check_connection_comport(previouslySelectedComport)
174             if not previousComportAmongCurrentPorts:
175                 if not self.comportConnectionErrorboxOpen:
176                     self.serial_port_connection_lost_error(previouslySelectedComport)
177             else:
```

```
178                 self.select_comport(previouslySelectedComport)
179             else:
180                 self.select_comport(self.comportsLongNames[0])
181             self.after(2500, self.update_serial_port_list)
182 
183         ##  CHECK IF THE SELECTED SERIAL PORT HAS BECOME UNAVAILABLE.
184         def check_connection_comport(self, longComportName):
185             comports = list(serial.tools.list_ports.comports(include_links=False))
186             comportFound = False
187             for comport in comports:
188                 if comport[1] == longComportName:
189                     comportFound = True
190             self.select_comport(self.comportsLongNames[0])
191             return comportFound
192 
193         ##  ERROR BOX: THE SELECTED SERIAL PORT HAS BECOME UNAVAILABLE
194         def serial_port_connection_lost_error(self, longComportName):
195             warningMessage = 'The serial port "' + longComportName + '" has been
                disconnected.\r\nPlease check the connection or use another serial port.'
196             tk.messagebox.showwarning("Connection lost",warningMessage,
                command=self.registerComportConnectionErrorboxState(True))
197             self.registerComportConnectionErrorboxState(False)
198 
199         ##  ERROR BOX: NO SERIAL PORT AVAILABLE.
200         def serial_port_missing_error(self):
201             tk.messagebox.showerror("No connection","No serial port availabe for data
                capture.\r\nPlease connect to a serial port and try again.")
202 
203         ##  ERROR BOX: THE SELECTED SERIAL PORT IS CONNECTED, BUT COULD NOT BE OPENED.
204         def serial_port_could_not_open_error(self, longComportName):
205             errorMessage = 'The serial port "' + longComportName + '" could not be
                opened.\r\nPlease close any other programs that might be using this serial
                port, and try again.'
206             tk.messagebox.showerror("Serial port error",errorMessage)
207 
208         ##  ERROR BOX: NO FILENAME SPECIFIED
209         def output_filename_missing_error(self):
210             tk.messagebox.showerror("Missing filename","Please enter a filename.")
211 
212         ##  DIALOG FOR SPECIFYING FILENAME FOR OUTPUT DATA.
213         def save_as_filename(self):
214 
215             #   If no filename has yet been defined (i.e., this is the first time the
                dialog is opened), seed the function.
216             if len(self.outputFilename) == 0:
217                 if self.firstTimeExecuted == True:
218                     self.initialDir = r'\\'
219                 initialFile = ''
220                 possibleFiletypes = self.tabularFiletypes
221 
222             #   If a filename also already been defined (i.e., this is not the first time
                that the dialog has been opened), take the old information into account when
                opening the dialog.
223             else:
224                 #   The initial folder should be the same as the folder that the user
                    navigated to the last time (unless it no longer exists).
225                 self.initialDir = path.dirname(self.outputFilename)
226                 if not path.isdir(self.initialDir):
227                     self.initialDir = r'\\'
228 
229                 #   Re-use the old filename
230                 initialFile = path.basename(self.outputFilename)
231 
232                 #   Re-arrange the list of filetypes so that the previously selected
                    filetype is the first item (meaning it will be pre-selected in the
                    drop-down menu).
233                 possibleFiletypes = self.tabularFiletypes
```

```
234             oldExtension = list(path.splitext(initialFile))[1]
235             extensionIndex = -1
236             for index in range(len(possibleFiletypes)):
237                 if possibleFiletypes[index][1] == '*' + oldExtension:
238                     extensionIndex = index
239             if extensionIndex == -1:
240                 for index in range(len(possibleFiletypes)):
241                     if possibleFiletypes[index][1] == '*.*':
242                         extensionIndex = index
243             firstFiletype = possibleFiletypes.pop(extensionIndex)
244             possibleFiletypes.insert(0, firstFiletype)
245 
246         #   Set the default extension.
247         if possibleFiletypes[0][1] != '*.*':
248             defaultExtension = possibleFiletypes[0][1]
249         else:
250             defaultExtension = ''
251 
252         #   Open the dialog, parse the filename that was input and display it on the
            settings screen.
253         f = filedialog.asksaveasfilename(initialdir = self.initialDir, initialfile =
            initialFile, title = "Save as", filetypes = possibleFiletypes,
            defaultextension=defaultExtension)
254         if f is not None and f != '':
255             self.setOutputFilename(f)
256 
257     ##  SET THE OUTPUT FILENAME AND DISPLAY IT IN THE APPROPRIATE BOX.
258     def setOutputFilename(self, outputFilename):
259         self.outputFilename = outputFilename
260         self.outputFilenameLabel.config(state='NORMAL')
261         self.outputFilenameLabel.delete(0,'end')
262         self.outputFilenameLabel.insert(0,self.outputFilename)
263         self.outputFilenameLabel.config(state='readonly')
264 
265     ##  SET THE CONNECTION TIMEOUT IN SECONDS.
266     def set_timeoutSeconds(self, dummy='dummy'):
267         timeoutNumber = int(self.timeoutString.get().split(' ')[0])
268         if timeoutNumber == 30:
269             self.timeoutSeconds = timeoutNumber
270         else:
271             self.timeoutSeconds = timeoutNumber*60
272 
273     ##  Complain if necessary modules are missing.
274     def modules_not_found_error(self):
275         tk.messagebox.showerror("Missing modules","One or more of the following
            necessary modules were not found:\n\npyserial\nXlsxWriter\nxlwt\n\nPlease close
            the program and install all of these modules before restarting.\nThe easiest
            way to perform the installation is to execute the script 'src/setup.py'.")
276 
277     ##  OPEN THE WINDOW FOR RECORDING/PARSING DATA FROM DEVICE AND WRITING IT TO A FILE.
278     def recordData(self):
279 
280         #   Prepare variables
281         comportShortName = self.comportShortName
282         comportLongName = self.comportLongName.get()
283         timeoutSeconds = self.timeoutSeconds
284         outputFilename = self.outputFilename
285         tabularFiletypes = self.tabularFiletypes
286 
287         #   Complain if necessary modules are missing.
288         if modulesNotFound == True:
289             self.modules_not_found_error()
290 
291         #   Complain if no serial port is available.
292         elif comportShortName == '-':
293             self.serial_port_missing_error()
294 
```

```
295             #   Complain if no filename has been entered.
296             elif outputFilename == '':
297                 self.output_filename_missing_error()
298             else:
299                 #   Try to open the serial port.
300                 try:
301                     serReceiver = serial.Serial(port=comportShortName, baudrate=9600,
                        bytesize=serial.EIGHTBITS, parity=serial.PARITY_NONE,
                        stopbits=serial.STOPBITS_ONE, timeout=None)
302
303                 #   Complain if the serial port cannot be opened.
304                 except serial.serialutil.SerialException:
305                     comportConnected = self.check_connection_comport(comportLongName)
306                     if comportConnected:
307                         self.serial_port_could_not_open_error(comportLongName)
308                     else:
309                         self.serial_port_connection_lost_error(comportLongName)
310                     return
311
312                 #   Set custom buffer size to avoid losing data during transmission. 200kB
                    should always be sufficient.
313                 serReceiver.set_buffer_size(rx_size = 204800, tx_size = 204800)
314
315                 #   During data download, a custom dialog box is displayed if there is a
                    problem with the serial connection.
316                 #   We make sure that this is the only error message that can be displayed
                    during data download.
317                 self.registerComportConnectionErrorboxState(True)
318
319                 #   Open the window for downloading and processing data.
320                 s = dataProcessingWindow(self, 'MyTest', comportShortName, comportLongName,
                    timeoutSeconds, serReceiver, outputFilename, tabularFiletypes)
321
322                 #   Wait for the window to close.
323                 self.master.wait_window(s)
324
325                 #   When the window has closed, re-able the settings window's ability to
                    display error messages about the serial connection.
326                 self.registerComportConnectionErrorboxState(False)
327
328                 #   Clear the output filename to avoid overwriting data by mistake.
329                 self.firstTimeExecuted = False
330                 self.initialDir = path.dirname(self.outputFilename)
331                 if not path.isdir(self.initialDir):
332                     self.initialDir = r'\\'
333                 self.setOutputFilename('')
334
335         ##  OPEN HELP WINDOW
336         def showHelpBox(self,helpType):
337             s = helpWindow(self, 'MyTest', helpType)
338             self.master.wait_window(s)
339
340     ##  WINDOW FOR RECORDING/PARSING DATA AND WRITING FILE.
341     class dataProcessingWindow(simpledialog.Dialog):
342
343         ## INITIALIZE THE WINDOW.
344         def __init__(self, parent, name, comportShortName, comportLongName, timeoutSeconds,
            serReceiver, outputFilename, tabularFiletypes):
345             tk.Toplevel.__init__(self, master=parent)
346             self.name = name
347             self.length = 400
348
349             #   Grab important variables.
350             self.comportShortName = comportShortName
351             self.comportLongName = comportLongName
352             self.timeoutSeconds = timeoutSeconds
353             self.serReceiver = serReceiver
```

```
354             self.outputFilename = outputFilename
355             self.tabularFiletypes = tabularFiletypes
356 
357             #   Create window and widgets.
358             self.create_window()
359             self.create_widgets()
360 
361         #   Create window.
362         def create_window(self):
363             #   Set focus on the new window.
364             self.focus_set()
365 
366             #   Make sure that focus stays on the new window.
367             self.grab_set()
368 
369             #   Only show one window in the taskbar.
370             self.transient(self.master)
371 
372             #   Configure layout and position over parent window.
373             self.title(u'Download data')
374             self.resizable(False, False)
375             dx = (self.master.master.winfo_width() >> 1) - (self.length >> 1)
376             dy = (self.master.master.winfo_height() >> 1) -75
377             self.geometry(u'+{x}+{y}'.format(x = self.master.winfo_rootx() + dx,
378                                              y = self.master.winfo_rooty() + dy))
379 
380             # No matter how the window is destroyed, we will shut down cleanly using the
                function self.close()
381             self.protocol(u'WM_DELETE_WINDOW', self.cancelTransfer)
382 
383             #   If the user presses the ESCAPE button, the process will cancel.
384             self.bind(u'<Escape>', self.cancelTransfer)
385 
386         #   Create widgets.
387         def create_widgets(self):
388             self.bytesReceivedString = tk.StringVar()
389             self.countdownString = tk.StringVar()
390             self.secondsPassed = 0
391             self.timeUntilTimeout = self.timeoutSeconds
392             self.progressbarValue = tk.IntVar()
393             self.timeoutTimeUnit = tk.StringVar()
394             self.maximum = self.timeoutSeconds
395             self.bytesReceived = 0
396             self.binaryBuffer = b''
397 
398             ttk.Label(self, textvariable=self.bytesReceivedString).pack(anchor='w',
                padx=10, pady=10)
399             self.progress = ttk.Progressbar(self, maximum=self.maximum, orient='horizontal',
400                                             length=self.length,
                                                variable=self.progressbarValue,
                                                mode='determinate')
401             self.progress.pack(padx=10)
402             ttk.Label(self, textvariable=self.countdownString).pack(side='left',
                padx=(10,0), pady=10)
403             ttk.Button(self, text='Cancel', command=self.cancelTransfer).pack(anchor='e',
                padx=(0,10), pady=10)
404             #
405             self.waitOneSecond()
406 
407         ## ENTER A ONE-SECOND LOOP, RECORDING DATA. AT THE SAME TIME, DISPLAY A PROGRESSBAR
            OF THE TIME TO CONNECTION TIMEOUT.
408         def waitOneSecond(self):
409             try:
410                 #   Attempt to read data from the serial port.
411                 self.getSerialData()
412 
413                 #   Update the progress bar. Ignore single bytes of FF (hexadecimal,
```

```
                control character sent by HbA1c 501 device when cable is connected).
414             if self.binaryStringReceived == b'' or self.binaryStringReceived == b'\xff':
415                 self.bytesReceivedString.set('Number of bytes received: ' +
                    str(self.bytesReceived))
416                 self.secondsPassed += 1
417                 self.timeUntilTimeout = self.timeoutSeconds-self.secondsPassed
418                 if self.binaryBuffer == b'':
419                     self.progressbarValue.set(self.timeUntilTimeout)
420                     countdownString = 'Waiting. Timeout in '
421                     minutesLeft = self.timeUntilTimeout // 60
422                     secondsLeft = self.timeUntilTimeout - 60*minutesLeft
423                     if minutesLeft > 0:
424                         countdownString += str(minutesLeft) + ' minute'
425                         if minutesLeft > 1:
426                             countdownString += 's'
427                         countdownString += ' and '
428                     countdownString += str(secondsLeft) + ' second'
429                     if secondsLeft > 1 or secondsLeft == 0:
430                         countdownString += 's'
431                     countdownString += '.'
432                     self.countdownString.set(countdownString)
433             else:
434                 self.bytesReceivedString.set('Number of bytes received: ' +
                    str(self.bytesReceived))
435                 self.countdownString.set('Transmission in progress. Countdown halted.')
436                 if self.binaryBuffer == self.binaryStringReceived:
437                     self.progress.configure(mode='indeterminate')
438                     self.progress.start()
439                 self.secondsPassed = self.timeoutSeconds - 6
440
441             #   If the countdown is not complete, take another round in the loop after
                1 second.
442             if self.timeUntilTimeout > 0:
443                 self.after(1000, self.waitOneSecond)
444
445             #   When the connection times out, close the progress window.
446             else:
447                 self.close()
448
449                 #   If no data was received, give the user an error message.
450                 if self.binaryBuffer == b'':
451                     self.connectionTimedOutError()
452
453                 #   If data was recived, process it.
454                 else:
455                     self.saveHbA1cData()
456
457         #   In case of serial port communication errors, close the progress window and
            display an error message.
458         except serial.serialutil.SerialException:
459             self.close()
460             self.serialPortCommError()
461
462     ##  FUNCTION TO READ DATA FROM SERIAL PORT
463     def getSerialData(self):
464         #   Bugfixing mode: If you set "self.bugfixing" to 'True' instead of 'False'
            and run the program with a python terminal open,
465         #   the program will request a path to a binary dump file containing HbA1c
            data. The bytestring in this file will then
466         #   be treated as if it had been received over serial. Furthermore, the program
            will print each row of data as it is
467         #   parsing it.
468         self.bugfixing = False
469
470         if self.bugfixing == False:
471             self.binaryStringReceived =
                self.serReceiver.read(self.serReceiver.in_waiting)
```

```
472
473             if self.bugfixing == True:
474                 if self.binaryBuffer == b'':
475                     bugfixingDataSource = ''
476                     while bugfixingDataSource == '' or not path.isfile(bugfixingDataSource):
477                         bugfixingDataSource = input("\nBUGFIXING MODE: Please write path to
                            binary file containing dump of HbA1c data.\n")
478                         bugfixingDataSource = bugfixingDataSource.strip('\"')
479                     with open(bugfixingDataSource,'rb') as f:
480                         self.binaryStringReceived = f.read()
481                 else:
482                     self.binaryStringReceived = b''
483
484             if self.binaryStringReceived != b'\xff':
485                 self.bytesReceived += len(self.binaryStringReceived)
486                 self.binaryBuffer += self.binaryStringReceived
487
488         ##  ERROR BOX: SERIAL CONNECTION ERROR.
489         def serialPortCommError(self):
490             warningMessage = ('Data transmission was interrupted due to a problem with the
                serial port "' + self.comportLongName + '".\r\n\r\n')
491             if self.binaryBuffer != b'':
492                 warningMessage += 'Do you want to save a copy of the raw binary data so you
                    can process it manually?'
493                 saveBinaryDataAnswer =
                    tk.messagebox.askyesno("Error",warningMessage,icon='error',default='yes')
494                 if saveBinaryDataAnswer == True:
495                     self.saveBinaryData()
496             else:
497                 warningMessage += 'No data was received before the interruption.'
498                 errorBox = tk.messagebox.showerror("Error",warningMessage)
499
500         ##  FUNCTION EXECUTED IF THE USER CANCELS DATA RECORDING.
501         def cancelTransfer(self, dummy='dummy'):
502             self.close()
503             if self.binaryBuffer != b'':
504                 title = 'Cancelled'
505                 question = ('Operation cancelled by user.\r\n\r\nSave a copy of the raw
                    binary data that had already been received?')
506                 saveBinaryDataAnswer = tk.messagebox.askyesno(title,question,default='yes')
507                 if saveBinaryDataAnswer == True:
508                     self.saveBinaryData()
509
510         ##  ERROR BOX: CONNECTION TIMED OUT
511         def connectionTimedOutError(self):
512             warningMessage = 'Connection timed out before any data was received.'
513             tk.messagebox.showerror("Timeout",warningMessage)
514
515         ##  CLOSE THE WINDOW CONTAINING THE PROGRESS BAR, AND CLOSE THE SERIAL CONNECTION.
516         def close(self, event=None):
517             self.serReceiver.close()
518             self.master.focus_set()  # put focus back to the parent window
519             self.destroy()  # destroy progress window
520
521         ##  CHECK THE INTEGRITY OF THE BINARY DATA RECORDED.
522         def checkDataIntegrity(self):
523             #   Get the string representation of the bytes.
524             receivedData = str(self.binaryBuffer)
525
526             #   Remove the leading 'b'.
527             try:
528                 receivedData = receivedData[2:len(receivedData)-1]
529             except IndexError:
530                 self.setUnknownDataStructure()
531                 return
532
533             #   Split the string into list items, based on the occurrrence of the string
```

```
            'Data No.:'.
534         self.receivedDataRows = receivedData.split(r'Data No.: ')
535
536         #   Now split based on line shifts.
537         rowCounter = 0
538         for row in self.receivedDataRows:
539             self.receivedDataRows[rowCounter] =
            str(self.receivedDataRows[rowCounter]).split(r'\r\n')
540             rowCounter += 1
541
542         #   If the data is from a HemoCue® HbA1c 501 device, it *must* contain the
            string 'HEMOCUE HbA1c 501' or 'HEMOCUE HbA1c501' (depending on whether it
            printed a single or multiple pieces of data).
543         try:
544             if ('HEMOCUE HbA1c 501' in self.receivedDataRows[0][1]) or ('HEMOCUE
                HbA1c501' in self.receivedDataRows[0][1]):
545                 self.dataType = 'HbA1c'
546             else:
547                 self.setUnknownDataStructure()
548                 return
549         except IndexError:
550             self.setUnknownDataStructure()
551             return
552
553         #   If transmission is complete, it will end with binary string b'\x1bd\x03'
554         lastRow = self.receivedDataRows[len(self.receivedDataRows)-1]
555         lastItem = lastRow[len(lastRow)-1]
556         if lastItem == r'\x1bd\x03':
557             self.transmissionCompleted = True
558         else:
559             self.transmissionCompleted = False
560
561         #   Detect if transmission was briefly interrupted and then resumed before the
            interruption could be detected, resulting in data that cannot be parsed (or
            results in invalid characters).
562         try:
563             self.parseHbA1cData()
564             for row in self.parsedHbA1cData[1:]:
565                 for cell in row:
566                     if (r'\x' in cell) and (r'\\x' not in cell):
567                         self.transmissionCompleted = False
568                 self.cellCounter = -3
569                 for cell in row[-2:]:
570                     self.cellCounter += 1
571                     try:
572                         float(cell)
573                     except ValueError:
574                         if not ((self.cellCounter == -2 and cell == '<4') or
                            (self.cellCounter == -2 and cell == '>14') or (self.cellCounter
                            == -1 and cell == '')):
575                             self.transmissionCompleted = False
576                 try:
577                     int(row[0])
578                 except ValueError:
579                     self.transmissionCompleted = False
580
581         except IndexError:
582             self.transmissionCompleted = False
583
584         #   Detect if the number of observations is consistent with the values listed
            in "Data no."
585         #   If there is an inconsistency, it is because there was some loss of data
            during transmission, but by chance it resulted in data that could still be
            parsed.
586         if self.transmissionCompleted == True:
587             try:
588                 lowestDataNo = int(self.parsedHbA1cData[1][0])
```

```
589                 highestDataNo = int(self.parsedHbA1cData[len(self.parsedHbA1cData)-1][0])
590                 if not (len(self.parsedHbA1cData) - 1 == highestDataNo - lowestDataNo +
                    1):
591                     self.transmissionCompleted = False
592                 if self.bugfixing == True:
593                     print('\n\nNumber of observations in binary data received
                        =',len(self.parsedHbA1cData) - 1)
594                     print('Expected number of observations, based on values listed in
                        "Data no." =',highestDataNo - lowestDataNo + 1)
595             except ValueError:
596                 self.transmissionCompleted = False
597
598     ##  IF THE BINARY DATA RECEIVED HAS AN UNKNOWN STRUCTURE, RECORD THIS.
599     def setUnknownDataStructure(self):
600         self.dataType = 'unknown'
601         self.transmissionCompleted = None
602         self.receivedDataRows = None
603
604     ##  PARSE THE BINARY DATA SO THAT IT BECOMES A WELL-DEFINED LIST OF LISTS
        (CORRESPONDING TO A MATRIX DATASET OF ROWS AND COLUMNS).
605     def parseHbA1cData(self):
606         self.parsedHbA1cData = []
607         for dataRow in self.receivedDataRows[1:]:
608             if self.bugfixing == True:
609                 print('\n\nNow parsing this row of data:\n',dataRow)
610
611             dataID = dataRow[0]
612             date = dataRow[1]
613             time = dataRow[2].replace('Time:','')
614
615             inputDateTimeFormat = ''
616             if r'[Y/M/D]' in date:
617                 inputDateTimeFormat += '%y/%m/%d'
618                 date = date.replace(r'[Y/M/D]','')
619             elif r'[M/D/Y]' in date:
620                 inputDateTimeFormat += '%m/%d/%y'
621                 date = date.replace(r'[M/D/Y]','')
622             elif r'[D/M/Y]' in date:
623                 inputDateTimeFormat += '%d/%m/%y'
624                 date = date.replace(r'[D/M/Y]','')
625
626             if ('AM' in time) or ('PM' in time):
627                 inputDateTimeFormat += ' %p %I:%M'
628             else:
629                 inputDateTimeFormat += ' %H:%M'
630
631             dateTimeString = re.sub(' +',' ', date + time)
632
633             try:
634                 dateTime = datetime.datetime.strptime(dateTimeString,
                    inputDateTimeFormat)
635
636                 dateTimeString = dateTime.strftime("%Y-%m-%dT%H:%M")
637                 hbA1cValues =
                    dataRow[4].replace('HbA1c','').replace('mmol/mol','').replace('
                    ','').split('%')
638                 hba1cPercent = hbA1cValues[0]
639                 hba1cMmol = hbA1cValues[1]
640
641                 #   If the data contains barcode information on operator ID, delete it
                    before extracting operator ID.
642                 if dataRow[7] != '':
643                     del dataRow[7]
644                 operatorID = dataRow[7].replace(r'\x00','')
645
646                 #   If the data contains barcode information on patient ID, delete it
                    before extracting patient ID.
```

```
647                 if dataRow[9] != '':
648                     del dataRow[9]
649                 patientID = dataRow[9].replace(r'\x00','')
650 
651 
                    self.parsedHbA1cData.append([dataID,dateTimeString,operatorID,patientID,h
                    ba1cPercent,hba1cMmol])
652             except:
653                 self.transmissionCompleted = False
654 
655         try:
656             self.parsedHbA1cData.sort(key=lambda number: number[0])
657         except:
658             self.transmissionCompleted = False
659 
            self.parsedHbA1cData.insert(0,['data_id','datetime','operator_id','patient_id','h
            ba1c_percent','hba1c_mmol_per_mol'])
660 
661     ##  ERROR BOX: THE DATA HAS UNKNOWN STRUCTURE.
662     def unknownDataStructureError(self):
663         title = 'Unknown data format'
664         question = ('Cannot decode the data received. Either an error happened during
            transmission, ' +
665                     'or the data is not from a HemoCue® HbA1c device.\r\n\r\n' +
666                     'Do you want to save a copy of the raw binary data so you can
                    process it manually?')
667         saveBinaryDataAnswer =
            tk.messagebox.askyesno(title,question,default='yes',icon='error')
668         if saveBinaryDataAnswer == True:
669             self.saveBinaryData()
670 
671     ##  ERROR BOX: DATA TRANSMISSION WAS INCOMPLETE.
672     def incompleteTransmissionError(self):
673         title = 'Transmission incomplete'
674         question = ('Data transmission was interrupted, probably because the HemoCue®
            HbA1c 501 device lost power, ' +
675                     'or because of a problem with the serial cable.\r\n\r\n' +
676                     'Do you want to save a copy of the raw binary data so you can
                    process it manually?')
677         saveBinaryDataAnswer =
            tk.messagebox.askyesno(title,question,default='yes',icon='error')
678         if saveBinaryDataAnswer == True:
679             self.saveBinaryData()
680 
681     ##  WRITE DATA (AS EITHER TABULAR OR BINARY DATA).
682     def outputFileWriter(self,mode='tabular'):
683 
684         #   Tabular data
685         if mode == 'tabular':
686 
687             #   Excel format
688             if list(path.splitext(self.outputFilename))[1] == '.xlsx':
689                 #   Find number of columns.
690                 numberOfColumns = 0
691                 for row in self.parsedHbA1cData:
692                     if len(row) > numberOfColumns:
693                         numberOfColumns = len(row)
694 
695                 #   Create a dictionary of column numbers and the minimum column width
                    in characters.
696                 columnNumberOfChars = {}
697                 for columnNumber in range(numberOfColumns):
698                     columnNumberOfChars.update({columnNumber:1})
699 
700                 #   Open workbook
701                 workbook = xlsxwriter.Workbook(self.outputFilename)
702                 worksheet = workbook.add_worksheet()
```

```
703                 rowNumber = 0
704                 colNumber = 0
705
706                 #   Loop over the HbA1c data and write it to the cells of the
                    worksheet. When a cell with contents wider than the minimum column
                    width is encountered, update the mimimum width to accomodate the content.
707                 for row in self.parsedHbA1cData:
708                     for cellContent in row:
709                         worksheet.write(rowNumber, colNumber, cellContent)
710                         if len(cellContent) > columnNumberOfChars[colNumber]:
711                             columnNumberOfChars[colNumber] = len(cellContent)
712                         colNumber += 1
713                     colNumber = 0
714                     rowNumber += 1
715
716                 #   Write the column widths to the file.
717                 for columnNumber in range(len(columnNumberOfChars)):
718                     worksheet.set_column(columnNumber, columnNumber,
                        columnNumberOfChars[columnNumber]+2)
719
720                 #   Close the Excel file.
721                 workbook.close()
722
723             #   Excel 97-2003 format
724             elif list(path.splitext(self.outputFilename))[1] == '.xls':
725                 #   Find number of columns.
726                 numberOfColumns = 0
727                 for row in self.parsedHbA1cData:
728                     if len(row) > numberOfColumns:
729                         numberOfColumns = len(row)
730
731                 #   Create a dictionary of column numbers and the minimum column width
                    in characters.
732                 columnNumberOfChars = {}
733                 for columnNumber in range(numberOfColumns):
734                     columnNumberOfChars.update({columnNumber:1})
735
736                 #   Open workbook.
737                 oldWorkbook = xlwt.Workbook()
738                 sheet1 = oldWorkbook.add_sheet("Sheet1")
739
740                 #   Loop over the HbA1c data and write it to the cells of the
                    worksheet. When a cell with contents wider than the minimum column
                    width is encountered, update the mimimum width to accomodate the content.
741                 for rowNumber in range(len(self.parsedHbA1cData)):
742                     row = sheet1.row(rowNumber)
743                     for columnNumber in range(len(self.parsedHbA1cData[rowNumber])):
744
                            row.write(columnNumber,self.parsedHbA1cData[rowNumber][columnNumb
                            er])
745                         if len(self.parsedHbA1cData[rowNumber][columnNumber]) >
                            columnNumberOfChars[columnNumber]:
746                             columnNumberOfChars[columnNumber] =
                                len(self.parsedHbA1cData[rowNumber][columnNumber])
747
748                 #   Write the column widths to the file.
749                 for columnNumber in range(len(columnNumberOfChars)):
750                     sheet1.col(columnNumber).width =
                        (columnNumberOfChars[columnNumber]+2) * 256
751
752                 #   Close the Excel file.
753                 oldWorkbook.save(self.outputFilename)
754
755             #   Tab-Separated Values
756             elif list(path.splitext(self.outputFilename))[1] == '.tsv':
757                 with open(self.outputFilename, 'w', newline='') as f:
758                     writer = csv.writer(f, delimiter='\t')
```

```
759                     writer.writerows(self.parsedHbA1cData)
760
761             #   Comma-Separated Values (if the user specifies an unknown file
                extension, data is also saved as CSV).
762             else:
763                 with open(self.outputFilename, 'w', newline='') as f:
764                     writer = csv.writer(f)
765                     writer.writerows(self.parsedHbA1cData)
766
767         #   Raw binary data
768         else:
769             with open(self.outputFilename,'bw') as f:
770                 f.write(self.binaryBuffer)
771
772     ##  DIALOG BOX: DATA SUCCESSFULLY WRITTEN TO DISK.
773     def fileSuccessfullyWritten(self):
774         myMessage = 'Successfully saved data in the file "' + self.outputFilename + '"'
775         tk.messagebox.showinfo('Data saved',myMessage)
776
777     ##  DIALOG BOX [USED IF WE CANNOT WRITE TO THE FILE THAT THE USER SPECIFIED]: ASK
        THE USER IF THEY WANT TO SAVE TO A DIFFERENT FILE.
778     def doesUserStillWantToSaveData(self):
779         title = 'Error'
780         question = ('Could not save to the file "' + self.outputFilename +
781                     '".\r\n\r\n' +
782                     'Do you want to save to a different file?')
783         self.userWantsToSaveData =
            tk.messagebox.askyesno(title,question,default='yes',icon='error')
784
785     ##  DIALOG FOR SPECIFYING A NEW FILENAME, IN CASE WE CAN'T WRITE TO THE ONE
        SPECIFIED BY THE USER.
786     def defineNewOutputFilename(self, mode='tabular', firstTime=False):
787         #   Define the initial directory for the dialog box.
788         initialDir = path.dirname(self.outputFilename)
789         if not path.isdir(initialDir):
790             initialDir = '\\'
791
792         #   Get list of the filetypes that the user can choose (depending on whether
            we're saving tabular or binary data), and
793         if mode == 'tabular':
794             possibleFiletypes = self.tabularFiletypes
795         else:
796             possibleFiletypes = [("Binary files", "*.bin"),('All files','*.*')]
797
798         #   Do the following, unless it is the first time asking for a filename for a
            binary file:
799         if not (mode=='binary' and firstTime==True):
800             #   Re-use the old filename.
801             initialFile = path.basename(self.outputFilename)
802
803             #   Re-arrange the list of filetypes so that the previously selected
                filetype is the first item (meaning it will be pre-selected in the
                drop-down menu).
804             oldExtension = list(path.splitext(initialFile))[1]
805             extensionIndex = -1
806             for index in range(len(possibleFiletypes)):
807                 if possibleFiletypes[index][1] == '*' + oldExtension:
808                     extensionIndex = index
809             if extensionIndex == -1:
810                 for index in range(len(possibleFiletypes)):
811                     if possibleFiletypes[index][1] == '*.*':
812                         extensionIndex = index
813             firstFiletype = possibleFiletypes.pop(extensionIndex)
814             possibleFiletypes.insert(0, firstFiletype)
815         #   If we are asking for a binary file name for the first time, do this instead:
816         else:
817             initialFile = ''
```

```
818
819             #   Set the default extension.
820             if possibleFiletypes[0][1] != '*.*':
821                 defaultExtension = possibleFiletypes[0][1]
822             else:
823                 defaultExtension = ''
824
825             #   Open the dialog, parse the filename that was input and change output
                filename as appropriate.
826             f = filedialog.asksaveasfilename(initialdir = initialDir, initialfile =
                initialFile, title = "Save as", filetypes = possibleFiletypes,
                defaultextension=defaultExtension)
827             if f is None or f == '':
828                 self.userWantsToSaveData = False
829             else:
830                 self.outputFilename = f
831
832         ##  WRAPPER FUNCTION TO TEST THE INTEGRETY OF THE HBA1C DATA, PARSE IT AND WRITE IT
            TO DISK.
833         def saveHbA1cData(self):
834             self.checkDataIntegrity()
835             if self.dataType == 'unknown':
836                 self.unknownDataStructureError()
837             elif self.transmissionCompleted == False:
838                 self.incompleteTransmissionError()
839             else:
840                 self.userWantsToSaveData = True
841                 self.saveOrAskForFilenameLoop(mode='tabular')
842
843         ##  WRITE RAW BINARY DATA TO DISK.
844         def saveBinaryData(self):
845             self.userWantsToSaveData = True
846             self.defineNewOutputFilename(mode='binary', firstTime=True)
847             self.saveOrAskForFilenameLoop(mode='binary')
848
849         ##  ENDLESS LOOP ASKING FOR A NEW FILENAME UNTIL IT IS EITHER POSSIBLE TO WRITE TO
            DISK, OR THE USER GIVES UP.
850         def saveOrAskForFilenameLoop(self, mode='tabular'):
851             while self.userWantsToSaveData == True:
852                 try:
853                     self.outputFileWriter(mode=mode)
854                     self.fileSuccessfullyWritten()
855                     self.userWantsToSaveData = False
856                 except (PermissionError, FileNotFoundError):
857                     self.doesUserStillWantToSaveData()
858                     if self.userWantsToSaveData == True:
859                         self.defineNewOutputFilename(mode=mode, firstTime=False)
860
861     ##  WINDOW FOR DISPLAYING HELP INFORMATION (INSTRUCTIONS FOR USE, ABOUT OR LICENSE INFO).
862     class helpWindow(simpledialog.Dialog):
863
864         ## INITIALIZE THE WINDOW.
865         def __init__(self, parent, name, helpType):
866             tk.Toplevel.__init__(self, master=parent)
867             self.name = name
868
869             #   Grab important variable.
870             self.helpType = helpType
871
872             #   Create window and widgets.
873             self.create_window()
874             self.create_widgets()
875
876         #   Create window.
877         def create_window(self):
878             #   Set focus on the new window.
879             self.focus_set()
```

```
880
881             #   Make sure that focus stays on the new window.
882             self.grab_set()
883
884             #   Only show one window in the taskbar.
885             self.transient(self.master)
886
887             #   Configure layout and position over parent window.
888             self.helpBoxTitle = "HemoDownloader 1.1 - " + self.helpType
889             self.title(self.helpBoxTitle)
890             self.resizable(False, False)
891             self.geometry(u'+{x}+{y}'.format(x = self.master.winfo_rootx(),
892                                              y = self.master.winfo_rooty()))
893
894             # Load text to be inserted into the box.
895             if self.helpType == "Instructions for use":
896                 self.text_to_be_inserted =  root.help_text
897             elif self.helpType == "About HemoDownloader":
898                 self.text_to_be_inserted =  root.about_text
899             elif self.helpType == "License information":
900                 self.text_to_be_inserted = root.license_text
901
902             # Calculate height of text box.
903             self.textbox_height = self.text_to_be_inserted.count('\n') + 1
904             if self.textbox_height > 35:
905                 self.textbox_height = 35
906
907             # Draw text box ith contents.
908             self.textField = tk.Text(self,wrap=tk.WORD, height=self.textbox_height,
                width=80, padx=15, pady=15)
909             self.textField.insert(tk.END, self.text_to_be_inserted)
910             self.textField.grid(row = 0, column=0, sticky='W')
911
912             # Configure scroll bar
913             self.scrollbar = tk.Scrollbar(self, command=self.textField.yview,
                orient=tk.VERTICAL)
914             self.scrollbar.config(command=self.textField.yview)
915             self.textField.configure(yscrollcommand=self.scrollbar.set)
916             self.scrollbar.grid(row = 0, column=1, sticky='NS')
917
918             #   If the user presses the ESCAPE button, the window will close.
919             self.bind(u'<Escape>', self.closeHelpWindow)
920
921         #   Creae a button for closing the window.
922         def create_widgets(self):
923             ttk.Button(self, text='Close', command=self.closeHelpWindow).grid(row=1,
                column=0,columnspan=2, pady=0)
924
925         ##  Function for closing the help box.
926         def closeHelpWindow(self, dummy='dummy'):
927             self.destroy()
928
929     ##  DEFINE MAIN WINDOW
930     root = tk.Tk()
931     feedback = settingsWindow(root)
932
933
934     ##   HELP TEXT
935     root.help_text ="""HemoDownloader 1.1
936     A GUI utility for downloading data from HemoCue® HbA1c 501 devices
937
938     This program allows biochemical data to be exported from the device HemoCue® HbA1c 501
        and saved in a structured database for further processing in statistical packages.
939
940     PLEASE NOTE THAT HEMODOWNLOADER IS INTENDED FOR USE IN EPIDEMIOLOGICAL STUDIES ONLY.
        THE SOFTWARE IS *NOT* APPROVED AS A MEDICAL DEVICE AND MUST NOT BE USED AS SUCH. THAT
        MEANS THE SOFTWARE MUST NOT BE USED ON HUMAN BEINGS FOR DIAGNOSIS, PREVENTION,
```

```
    MONITORING, PREDICTION, PROGNOSIS, TREATMENT OR ALLEVIATION OF DISEASE OR ANY OTHER
    MEDICAL PURPOSES.


How to transfer all results in device memory:
 1. Click 'RECEIVE DATA'.
 2. Connect HemoCue® HbA1c 501 device to computer using RS232 null modem cable.
 3. Turn on the device and wait for it to go into stand-by mode.
 4. Press 'MODE' button for 3 seconds.
 5. The device will display 'Set up' and 'Data'.
 6. Press 'DOWN' (▼) or 'UP' (▲) button to select 'Data'.
 7. Press 'MODE' button to display the results in memory.
 8. Press 'PRINTER' button.
 9. Using 'DOWN' (▼) or 'UP' (▲) button, select 'All'.
10. Press 'MODE' button.
11. Wait for data to be transferred to computer.


How to transfer individual results:
 1. Click 'RECEIVE DATA'.
 2. Connect HemoCue® HbA1c 501 device to computer using RS232 null modem cable.
 3. Turn on the device and wait for it to go into stand-by mode.
 4. Press 'MODE' button for 3 seconds.
 5. The device will display 'Set up' and 'Data'.
 6. Press 'DOWN' (▼) or 'UP' (▲) button to select 'Data'.
 7. Press 'MODE' button to display the results in memory.
 8. Scroll through test results using 'DOWN' (▼) and 'UP' (▲) buttons.
 9. When the desired record is shown, press 'PRINTER' button.
10. Using 'DOWN' (▼) or 'UP' (▲) button, select 'Current'.
11. Press 'MODE' button.
12. Wait for data to be transferred to computer."""

##   ABOUT TEXT
root.about_text = """HemoDownloader 1.1
A GUI utility for downloading data from HemoCue® HbA1c 501 devices

Copyright © 2018-2019 Martin Rune Hassan Hansen

To show software license, select 'Help' > 'License information'.


Contact information

email address: martinrunehassanhansen@ph.au.dk

Physical addresses:

  Section for Environment, Work and Health
  Att: Martin Rune Hassan Hansen
  Department of Public Health
  Aarhus University
  Bartholins Allé 2
  DK-8000 Aarhus C
  Denmark

  and

  The National Research Centre for the Working Environment
  Att: Martin Rune Hassan Hansen
  Lersø Parkallé 105
  DK-2100 København
  Denmark"""

##   LICENSE TEXT
root.license_text =
    """*****************************************************************************
*** HemoDownloader 1.1                                                      ***
```

```
********************************************************************************
```


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HemoDownloader uses the following third-party python modules that are covered by their own licenses listed below:
* pyserial
* XlsxWriter
* xlwt

The compiled versions of HemoDownloader 1.1 were created using the program PyInstaller that is distributed under a modified GPL license, also listed below.

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```
********************************************************************************
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********************************************************************************
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Version 3, 29 June 2007

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##   START THE MAIN GUI
root.mainloop()
```

Paper IV

Correlation between low red blood cell acetylcholine esterase and low blood glucose level in a population of small-scale Ugandan farmers: A short-term follow-up study

Hansen MRH, Jørs E, Sandbæk A, Sekabojja D, Ssempebwa J, Mubeezi R, Staudacher P, Fuhrimann S, Burdorf A, Bibby BM, Schlünssen V.

Submitted to Occupational and Environmental Medicine.

Paper IV has not been published yet and has therefore been removed from this version of the document.

Paper V

Organophosphate and carbamate insecticide exposure is related to decreased pulmonary function among smallholder farmers in Uganda: A short-term follow-up study

Hansen MRH, Jørs E, Sandbæk A, Sekabojja D, Ssempebwa J, Mubeezi R, Staudacher P, Fuhrimann S, Sigsgaard S, Burdorf A, Bibby BM, Schlünssen V.

Submitted to Thorax.

Paper V has not been published yet and has therefore been removed from this version of the document.

Paper VI

Pyridostigmine impairs pulmonary function in asthmatic subjects: Re-analysis of results from an observational study

Hansen MRH, Schlünssen V.

Submitted to Military Medicine.

Title

Pyridostigmine impairs pulmonary function in asthmatic subjects: Re-analysis of results from an observational study

Short title

Pyridostigmine impairs pulmonary function in asthmatic subjects

Keywords

Vital capacity; FVC; spirometry; pyridostigmine; cholinesterase inhibitors

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Pyridostigmine impairs pulmonary function in asthmatic subjects: Re-analysis of results from an observational study

Introduction

Compounds inhibiting the enzyme cholinesterase are used clinically (e.g., pyridostigmine[1]) and as insecticides (organophosphate and carbamate insecticides[2]), and other compounds have been used as chemical warfare agents (e.g., sarin[3]). Respiratory failure is a well-known feature of acute intoxication with cholinesterase-inhibiting insecticides[3] [4] and chemical warfare agents,[3] and epidemiological studies suggest that lower-dose exposure to cholinesterase-inhibiting insecticides may be associated with impaired lung function.[5]

Pyridostigmine is a reversible cholinesterase inhibitor that is used as prophylaxis against nerve agents.[1] *Military Medicine* has previously published an observational study on the pulmonary effects of pyridostigmine administration to both asthmatic and healthy subjects (Gouge *et al* 1994[1]). Gouge *et al* concluded that they "(…) found no changes in forced vital capacity in any of the soldiers, but observed exacerbation of asthma symptoms in seven of the asthmatics." We respectfully disagree with the statistical approach in their paper. In this paper, we briefly summarize the experimental setup of the study by Gouge *et al*, and using the data from the original study, we demonstrate that pyridostigmine does impair lung function among asthmatics, and that the change is both statistically and clinically significant.

Methods

Data collection has previously been described in detail by Gouge *et al*.[1] Briefly, sixteen American troops stationed in Saudi Arabia volunteered for a study on pulmonary function after pyridostigmine administration. The subjects included 10 asthmatics and 6 healthy soldiers. Forced expiratory volume (FVC) was measured at baseline (time 0). A 30 mg pyridostigmine tablet was administered to each subject, and FVC was measured again after 2, 4, 6 and 8 hours. FVC was presented as % of predicted, based on height and weight. The original paper contains the microdata from the experiment (see table 2 in Gouge *et al*[1]).

Gouge *et al* reported that "We found no consistent changes in VC in asthmatics or non-asthmatics and no differences between groups at any of the time intervals (all $p > 0.05$ by analysis of variance and unpaired *t* tests)."[1] However, an analysis of variance may not properly reflect the structure of the data, and may thus fail to demonstrate a true effect of pyridostigmine on the subjects. We used a mixed effect model of the following structure to analyze the data:

$$FVC = \beta_o + \beta_h \times h + \alpha + \varepsilon$$

where β_o is intercept, β_h is the effect of pyridostigmine h hours after the drug was administered (fixed effect), α is a random effect for subject ID, and ε is an error term. Each value of h (0, 2, 4, 6, 8) was modelled as a separate indicator variable, with 0 as the reference. We fitted this model for the entire study population, as well as stratified by disease status (asthmatics and healthy subjects). The analyses were performed using Stata 15 (StataCorp, College Station, Texas, USA). All output from the analyses are provided in online appendix 1, and the data and Stata syntax files can be found in online appendix 2.

Results and discussion

The mixed effect model showed that for the entire study population, FVC changed by -4.3 [-7.8; -0.9] % of predicted from baseline to 2 hours after pyridostigmine administration (Figure 1A). The point estimates indicated that the FVC did not return to baseline until 8 hours after the drug had been administered (Figure 1A).

Stratification of the analysis by asthma status clearly showed that pyridostigmine impaired FVC among asthmatics, but not among healthy subjects. Two hours after pyridostigmine administration, the asthmatic subjects had a mean FVC of -6.8 [-10.9; -2.8] %-points compared to baseline, and though the effect disappeared over the following 8 hours, the decrease was still statistically significant 4 and 6 hours after pyridostigmine (Figure 1B). In contrast, the FVC of the healthy subjects did not change 2 hours after pyridostigmine administration (mean difference from baseline -0.2 [-5.6; 5.3] %-points), and there was actually a small, statistically significant increase in FVC over the following six hours (Figure 1C).

As mentioned by Gouge *et al*,[1] the effects of pyridostigmine has also been tested in two blinded, randomized, placebo-controlled trials. One study did not find any differences in FEV_1 (forced expiratory volume in 1 second) and FVC after pyridostigmine 30 mg three times a day given to subjects who were either healthy or had mild asthma.[6] The other study showed that 60 mg pyridostigmine administered to healthy subjects caused a significant decrease in FEV_1 (but not in peak expiratory flow or FVC), and that the decrease in FEV_1 was strongly correlated with the degree of cholinesterase inhibition caused by the drug.[7] In the same study, spirometric indices did not change for asthmatics given 30 mg of pyridostigmine.[7]

The current re-analysis does not support the conclusions by Gouge *et al* that there was no objective change in lung function after pyridostigmine administration, nor their suggestion that subjective

symptoms among the subjects after pyridostigmine "could have been due to a placebo effect".[1] The results underline the need to exercise caution before administering pyridostigmine or other cholinesterase inhibitor drugs to asthmatics. Furthermore, they provide mechanistic support of the hypothesis that exposure to cholinesterase-inhibitor insecticides cause lung function impairment in humans,[5] a subject that cannot be investigated directly in an ethical manner, due to the high acute toxicity of these insecticides.

Conclusion

In contrast with the results in the original publication, we showed that administration of pyridostigmine 30 mg lowered FVC in asthmatic subjects, and the effect was both clinically and statistically significant. Such an effect was not demonstrated for healthy subjects. Caution should be exerted before prescribing pyridostigmine to asthmatics.

Funding

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Acknowledgements

None.

Conflicts of interest

None declared.

Ethics

We did not apply for ethical approval of the analyses. The anonymous microdata used were publically available.[1] In Denmark, ethical approval is not required for "Research projects that involve only anonymous biological material (…) which has been collected in compliance with applicable local legislation (…)"[8]

Data availability

All raw data and Stata syntax files used for the analyses in this paper are provided in online appendix 2.

Figure 1: Change in FVC after pyridostigmine administration

Figure 1A: All subjects (n = 16)

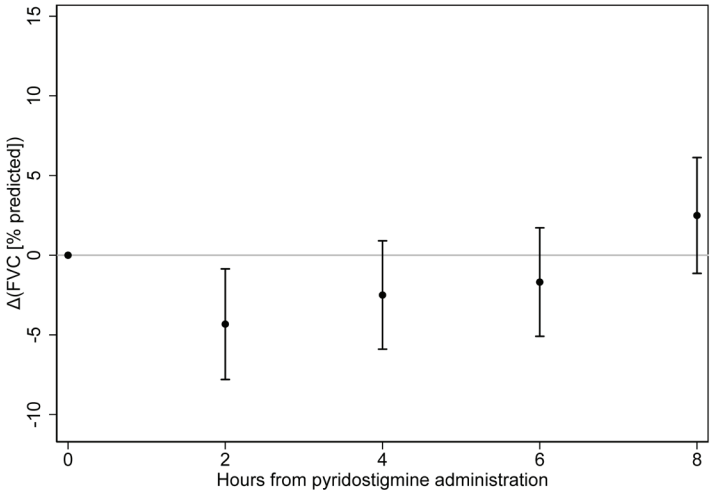


Figure 1B: Asthmatics only (n = 10)

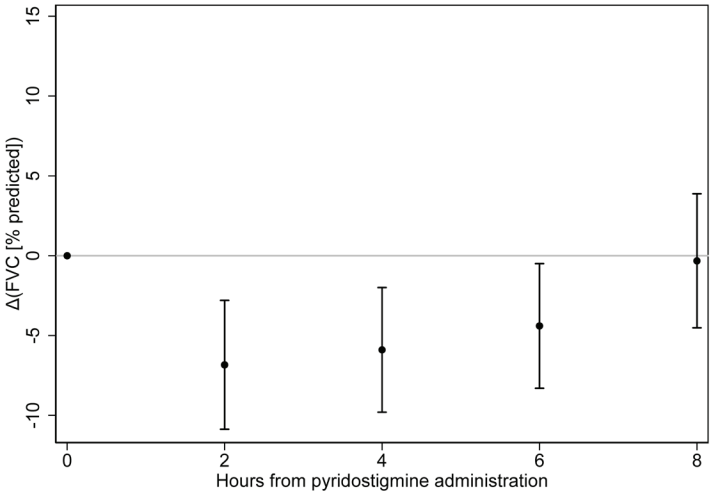
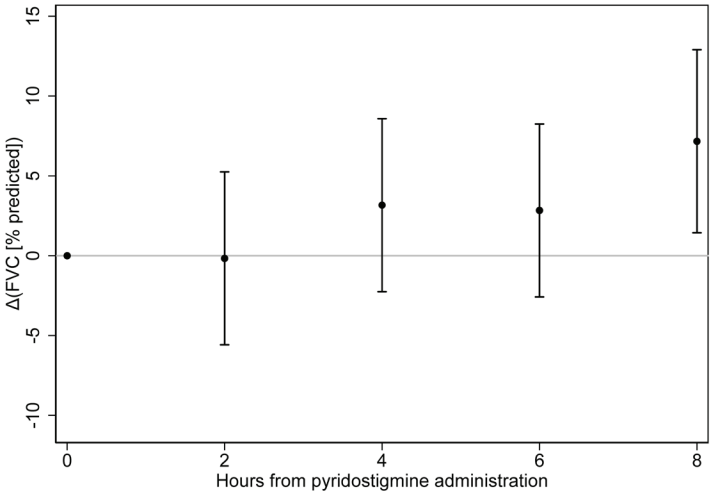


Figure 1C: Healthy subjects only (n = 6)



Dots are point estimates of difference from baseline; bars are 95% confidence intervals.

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Supplementary paper I

Awareness, prevalence, treatment, and control of type 2 diabetes in a semi-urban area of Nepal: Findings from a cross-sectional study conducted as a part of COBIN-D trial

Gyawali B, Hansen MRH, Povlsen MB, Neupane D, Andersen PK, McLachlan CS, Sandbæk A, Kallestrup P.

PloS ONE. 2018; 13(11).

The supplementary paper does not form part of the dissertation.

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RESEARCH ARTICLE

Awareness, prevalence, treatment, and control of type 2 diabetes in a semi-urban area of Nepal: Findings from a cross-sectional study conducted as a part of COBIN-D trial

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Abstract

Background

Type 2 diabetes is an escalating public health problem in Nepal. The current study aims to assess the prevalence, associated factors, awareness, treatment, and control of type 2 diabetes in a semi-urban area of Nepal.

Methods

A population-based cross-sectional survey was conducted including 2,310 adults aged 25 years or above from a semi-urban area of Lekhnath Municipality of Nepal, during October 2016 to April 2017 using the World Health Organization (WHO) STEPS approach. Data on demographics, behavioral risk factors, blood pressure, anthropometric measurements (weight, height, waist and hip circumference), and fasting blood glucose were collected by face-to-face interviews during a door-to-door visit. Participants were considered to have type 2 diabetes if they had previously been diagnosed by a physician and/or were on antidiabetic medications and/or had fasting blood glucose $\geq$ 7.0 mmol/L. Participants were classified as being aware of their diabetes conditions if they had earlier been told that they had type 2 diabetes. Treatment of diabetes among those aware was if participants received any kind of medication treatment or counseling, and control of diabetes among those treated was defined as fasting blood glucose level was <7.0 mmol/L. Odds Ratio (OR) with 95% Confidence Interval (CI) was used to determine the strength of association.

Results

The prevalence of type 2 diabetes was 11.7% (95% CI: 10.5–13.1). Among type 2 diabetes participants, 65% were aware of their disease, 94% of those who were aware received

funding. There was no additional external funding received for this study.

Competing interests: The authors have declared that no competing interests exist.

treatment, and 21% of the treated subjects had their diabetes under control. Factors significantly associated with type 2 diabetes were older age (OR = 3.2 for age group 45–54 years, OR = 3.8 for age group 55–64 years), Janajati ethnicity (OR = 1.4), abdominal obesity (OR = 2.3), being overweight or obese (OR = 1.4), and hypertension (OR = 2.0), while protective factors included being a female (OR = 0.4), medium physical activity (OR = 0.3), high physical activity (OR = 0.2), and not having family history of diabetes (OR = 0.3).

Conclusions

The study revealed a high prevalence of type 2 diabetes among adults. Older age, male gender, Janajati ethnicity, abdominal obesity, overweight or obesity, hypertension, low physical activity, and family history of diabetes were associated with type 2 diabetes. Immediate public health and individual measures are warranted to reduce further burden of type 2 diabetes.

Introduction

Global estimates of diabetes prevalence in 2014 suggested that the number of people with type 2 diabetes was 422 million and this number is projected to increase to 642 million by 2035 [1]. There is an increasing trend in the prevalence of type 2 diabetes in low-and middle-income countries (LMICs), and more than 75% adults with type 2 diabetes are now living in developing countries [1]. Furthermore, the population with prediabetes-a marker for development of type 2 diabetes-has reached approximately 318 million worldwide, equal to 6.7% of the global adult population [2]. Currently, South Asia is experiencing an increasing burden of type 2 diabetes and its complications [3]. Approximately one-fifth of all adults with type 2 diabetes in the world live in the South-East Asia Region.

Nepal is a low-income country in South Asia. While communicable diseases remain an important public health issue in Nepal, there is also a rapidly increasing burden of non-communicable diseases (NCDs), including type 2 diabetes, posing an additional burden on a resource-poor health systems. However, there is limited demographic knowledge of type 2 diabetes and its risk factors in Nepal. A 2015 systematic review and meta-analysis estimated that the prevalence of type 2 diabetes in Nepal was 8.4% (95% CI: 6.2–10.5%) [4], but the quality of the included studies were generally low, and results may not be representative for the population of Nepal as a whole.

Type 2 diabetes is mainly associated with a number of lifestyle behaviors, including daily smoking, heavy alcohol drinking, obesity, and reduced physical activity [5]. It has been revealed that behavioral risk factors are responsible for a large number of premature mortality due to cardiovascular diseases, followed by stroke [6]. More than 70% of diabetes patients die of cardiovascular events, leading to an epidemic of diabetes-related cardiovascular diseases [7]. In Nepal, risk factors for type 2 diabetes have so far rarely been investigated. However, our recent review found a number of modifiable and non-modifiable risk factors for type 2 diabetes in Nepal such as high socio-economic status, high body mass index (BMI), lack of physical activity, hypertension, alcohol and tobacco use [8]. Statistical analysis of the predictors of type 2 diabetes in Nepal has been lacking, and further research is hence needed. Moreover, diabetes awareness, treatment and control in Nepal has not received attention [8]. Our aim is to conduct a population-based study of type 2 diabetes in the semi-urban area of Lekhnath

Municipality of Nepal, especially to estimate prevalence, associated factors, awareness, treatment and control of type 2 diabetes at the population level, which could help in further planning of diabetes health systems management in Nepal.

Materials and methods

Ethics statement

This study conformed to the Helsinki Declaration and was approved by the Nepal Health Research Council, Kathmandu, Nepal (Reg. no. 263/2016). Written informed consent was obtained from each participant before enrolling in the survey. If the participants were unable to write, then fingerprinting was used. Participants were assured verbally and in writing that all information provided would be kept strictly confidential and only used for the purpose of this study. Participants diagnosed with type 2 diabetes were referred to the nearest health facility for further treatment and follow-up.

Study setting, design and population

This cross-sectional population-based study is a part of the Community Based Intervention for Management of Diabetes in Nepal (COBIN-D) trial (**Trial registration:** ClinicalTrial.gov: NCT03304158) [9], which was initiated in the semi-urban area of Lekhnath Municipality of Nepal (now named the Pokhara Metropolitan City due to recent restructuring of the state of Nepal according to the concept of a democratic federal system) situated 180 km west of the capital city Kathmandu. This semi-urban area has a total population of 58,816 with 14,937 households. The study was conducted from October 2016 to April 2017 among the participants recruited for the Community Based Management of Non-Communicable Diseases in Nepal (COBIN) study, the full details of which have been described by Neupane et al [10]. In brief, a systematic random sampling method was used to select a representative sample of the general population aged 25 years or above. A population framework of all eligible persons was prepared using the election voter's list for 2007 (Lekhnath). The voter list contained information about the household. If there was more than one person from the same household eligible to participate in the study at the time of data collection, the Kish method was adopted to select the participant [11]. Selected persons who did not sign the written consent or were not able to complete the questionnaire were excluded.

Sample size

The sample size was calculated based on an estimated prevalence of type 2 diabetes in Nepal of 9.5% [12], a 95% CI and the level of significance of 0.05 as recommended by the STEPS manual [13]. The total sample size estimate was adjusted using a design effect of 2. Using these values a sample size of 2,113 was derived which was adequate to provide results by 4 age groups (25–34 years, 35–44 years, 45–54 years, and 55–64 years) for each sex (total strata = 8). Assuming a response rate of 80%, the sample size was raised to 2,643 for this study.

Study instruments

A culturally adapted, Nepali (local language) translated and previously validated World Health Organization (WHO) Stepwise Surveillance (STEPS) questionnaire was used [14]. The questionnaire is an instrument developed by WHO for collection of surveillance data on NCDs in resource poor settings, which includes socio-demographic information (age, gender, ethnicity, education, marrital status, occupation, income), behavioral characteristics (dietary habits, harmful alcohol use, current smoking, physical activity, hypertension, family history of

diabetes), anthropometric measurements (height, weight, waist and hip circumference), blood glucose measurement and blood pressure measurements [15].

Data collection

Data collection and training was carried out in accordance with the WHO STEPS approach recommended for NCD surveillance [16]. Prior to data collection, the questionnarie was pre-tested in a nearby non-study area. Necessary revisions were made to each questionnaire on the results of the pretest. Data were collected in face-to-face interviews by eight specifically trained field investigators with a background in health during a door-to-door visit.

Blood glucose measurements

Fasting blood glucose for the subjects was estimated using a standardised digital glucometer, using the capillary finger prick method (fasting being defined as no caloric intake for at least eight hours). Participants were considered to have type 2 diabetes if they had previously been diagnosed by a physician and/or were on antidiabetic medications and/or had fasting blood glucose $\geq$ 7.0 mmol/L (126 mg/dL). Participants were classified as prediabetic if their fasting blood glucose levels were $\geq$ 6.1 mmol/L (110 mg/dL) and $<$ 7.0 mmol/L (126mg/dL). The cut-off values were based on the 2006 WHO guidelines [17]. The fasting blood glucose test was conducted in the morning on a predetermined date. Participants were requested to fast overnight (including no smoking or drinking tea in the morning) and were reminded by telephone a day before the test. Fasting was confirmed verbally by the participants immediately before collecting the blood sample.

Blood pressure measurements

Blood pressure was measured using a digital sphygmomanometer. Three readings of the systolic and diastolic blood pressure were taken with three-minute rest between each reading. In accordance with the WHO recommendation the mean systolic and diastolic blood pressure from the second and third readings were used for analysis. Participants were classified as hypertensive if their average systolic blood pressure was $\geq$ 140 mm Hg and/or their average diastolic blood pressure was $\geq$ 90 mm Hg, or if they reported being on regular anti-hypertensive therapy [18].

Socio-demographic variables

Socio-demographic variables included in the study were age group in years (25–34, 35–44, 45–54, 55–64), gender (male, female), ethnicity (Upper caste, Janajati, Others- based on the classification by the Department of Health Services of Nepal [19], marital status (unmarried, married), education (low: up to primary schooling, medium: upto secondary and high schooling, high: college or university education), occupation (employee, housemaker, agriculture, labor, others), monthly household income ($<$20,000 Nepali Rupees (NPR) or $<$200 US Dollars (1 NPR = 0.01 US Dollar, August 2017), $\geq$20,000 NPR or $\geq$200 US Dollars), current smoking (yes, no), harmful alcohol use (yes, no), $\geq$5 servings of fruits and vegetables weekly (yes, no), abdominal obesity defined by waist-hip ratio (normal, high), overweight or obesity defined by BMI (yes, no), physical activity level (low, medium, high), and family history of diabetes (yes, no).

Type 2 diabetes awareness, treatment, and control variables

Participants who reported that a physician ever told them they had type 2 diabetes were considered aware of their diabetic conditions. Participants were categorised as undergoing treatment if they received any kind of treatment such as insulin or anti-diabetic medications or counselling, and categorised as having good glycemic control if their fasting blood glucose level was lower than 7.0 mmol/L.

Behavioral variables

Current smoking was defined as smoking at least one cigarette per day. Harmful alcohol use was determined from self-reported alcohol consumption during the last 30 days, and was defined as drinking 8 standard drinks or more in a single occasion per week among females and drinking 15 or more standard drinks in a single occasion per week among males. Pictorial cards showing different kinds of glasses and bowls most commonly used in Nepal were used to help the participants recall the amount of alcohol consumed. The amount, as identified by the respondent, was then used to estimate the number of standard drinks of alcohol use (one standard drink being defined as 10 grams of ethanol). Physical activity level was determined from questions on number of days and time spent on vigorous and/or moderate activities for work, travel and leisure activities. Using standard formula from the WHO STEPS, the number of Metabolic Equivalent of Task (MET) minutes per weeks were calculated and categorized as low (<600 MET minutes per week), moderate (> = 600 but <3000 MET minutes per week), and high physical activity (> = 3000 MET minutes per week). Participants self-reported their fruit and vegetable consumption in a typical week. One serving of vegetable was considered to be one cup of raw green leafy vegetables or 1/2 cup of other vegetables (cooked or chopped raw). One serving of fruit was considered to be one medium size piece of apple, banana or orange, 1/2 cup of chopped, canned fruit or 1/2 cup of fruit juice.

Anthropometric measurements

Weight was measured using a digital scale, and height using a portable standard stature scales. BMI was calculated using the formula weight (kg)/(height^2)(m^2). A person was considered to be overweight or obese if BMI $\geq$24 kg/m^2 (the cut-off levels for South Asians) [20]. Waist and hip circumferences were measured by John's nonstretchable measuring tape. BMI was calculated using Central/abdominal obesity was defined by waist circumference $\geq$90 cm in males and $\geq$85 cm in females (undefined for pregnant women).

Quality control

To ensure the validity and reliability of the data, strict protocols were implemented. All data enumerators were uniformly trained to conduct the face-to-face questionnaire interviews and to use the measurement instruments for five consecutive days. Completed questionnaires were validated in telephone interviews with selected participants. Repeated interviews or examinations were conducted if missing information was found. To ensure standardized measurements, all glucometers, sphygmomanometers, weighing scales and tape measures were assessed weekly by taking measurements on one person with each of the instruments. Moreover, for all participants who self-reported an earlier diagnosis of diabetes, the information was validated using their medical records.

Data management and analysis

The completed questionnares were checked for completeness, sorted, and entered into the Epidata 3.1 software, and exported to the STATA statistical software version 14.1. Frequencies and percentages were calculated to identify the distribution of sociodemographic information. Chi-square test was conducted for comparing proportions of categorical variables. Univariate and multiple logistic regressions were performed to identify the associations between type 2 diabetes and its risks factors, and we calculated odds ratios (OR) with 95% confidence intervals (CIs). The covariates in the multivariate model were selected a priori based on literature, which will allow for better confounding adjustment. We also performed sensitivity analyses with the most significant variables, and there was no change in the significance of the variables. All statistical tests were two-tailed, and associations were considered to be statistically significant for a $P<0.05$. In all logistic regression models, we adusted for age, gender, ethnicity, marital status, education, occupation, monthly income, current smoking, harmful alcohol use, fruits and vegetable servings weekly, abdominal obesity, overweight/obesity, and physical activity, hypertension, and family history of diabetes.

Results

Socio-demographic characteristics of the study participants

The study invited 2,643 participants with a response rate of 87.4%. The total sample studied was 2,310, of which 1,574 (68%) were females and 736 (32%) were males. Table 1 shows the socio-demographic and behavioral characteristics of the study participants. The median age (±SD) of the study group was 47.37 (±9.95) years. In total, 31% of the study participants were in the 45-54-year age group. The majority of the study population had low education (53%), were from the Upper caste (54%), and were married (91%). We observed that 36% of the participants were engaged in agriculture. In total, 35% of the participants had hypertension. Of the study participants, 16% were current smokers, and around 13% consumed harmful amounts of alcohol.

Prevalence of type 2 diabetes

The overall prevalence of type 2 diabetes was found to be 11.7% (95% CI: 10.4–13.1), and the prevalence of prediabetes was 13.0% (95% CI: 11.8–14.5). Table 2 presents prevalence of type 2 diabetes and prediabetes stratified by age and gender. Fig 1 shows the prevalence of diabetes by administrative units (wards) of the study area.

Factors associated with type 2 diabetes

Table 3 presents the results of univariate and multivariate logistic regression analysis to identify the factors associated with type 2 diabetes. On univariate analysis, the prevalence of type 2 diabetes was found to be significantly higher among those who: a) were 55–64 years (18.2%), b) were males (15.3%), c) were of Janajati ethnicity (14.9%), d) were abdominally obese (13.5%), e) were overweight or obese (14.4%), f) had low physical activity (30.0%), g) had hypertension (19.6%), or h) had a family history of diabetes (23.5%). No difference was found in prevalence by marital status, education, monthly income, current smoking, harmful alcohol use or consumption of fruits and vegetables. When these variables were entered in a multivariate logistic model, older age (both 45–54 and 55–64 years), Janajati ethnicity, abdominal obesity, overweight or obesity, and hypertension turned out to be significant risk factors ($OR>1$) of type 2 diabetes. Female gender, medium and high physical activity, and not having family history of diabetes were identified as significant protective factors ($OR<1$).

Table 1. Socio-demographic and behavioral characteristics of the study population.

Characteristics	N = 2,310 (%)
Age (years)	
25–34	288 (12)
35–44	676 (29)
45–54	727 (31)
55–64	619 (27)
Gender	
Male	736 (32)
Female	1,574 (68)
Ethnicity	
Upper caste	1,254 (54)
Janajati	742 (32)
Others	314 (14)
Marital status	
Married	2,093 (91)
Unmarried	217 (9)
Education	
Low	1,215 (53)
Medium	969 (42)
High	126 (5)
Occupation	
Employee	462 (20)
Housemaker	757 (33)
Agriculture	838 (36)
Labor	69 (3)
Others	184 (8)
Monthly income (NPR)	
<20,000	817 (35)
≥20,000	1,493 (65)
Current smoking[a]	
Yes	365 (16)
No	1,945 (84)
Harmful alcohol use[b]	
Yes	307 (13)
No	2,003 (87)
≥5 servings of fruits and vegetables weekly[c]	
Yes	122 (5)
No	2,188 (95)
Abdominal obesity[d]	
Normal	474 (21)
High	1,836 (79)
Overweight or Obese (Asian cut-off)[e]	
Yes	1,422 (62)
No	888 (38)
Physical activity[f]	
Low	43 (2)
Medium	221 (10)
High	2,046 (88)

(*Continued*)

Table 1. (Continued)

Characteristics	N = 2,310 (%)
Hypertension[g]	
Yes	797 (35)
No	1,513 (65)
Family history of diabetes	
Yes	455 (20)
No	1,855 (80)

Note: *N* group size, *NPR* Nepalese Rupee

[a]Smoking at least one cigarette per day

[b]Drinking 8 standard drinks or more in a single occasion per week among females and drinking 15 or more standard drinks in a single occasion per week among males

[c]One serving of fruit was considered to be one medium size piece of apple, banana or orange, 1/2 cup of chopped, canned fruit or 1/2 cup of fruit juice

[d]Waist circumference ≥90 cm in males and ≥85 cm in females

[e] BMI ≥24 kg/m^2

[f] Low (< 600 MET minutes per week), moderate (> = 600 but <3000 MET minutes per week), and high physical activity (> = 3000 MET minutes per week).

[g]Average systolic blood pressure was ≥140 mm Hg and/or average diastolic blood pressure was ≥90 mm Hg, or if reported being on regular anti-hypertensive therapy

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Awareness, treatment, and control status of type 2 diabetes

Among all individuals identified as having type 2 diabetes, nearly two-fifths (35%) were unaware of their disease. Nearly 94% of those aware were receiving some kind of treatment such as insulin or oral anti-diabetic medications and counselling but the overall control rate was less than one quarter of those who were receiving treatment (21%) (Table 4).

Discussion

The current study, using a representative sample from the semi-urban area of Lekhnath Municipality of Nepal, showed that 11.7% of the participants had type 2 diabetes and 13.0%

Table 2. Prevalence of diabetes and prediabetes stratified by age group and gender.

Characteristics	N	Prevalence of prediabetes % (95% CI)	N	Prevalence of diabetes % (95% CI)
Age (years)				
25–34	19	6.5 (4.2–10.1)	11	4.1 (2.1–6.7)
35–44	81	11.9 (9.7–14.6)	49	7.2 (5.5–9.4)
45–54	109	14.9 (12.5–17.7)	98	13.4 (11.1–16.1)
55–64	93	14.9 (12.4–18.0)	113	18.2 (15.4–21.4)
Gender				
Male	90	12.2 (10.0–14.7)	113	15.3 (12.9–18.1)
Female	212	13.4 (11.8–15.2)	158	10.0 (8.7–11.6)
Overall	302	13.0 (11.7–14.5)	271	11.7 (10.2–12.8)

Diabetes is defined as individuals diagnosed by a physician and/or were on antidiabetic medications and/or those who had fasting blood glucose ≥7.0 mmol/L (≥126 mg/dL); prediabetes is defined as individuals who had fasting blood glucose levels ≥ 6.1 mmol/L (≥110 mg/dL) and <7.0 mmol/L (126mg/dL). The figures in the parentheses are expressed as percentages with 95% CIs.

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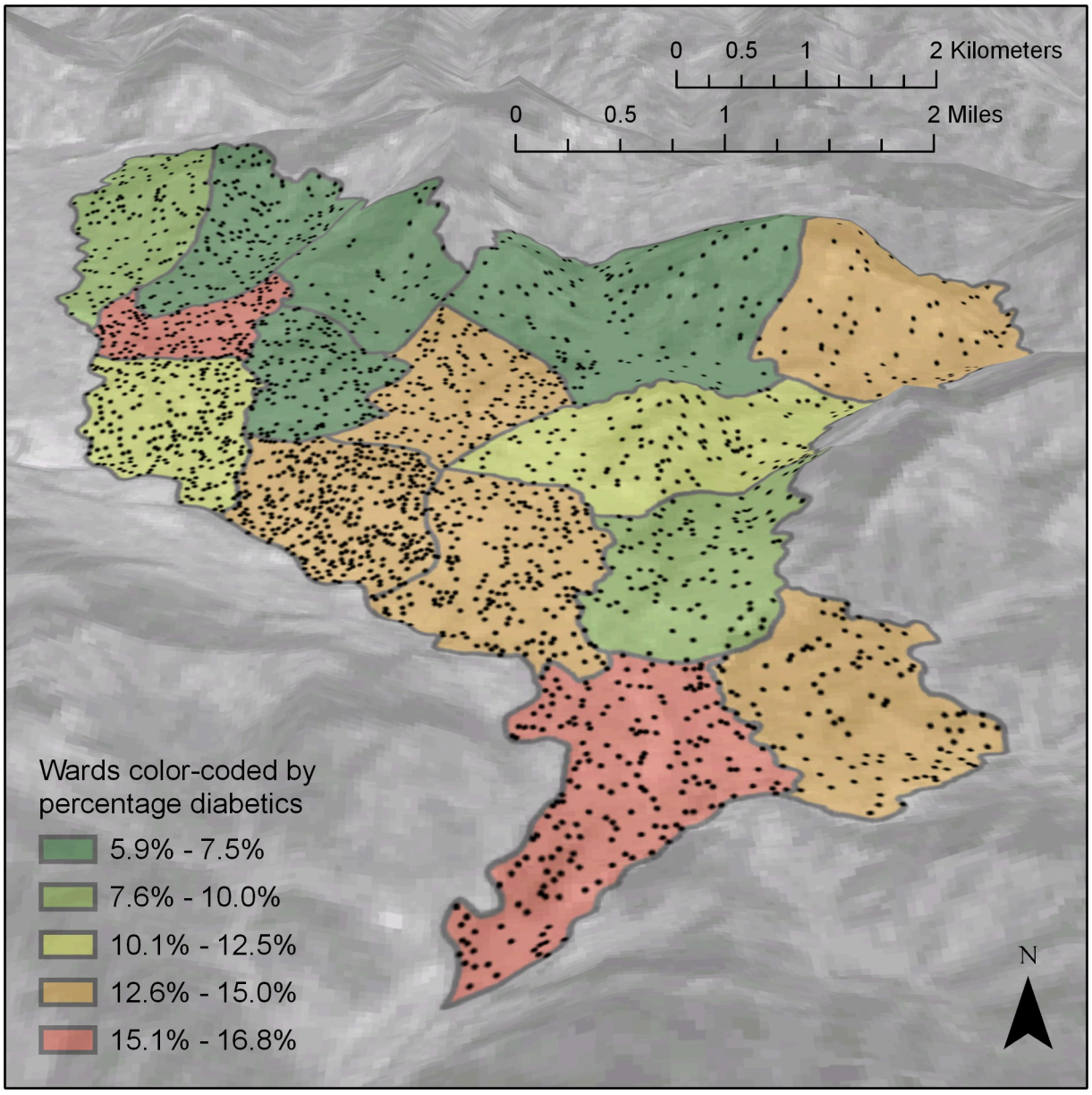

Fig 1. Map showing diabetes prevalence by project area. Wards in the project area are shown as individual polygons each containing a number of randomly placed dots equal to the number of participants from that ward. Map created using Esri ArcGIS Pro (Esri, Redlands, California, USA). Elevation exaggerated by a factor of 2.5. Data sources: Elevation and satellite image data available from the U.S. Geological Survey. Ward outlines from the Survey Department, National Geographic Information Infrastructure Project (Kathmandu, Nepal).

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of the participants had prediabetes. The diabetes prevalence was higher in the urbanized, low-land wards than in the more rural highland wards. Older age, male gender, Janajati ethnicity, abdominal obesity, overweight or obesity, hypertension, low physical activity, and family history of diabetes were associated with type 2 diabetes. Despite the high burden of type 2 diabetes, only two-thirds (65%) of participants were aware of their condition, 94% of those aware were receiving the treatment, but only about one-fifths of those on treatment had their blood glucose under control according to recommendations.

Table 3. Odds ratios for type 2 diabetes according to socio-demographic, behavioral and anthropometric measurement characteristics among the study population.

Characteristics	Diabetes	p-value	OR	95% CI	p-value
Age (years)		$p< 0.001$			
25–34	11 (3.8)		Ref		
35–44	49 (7.2)		1.7	(0.9–3.5)	0.115
45–54	98 (13.4)		3.0	(1.6–6.0)	0.001
55–64	113 (18.2)		3.6	(1.9–7.4)	$p< 0.001$
Gender		$p< 0.001$			
Male	113 (15.3)		Ref		
Female	158 (10.0)		0.4	(0.3–0.7)	$p< 0.001$
Ethnicity		0.004			
Upper caste	127 (10.1)		Ref		
Janajati	111 (14.9)		1.4	(1.0–1.9)	0.035
Others	33 (10.5)		1.2	(0.8–2.0)	0.382
Marital status		0.935			
Unmarried	25 (11.5)		Ref		
Married	246 (11.7)		1.0	(0.6–1.7)	0.838
Education		0.13			
Low	158 (13.0)		Ref		
Medium	100 (10.3)		0.7	(0.5–1.1)	0.103
High	13 (10.3)		0.6	(0.3–1.3)	0.199
Occupation		0.048			
Employee	46 (9.9)		Ref		
Housemaker	89 (11.7)		1.4	(0.9–2.3)	0.179
Agriculture	98 (11.6)		1.3	(0.9–2.0)	0.217
Labor	5 (7.2)		0.7	(0.3–2.1)	0.555
Others	33 (17.9)		1.2	(0.7–2.1)	0.477
Monthly income (NPR)		0.419			
<20,000	90 (11.0)		Ref		
> = 20,000	181 (12.1)		1.1	(0.8–1.4)	0.617
Current smoking		0.082			
Yes	33 (9.0)		1.5	(0.9–2.3)	0.06
No	238 (12.2)		Ref		
Harmful alcohol use		0.999			
Yes	36 (11.7)		0.7	(0.5–1.2)	0.215
No	235 (11.7)		Ref		
≥5 servings of fruits and vegetables weekly		0.121			
Yes	9 (7.3)	Ref			
No	262 (11.9)		1.7	(0.8–3.5)	0.135
Abdominal obesity		$p< 0.001$			
Normal	21 (4.4)		Ref		
High	250 (13.5)		2.2	(1.4–3.7)	0.001
Overweight or obese (Asian cut off)		$p< 0.001$			
Yes	206 (14.4)		1.4	(1.1–2.1)	0.023
No	65 (7.3)		Ref		
Physical activity		$p< 0.001$			
Low	13 (30.0)		Ref		
Medium	37 (16.7)		0.3	(0.2–0.8)	0.011
High	221 (10.8)		0.2	(0.1–0.5)	$p< 0.01$

(*Continued*)

Table 3. (Continued)

Characteristics	Diabetes	p-value	OR	95% CI	p-value
Hypertension		$p< 0.001$			
Yes	157 (19.6)		1.9	(1.4–2.6)	$p< 0.001$
No	114 (7.5)		Ref		
Family history of diabetes		$p< 0.001$			
Yes	107 (23.5)		Ref		
No	164 (8.8)		0.3	(0.2–0.4)	$p< 0.001$

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The prevalence of diabetes in our study population is consistent with a previous systematic review on diabetes in Nepal [4] that reported as pooled prevalence of 8.4%, but with prevalence estimates in individual studies ranging from 0.3% [21] to 19% [22]. Our findings are reasonably similar to diabetes prevalence estimates from studies in neighbouring South Asian countries, including India (11.1%) [23], 13.6% [24] and 18.6% [25], Bangladesh (11%) [26], Pakistan (11.1%) [27], Sri Lanka (10.3%) [28], and China (11.6%) [29]. We note that our age groups, different study populations, measurement methods, and choice of diagnostic criteria and definitions of diabetes influence prevalence estimation. Hence caution should be observed in comparisons of our findings to corresponding data from previous surveys.

In this study, age was significantly associated with type 2 diabetes and prevalence was highest among participants aged 55–64 years. A worldwide estimate for the prevalence of type 2 diabetes in 2030 predicts that in most developing countries, diabetes will be more prevalent in individuals between 45 and 64 years [30]. The importance of age as a risk factor is consistent with previous data, from various contexts [22, 26, 31].

Females were at a lower risk of type 2 diabetes compared to males in our study. This is in contrast to the findings from a meta-analysis suggesting that females were at higher risk of type 2 diabetes in Nepal (OR = 1.6; 95% CI = 1.3–1.9) [4]. Around 80% of adults in our study had abdominal obesity; and out of these 13.5% had diabetes. Similarly, more than three fifth of adults were overweight or obese (61.5%); out of which 14.5% were diabetics. Our findings underscore obesity is a risk factor for the development of type 2 diabetes as shown in previous studies [32–34]. It was reported that Asian populations are more likely to have abdominal obesity and overweight or obesity compared to their Western counterparts [35]. Similary, medium and high physical activity was associated with lower risk of type 2 diabetes in our

Table 4. Awareness, treatment and control status among diabetes patients.

Demographic variables	Total diabetics N	Awareness N (%)	On treatment N (%)	Good glycemic control N (%)
Total	**N = 271**	**N = 175 (65)**	**N = 164 (94)**	**N = 37 (21)**
Age (years)				
25–34	11	6 (55)	5 (83)	1 (20)
35–44	49	26 (53)	23 (88)	7 (30)
45–54	98	59 (60)	56 (95)	12 (21)
55–64	113	84 (74)	80 (95)	17 (21)
Gender				
Male	113	77 (68)	74 (96)	14 (19)
Female	158	98 (62)	90 (92)	23 (26)

Good glycemic control was defined as fasting blood glucose <7.0 mmol/L.

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study, corroborating findings of previous studies [36, 37]. Although there is a paucity of physical activity data in Nepal, one study revealed a high burden of physical inactivity in Nepal [38]. There is a pressing need to raise awareness on increasing physical activity and lifestyle modifications to decrease risk of type 2 diabetes.

The prevalence of type 2 diabetes in our study varied significantly with ethnicity and was highest among participants of Janajati descent. An earlier study from Nepal reported similarly a high prevalence of diabetes mellitus among Janajatis [39], and another study reported that Janajatis had the highest prevalence of overweight and obesity in Nepal [40]. Exposure to unhealthy lifestyle behaviours such as lack of physical activity and obesity as observed in our study and other studies [38] could be the contributing factors. Our study observed a positive association between hypertension and type 2 diabetes, which is consistent with a previous finding from other study conducted in a similar setting [41]. The coexistence of type 2 diabetes and hypertension might be due to sharing of common risk factors such as unhealthy lifestyle behaviors. Harmful alcohol use did not show any significant association with type 2 diabetes. This finding is consistent with studies conducted elsewhere [42, 43].

To the best of our knowledge there are no previously published studies on the awareness, treatment and control of diabetes in Nepal, and there is only limited evidence in the developing countries of South Asia. In this study, only 65% of individuals with type 2 diabetes were aware of their disease and among them, 94% were treated. Our findings were similar to findings reported in studies from Kazakhshtan [44], India [45], and Bangladesh [46]. Despite varying rates of awareness and treatment, the control rates were very low. Compared to our control rate of 21%, two different studies in China found control rates to be 27.2% and 44.2%, respectively [47, 48] and a study from Kazakhshtan found 27.7% [44]. Despite the availability of low-cost drugs for diabetes in Nepal, the overall control rate is not satisfactory. Management of diabetes is a major challenge in Nepal due to paucity of programmes to detect, manage, and prevent diabetes and its complications [8]. Nepal does not have specific guidelines regarding diabetes medication use and low medication adherence. This could be one of the barriers to proper management of diabetes. The considerably low control rate of type 2 diabetes suggests that intensive interventions and increased clinical attention should be urgently initiated among diabetics to decrease blood sugar levels.

This is one of the few studies, which surveyed prevalence and associated factors of type 2 diabetes in Nepal. The strengths of the study are random sampling of participants, interviews according to the validated STEPS questionnaire, and fasting blood glucose measurements according to the WHO recommendations. We acknowledge that the study also had a number of limitations. First, only fasting blood glucose, without other glycaemic indexes, including 2 hours post-prandial glucose or HbA1c, was used for the diagnosis of type 2 diabetes. On the other hand, this method has been reported by large cross-sectional studies conducted elsewhere [49–51]. While methods and timing of measurements may be variable, this may limit direct comparison with other published studies. It was not feasible to conduct oral glucose tolerance testing and HbA1c in the context of this large survey because of logistic and financial barriers. Consequently, we may have underestimated the true diabetes prevalence. However, all participants with known diabetes were confirmed through their medical records. The WHO considers that for epidemiological purposes, a single fasting plasma glucose estimation is acceptable [17]. Second, our study could not examine causal relationship between type 2 diabetes and demographic and behavioral factors, for which further longitudinal studies are needed. Third, the use of self-reported physical activity measures that are subjected to recall bias and over-reporting could have increased the possibility of exposure misclassification. This might have led to, for example, a higher number of individuals self reporting meeting the physical activity recommendations, thus altering the associations.

Despite the above-mentioned weaknesses, we have demonstrated a high prevalence of type 2 diabetes in Nepal that constitutes a tremendous burden to the country. The results underline the need for effective strategies for diabetes control—including prevention, surveillance and treatment. Policy makers should incorporate promotion of healthy diets and physical activity in national strategic plans to tackle NCDs, including type 2 diabetes. The study will serve as a useful tool in the planning of intervention programmes aimed at early detection of type 2 diabetes in Nepal.

Conclusions

This study found high prevalence of type 2 diabetes, medium awareness, a high treatment rate in diagnosed cases but a suboptimal control rate. Older age, male gender, Janajati ethnicity, abdominal obesity, overweight or obesity, hypertension, low physical activity, and family history of diabetes were risk factors for type 2 diabetes. Current findings suggest a high future burden of cardiovascular diseases in Nepal. Immediate planning and implementation of public health measures and individual interventions are needed to prevent the occurance and complications of type 2 diabetes.

Supporting information

S1 Table. Minimal data set.
(XLSX)

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Supplementary paper II

The burden and correlates of multiple cardiometabolic risk factors in a semi-urban population of Nepal: a community-based cross-sectional study

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The burden and correlates of multiple cardiometabolic risk factors in a semi-urban population of Nepal: a community-based cross-sectional study

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This study assessed the burden and correlates of three cardiometabolic risk factors, (hypertension, diabetes, and overweight/obesity), and their possible clustering patterns in a semi-urban population of Nepal. Data were obtained from a community-based management of non-communicable disease in Nepal (COBIN) Wave II study, which included 2,310 adults aged 25–64 years in a semi-urban area of Pokhara Metropolitan City of Nepal, using the World Health Organization-STEPS questionnaire. Unadjusted and adjusted binary logistic regression models were used to study the correlates of the individual risk factors and their clustering. The prevalence of hypertension, diabetes, and overweight/obesity was 34.5%, 11.7%, and 52.9%, respectively. In total, 68.2% of the participants had at least one risk factor and many participants had two risks in combination: 6.8% for 'hypertension and diabetes', 7.4% for 'diabetes and overweight/obesity' and 21.4% for 'hypertension and overweight/obesity'. In total, 4.7% had all three risk factors. Janajati ethnicity (1.4–2.1 times), male gender (1.5 times) and family history of diabetes (1.4–3.4 times) were associated with presence of individual risk factors. Similarly, Janajati ethnicity (aOR: 4.31, 95% CI: 2.53–7.32), current smoking (aOR: 4.81, 95% CI: 2.27–10.21), and family history of diabetes (aOR: 4.60, 95% CI: 2.67–7.91) were associated with presence of all three risk factors. Our study found a high prevalence of all single and combined cardiometabolic risk factors in Nepal. It underlines the need to manage risk factors in aggregate and plan prevention activities targeting multiple risk factors.

Cardiovascular disease (CVD), a leading cause of global morbidity and mortality, accounts for 17.9 million deaths worldwide annually[1]. In 2012, it was estimated that 7.4 million died due to coronary heart diseases and 6.7 million died due to stroke[2]. Over 75% of cardiovascular deaths take place in low-and middle-income countries (LMICs)[1]. Additionally, CVD contributes to the global economic burden by increasing health-care expenditures, lower productivity at work, increasing the number of sick days, causing permanent disability[3]. Nepal, a low-income country in South Asia, is experiencing a similar increasing trend in CVD morbidity and mortality. The mortality attributed to CVD in Nepal has increased rapidly from 22% to 25% between 2004 and 2008[4,5].

The public health care spending in Nepal is still focused on infectious diseases, and the resources allocated to fighting CVD have not kept up with its increasing burden. As is common in poor resource settings,

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cardiometabolic risk factors at subclinical stages are often not presented to health professionals until some serious symptoms of major CVD arise[6]. The resulting late diagnosis precludes the benefits of early diagnosis and management of the conditions and prevention of complications. Further, although patients with multiple risk factors may have preventive and clinical needs that are different than those with a single factor, disease management protocols in Nepal primarily focus on the treatment of symptoms presented to the health professionals, and often competing risk factors are not handled.

There is a dearth of information on the major drivers of the concurrent cardiometabolic risk factors among the Nepalese population. A previous study evaluated the prevalence and concurrence of cardiometabolic risk factors but was limited to Eastern parts of the country[7]. A recent study evaluated the prevalence and determinants of metabolic syndrome among nationally representative Nepalese adults, however, failed to include some important socio-demographic determinants of CVD, such as monthly income[8]. To our knowledge, no studies in Nepal have adequately evaluated socio-demographic and lifestyle correlates of the prevalence of cardiometabolic risk profiles, individually as well as in combination as multiple cardiometabolic risk factors. The current study aimed to assess the burden and correlates of three cardiometabolic risk factors (hypertension, diabetes, and overweight/obesity), and their clustering patterns in a semi-urban population of Nepal.

Results

Socio-demographics and behavioural characteristics. Table 1 provides the socio-demographic and behavioural characteristics of the participants and has been previously reported[9]. Three-fifth of the participants were middle-aged (35–54 years). The majority of the participants were female (68%), Upper caste (54%), and had an average monthly income of 20,000 Nepali Rupees (NPR) or greater (65%).

A sizeable proportion of the participants smoked daily (16%) or used alcohol at harmful level (13%). Most of the participants displayed a high level of physical activity (89%) but poor fruit/vegetable intake; 95% did not consume the recommended five or more servings per week. One-fifth of the participants had a family history of diabetes.

Prevalence and correlates of cardiometabolic risk factors. More than 68% of the participants had at least one cardiometabolic risk factor. The prevalence of hypertension was 34.5%, diabetes was 11.7%, and overweight/obesity was 52.9%. Supplemental Table 1 shows the prevalence of the three risk factors by demographic and behavioural characteristics. The prevalence of diabetes and hypertension, showed an increasing trend across the age categories, whereas a peak in the prevalence of overweight/obesity was observed for the age group 35–54 years. The prevalence of diabetes, hypertension, and overweight/obesity, individually, was higher in females, Upper caste, and those with higher income (Supplemental Table 1).

In multivariate logistic regression analyses, hypertension and diabetes showed a positive dose-response relationship with the age category. Compared to males, females were less likely to be hypertensive (aOR: 0.62, 95% CI: 0.50–0.76) and diabetic (aOR: 0.63, 95% CI: 0.47–0.84) but more likely to be overweight/obese (aOR: 1.32, 95% CI: 1.07–1.62). Compared to the Upper caste, Janajati ethnicity had higher odds of being diabetic (aOR: 1.73, 95% CI: 1.29–2.32) and overweight/obese (aOR: 2.16, 95% CI: 1.75–2.66). Higher odds of overweight/obesity were seen for those with higher income (aOR 1.44, 95% CI 1.20–1.74), compared to those with lower income. Current smokers had higher odds of two risk factors, i.e., diabetes (aOR: 1.67, 95% CI: 1.10–2.53) and overweight/obesity (aOR: 2.66, 95% CI: 2.06–3.44). Higher physical activity was associated with reduced risk of diabetes (aOR: 0.63, 95% CI: 0.44–0.91) and overweight/obesity (aOR: 0.74, 95% CI: 0.66–0.99), and harmful use of alcohol was associated with increased odds of hypertension (aOR: 2.01, 95% CI: 1.50–2.69). A family history of diabetes was associated with increased odds of each of the three-individual risk factor whereas participants' history of heart disease was associated with increased odds of hypertension and diabetes. Risk factors for hypertension, diabetes and overweight/obesity are shown in Figs 1–3.

Two-way clustering: prevalence and correlates. A sizable proportion of the participants showed two-way unique combinations of risk factors; the prevalence ranging from 6.8% to 66.0% (hypertension and diabetes: 6.8%, hypertension or diabetes: 39.4%, hypertension and overweight/obesity: 21.4%, hypertension or overweight/obesity: 66.0%, diabetes and overweight/obesity: 7.4%; diabetes or overweight/obesity: 57.2%). Supplemental Table 1 shows the prevalence of the six-different two-way clustering of risk factors by demographic and behavioral characteristics. The prevalence of the two-way clustering of risk factors was higher in older age groups, female, Upper caste, those with higher monthly income ($\geq$20,000 NPR), and those having a history of heart diseases Supplemental Table 1.

In multivariate analyses (Table 2), the odds of any two-risk factors clustering showed a gradually increasing trend by age categories, except for the co-occurrence of diabetes and overweight/obesity where no significant association was reported across the higher age categories. Likewise, participants from the Janajati ethnicity and having a family history of diabetes were more likely to have any two-risk factors clustering compared to their respective counterparts. Female gender and history of heart disease were associated with lower odds of having either hypertension or diabetes or both. Smoking was associated with higher odds of having all six two-way unique combinations of risk factors (Table 2).

Three-way clustering: prevalence and correlates. A substantial proportion of study participants had at least one (68.2%) or all three (4.7%) cardiometabolic risk factors. Supplemental Table 1 shows the prevalence of the three-way clustering of the risk factors by demographic and behavioral characteristics. Among those with at least one of the risk factors, the prevalence of clustering was inverted J-shape with the highest prevalence (32%) observed for the age group 45–54 years. Within-group comparison suggest that prevalence of at least one cardiometabolic risk factor was highest for female (69%), Upper caste ethnic group (51%), and those with higher

Characteristics	n (%)
Age group (years)	
25–34	288 (13)
35–44	676 (29)
45–54	727 (31)
55–64	619 (27)
Gender	
Male	736 (32)
Female	1,574 (68)
Ethnicity	
Upper caste	1,254 (54)
Janajatis	742 (32)
Dalits and ethnic minorities	314 (14)
Monthly income (NPR)*	
<20,000	817 (35)
≥20,000	1,493 (65)
Current smoking	
Yes	365 (16)
No	1,945 (84)
Harmful alcohol use	
Yes	307 (13)
No	2,003 (87)
Physical activity	
Low	264 (11)
High	2,046 (89)
Servings of fruits/vegetables	
≥5 servings	122 (5)
<5 servings	2,188 (95)
History of heart diseases	
Yes	75 (3)
No	2,235 (97)
Family history of diabetes	
Yes	455 (20)
No	1,855 (80)
Diabetes	
Yes	271 (12)
No	2,039 (88)
Overweight/obesity	
Yes	1,222 (53)
No	1,088 (47)
Hypertension	
Yes	797 (35)
No	1,513 (65)

Table 1. Socio-demographic and behavioural characteristics of the study participants. Socio-demographic and behavioural characteristics of the study participants (N = 2,310). *100 NPR ~ 1 US dollar.

income (66%) compared to their respective counterparts (Supplemental Table 1). Likewise, the prevalence of all three cardiometabolic risk factors was higher in women (53%) compared to men (47%), and it increased linearly with age groups (Supplemental Table 1).

In multivariate analyses, the two types of three-way clustering pattern (at least one risk factors and all three risk factors), showed a gradual increase in the odds of risk factors with age categories, but the magnitude of the odds ratio was much higher for all three risk factors. Janajati ethnicity, current smoking, and family history of diabetes was associated with higher odds of having both all three and at least one risk factor. Females had lower odds (aOR: 0.48, 95% CI: 0.29–0.80) of having all three risk factors compared to men (Table 3).

Discussion

With an aim to assess the burden and correlates of three cardiometabolic risk factors, i.e., hypertension, diabetes and overweight/obesity, and their possible clustering patterns in a semi-urban population of Nepal, we found evidence of a high burden of the risk factors, individually and as clusters. In fact, 68.2% of the participants had at least one cardiometabolic risk factor, and 4.7% had all three risk factors. The risk factors, individually and clusters,

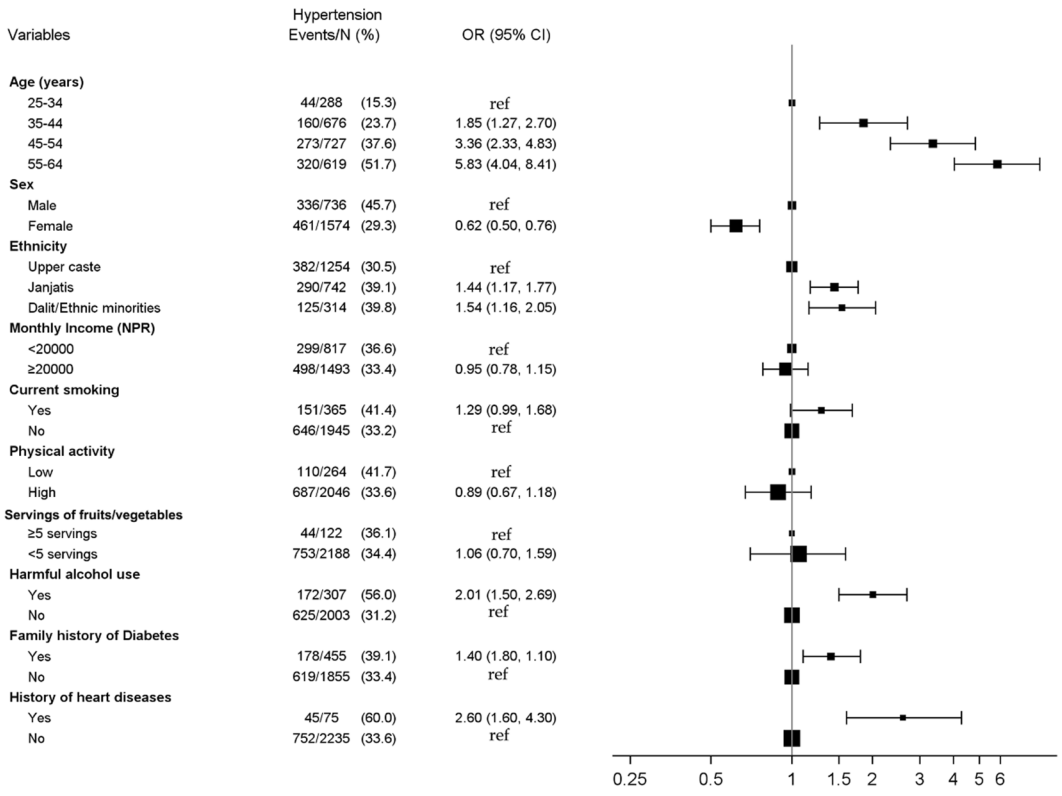

Figure 1. Risk factors for hypertension.

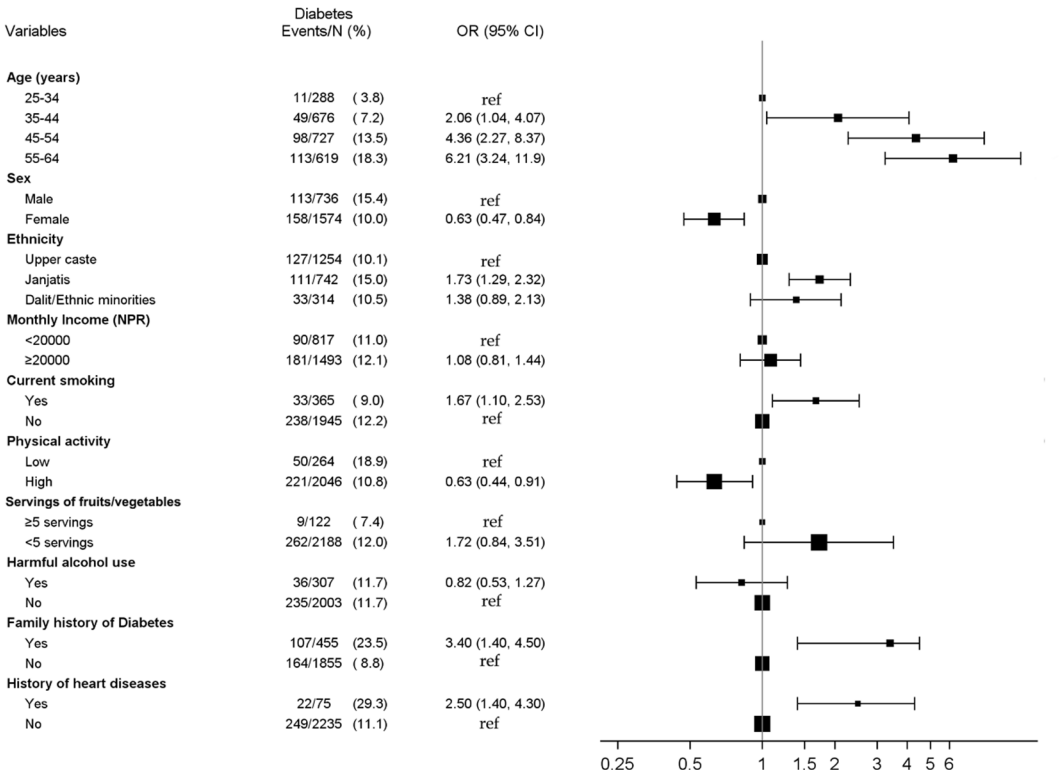

Figure 2. Risk factors for diabetes.

Demographic and behavioral characteristics	Hypertension and diabetes	Hypertension or diabetes	Hypertension and overweight/obesity	Hypertension or overweight/obesity	Diabetes and overweight/obesity	Diabetes or overweight/obesity
	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)
	Adjusted	Adjusted	Adjusted	Adjusted	Adjusted	Adjusted
Age group						
25–34	Ref	Ref	Ref	Ref	Ref	Ref
35–44	**4.86 (1.10–21.46)**	**1.89 (1.33–2.69)**	**2.07 (1.29–3.31)**	**1.54 (1.15–2.06)**	**3.10 (1.12–8.56)**	**1.36 (1.01–1.82)**
45–54	**17.06 (4.06–71.60)**	**3.56 (2.52–5.01)**	**3.10 (1.97–4.87)**	**1.62 (1.21–2.16)**	**8.02 (3.02–21.29)**	1.23 (0.92–1.64)
55–64	**42.02 (10.05–175.70)**	**5.92 (4.17–8.40)**	**3.95 (2.50–6.26)**	**1.85 (1.37–2.50)**	**9.66 (3.64–25.69)**	1.04 (0.77–1.40)
Sex						
Male	Ref	Ref	Ref	Ref	Ref	**Ref**
Female	**0.45 (0.30–0.67)**	**0.59 (0.48–0.73)**	0.87 (0.66–1.15)	1.12 (0.91–1.38)	0.75 (0.50–1.12)	**1.38 (1.13–1.70)**
Ethnicity						
Upper caste	Ref	Ref	Ref	Ref	Ref	**Ref**
Janajatis	**2.19 (1.46–3.28)**	**1.48 (1.20–1.81)**	**2.59 (1.97–3.41)**	**1.85 (1.50–2.28)**	**3.51 (2.35–5.26)**	**2.10 (1.72–2.58)**
Dalit/ethnic minorities	1.64 (0.87–3.10)	**1.66 (1.25–2.19)**	1.28 (0.88–1.86)	1.06 (0.81–1.39)	1.63 (0.93–2.86)	0.85 (0.65–1.11)
Monthly income (NPR)						
<20000	Ref	Ref	Ref	**Ref**	Ref	Ref
≥20000	1.18 (0.79–1.76)	0.92 (0.76–1.11)	1.11 (0.86–1.42)	**1.24 (1.03–1.49)**	1.35 (0.92–1.10)	**1.30 (1.09–1.56)**
Current smoking						
No	Ref	Ref	Ref	Ref	Ref	Ref
Yes	**2.45 (1.39–4.32)**	**1.33 (1.02–1.73)**	**2.57 (1.78–3.70)**	**1.80 (1.39–2.32)**	**3.11 (1.77–5.50)**	**2.28 (1.76–2.95)**
Physical activity						
Low	Ref	Ref	Ref	Ref	Ref	Ref
High	0.66 (0.40–1.09)	0.83 (0.63–1.10)	0.79 (0.54–1.15)	0.82 (0.62–1.10)	0.68 (0.41–1.15)	0.76 (0.58–1.01)
Servings of fruits/vegetables						
≥5 servings	Ref	Ref	Ref	Ref	Ref	Ref
<5 servings	2.20 (0.75–6.45)	1.10 (0.74–1.65)	1.07 (0.64–1.78)	1.24 (0.84–1.82)	1.59 (0.65–3.89)	1.19 (0.81–1.75)
Harmful alcohol use						
No	Ref	Ref	**Ref**	Ref	Ref	Ref
Yes	1.65 (0.94–2.90)	**1.75 (1.31–2.35)**	**1.63 (1.11–2.40)**	**1.43 (1.05–1.95)**	0.72 (0.40–1.31)	0.93 (0.69–1.24)
Family history of diabetes						
No	Ref	Ref	Ref	Ref	Ref	Ref
Yes	**3.57 (2.35–5.40)**	**1.97 (1.57–2.46)**	**1.91 (1.41–2.59)**	**1.70 (1.34–2.19)**	**5.16 (3.48–7.65)**	**1.99 (1.58–2.50)**
History of heart diseases						
No	Ref	Ref	Ref	Ref	Ref	Ref
Yes	**4.65 (2.19–9.87)**	**2.76 (1.64–4.62)**	**2.32 (1.22–4.39)**	1.44 (0.84–2.48)	1.45 (0.58–3.62)	1.5 (0.92–2.60)

Table 2. Two-way clustering of cardiometabolic risk factors by demographic and behavioural characteristics. Adjusted for all demographic and behavioral characteristics given in the table. Unadjusted estimates are provided in Supplemental Table 2. *100 NPR ~1 US dollar. Significant estimates are bolded.

showed increasing odds with age, male gender, and Janajati ethnicity. Similarly, the participant's family history of diabetes was associated with higher odds of any single or combined risk factors.

The high burden of hypertension (34.5%), overweight/obesity (52.9%), diabetes (11.7%), and their clustering observed in this study is concerning, although not surprising. Compared to the previous study from eastern Nepal, the prevalence of overweight/obesity (60.7%) was slightly higher, but previous estimates for hypertension (33.9%) and diabetes (6.3%) were slightly lower than ours[7]. The urban-rural disparity in the distribution of cardiometabolic risk factors, which is well documented in the Nepalese context[10] as well as globally[11,12] may explain the discrepancy in findings. Lifestyle changes, specifically access to heart non-healthy dietary choices and sedentary activities, as a consequence of rapid urbanization has particularly localized these risk factors to urban areas. The semi-urban locality of our study setting suggests our estimates to be in-between those from the rural and urban area.

The risk factors, individually, and as a cluster, showed increasing odds with age, male gender and Janajati ethnicity in our study which is consistent with the previous findings from Nepal[7,10] and globally[13]. The decline in cardiovascular health with aging was expected given that with increasing age, there is a gradual decrease in physiological functions, increased vulnerability to diseases, and a general decline in the capacity of the individual[14]. More importantly, the magnitude of the odds ratio increased gradually from a single risk factor to two-way clustering and reached maximum for three-way clustering. Older population exhibits a naturally higher burden of comorbidities due to biological aging[15], often exacerbated by the frequent clustering of risk factors in older adults[16]. It also hints towards the accumulation of risk factors over the life course, highlighting a longitudinal growth in CVD risk. Future studies with longitudinal designs have the opportunity to study pathways leading to risk factors clustering.

Demographic and behavioural characteristics	At least one risk factor (n = 1,575, 68.2%) (Diabetes or hypertension or overweight/obesity) OR (95% CI)		All three risk factors (n = 108, 4.7%) (Diabetes and hypertension and overweight/obesity) OR (95% CI)	
	Unadjusted	Adjusted	Unadjusted	Adjusted
Age group				
25–34	Ref	Ref	Ref	Ref
35–44	**1.54 (1.16–2.05)**	**1.56 (1.16–2.09)**	5.61 (0.71–44.39)	6.66 (0.82–54.33)
45–54	**1.56 (1.17–2.07)**	**1.67 (1.25–2.24)**	**21.06 (2.86–155.11)**	**26.71 (3.52–202.80)**
55–64	**1.74 (1.30–2.34)**	**1.96 (1.44–2.66)**	**38.11 (5.21–278.95)**	**59.61 (7.85–452.42)**
Sex				
Male	Ref	Ref	Ref	Ref
Female	1.07 (0.89–1.30)	1.07 (0.86–1.32)	**0.55 (0.36–0.82)**	**0.48 (0.29–0.80)**
Ethnicity				
Upper caste	Ref	Ref	Ref	Ref
Janajatis	**1.82 (1.48–2.24)**	**1.87 (1.51–2.32)**	**3.71 (2.38–5.78)**	**4.31 (2.53–7.32)**
Dalit/ethnic minorities	1.01 (0.78–1.30)	1.10 (0.84–1.45)	1.34 (0.69–2.59)	2.02 (0.96–4.26)
Monthly income (NPR)				
<20,000	Ref	Ref	Ref	Ref
≥20,000	**1.23 (1.03–1.48)**	1.18 (0.98–1.43)	1.43 (0.93–2.21)	1.24 (0.75–2.05)
Current smoking				
No	Ref	Ref	Ref	Ref
Yes	**1.64 (1.31–2.07)**	**1.85 (1.43–2.41)**	**2.53 (1.29–4.98)**	**4.81 (2.27–10.21)**
Physical activity				
Low	Ref	Ref	Ref	Ref
High	0.77 (0.57–1.02)	0.78 (0.58–1.06)	**0.50 (0.29–0.87)**	0.73 (0.38–1.43)
Servings of fruits/vegetables				
≥5 servings	Ref	Ref	Ref	Ref
<5 servings	1.32 (0.90–1.92)	1.28 (0.87–1.89)	2.34 (0.71–7.65)	2.19 (0.63–7.66)
Harmful alcohol use				
No	Ref	Ref	Ref	Ref
Yes	1.12 (0.87–1.46)	1.35 (0.98–1.84)	1.32 (0.75–2.32)	1.03 (0.49–2.15)
Family history of diabetes				
No	Ref	Ref	Ref	Ref
Yes	**1.96 (1.53–2.50)**	**1.98 (1.54–2.55)**	**4.40 (2.83–6.84)**	**4.60 (2.67–7.91)**
History of heart diseases				
No	Ref	Ref	Ref	Ref
Yes	**1.90 (1.07–3.37)**	1.76 (0.98–3.18)	**4.36 (1.86–10.23)**	2.89 (0.96–8.71)

Table 3. Three-way clustering of cardiometabolic risk factors by demographic and behavioural characteristics. Adjusted for all demographic and behavioral characteristics given in the table. *100 NPR ~1 US dollar. Significant estimates are bolded.

The findings on increased odds of risk factors for participants of Janajati ethnicity was interesting, but also not surprising. Based on historical association with the caste system, persistent in driving disparities between ethnic groups, three major ethnic groups are identified: Upper caste, Janajatis, and Dalits. These three ethnic groups would be expected to generally represent higher, medium, and lower social status, with the Dalit representing the most marginalized of all groups[17]. The government of Nepal has recognized Janajati and Dalit ethnic groups as marginalized groups and special provisions are in place to provide them with an equal opportunity for health, education, public jobs, and etc.[17] Although, Janajatis are generally considered less privileged, our participants do not fall into that category. Our Janajati participants are likely to have better socio-economic status, sedentary and unhealthy lifestyle because most of them are active in duty or retired Gurkha army personnel, employed in the workforce by the governments of Nepal, India, and Britain. A previous study has reported heavy alcohol use, tobacco smoking and sedentary lifestyle among Gurkha army personnel[18], which constitutes cardiometabolic risk factors. As a consequence, high prevalence of CVDs has been reported among people of Janajati ethnicity in Nepal[19,20]. The odds ratio for Janajatis are adjusted for the other risk factors in the study, but an association between Janajatis and CVD risk persists, likely because other unmeasured risk factors, such as total caloric intake and genetic factors also differ between the ethnic groups. Including the ethnic group in the analysis is thus also a way of handling unmeasured confounders.

Likewise, current smoking and harmful alcohol consumption were associated with cardiometabolic risk factors in our study, not surprisingly since they are well established modifiable factors in cardiology[21–23]. Lifestyle choices are important contributors to the global rise in chronic disease, including CVDs[24]. Moreover, having a family history of diabetes was significantly associated with cardiometabolic risk factors in our study. Our findings are in line with most of the earlier studies, which demonstrated familial aggregation of diabetes and related phenotypes[25].

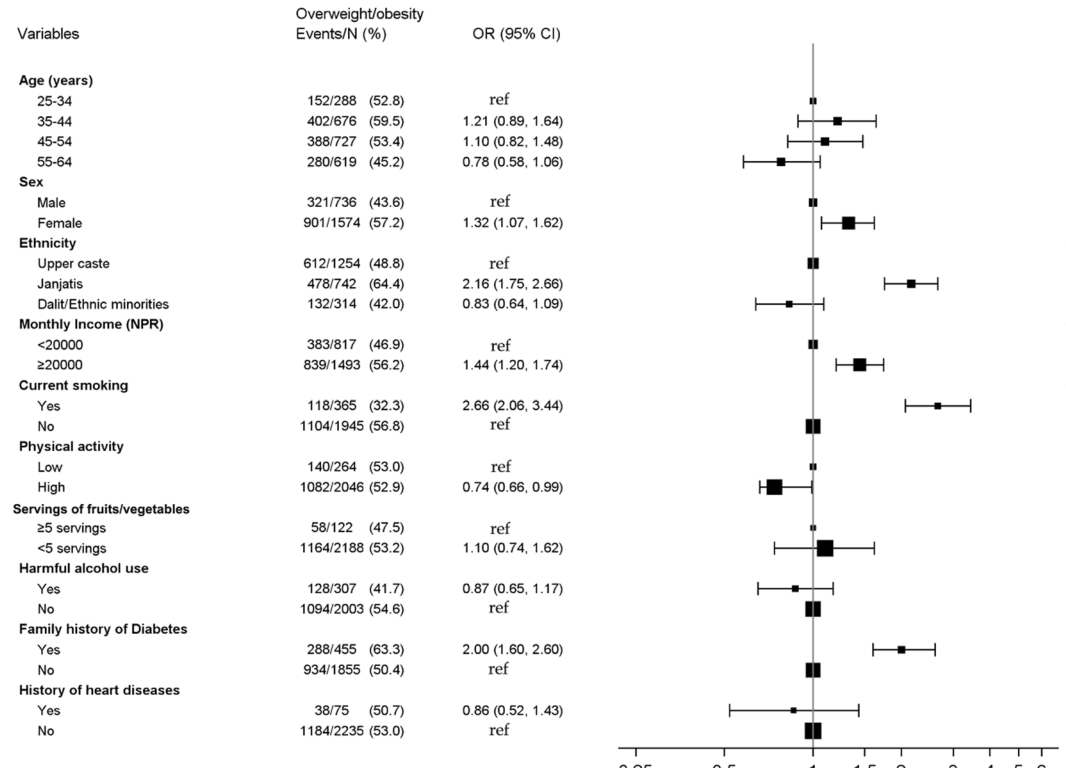

Figure 3. Risk factors for overweight/obesity.

Our finding that about two-thirds of the participants had at least one of the risk factors particularly concerns because risk factors together show a multiplicative, rather than merely additive, synergistic effect[26]. The high burden of risk factor clustering, on the one hand, supports the 2016 estimates of the Global Burden of Disease (GBD) for Nepal which established CVD as the leading cause of both mortality and morbidity[27]. On the other hand, it portends escalating rates of CVD-related morbidity and premature mortality in Nepal, and thus a need to curb these risk factors at the earliest onset. From policy and practice perspectives, the clustering highlights the maturation of cardiometabolic syndemics from single to multiple cardiometabolic risk factors in Nepal and posits a need to address these risk factors holistically in both policy and clinical practice.

Strengths and limitation. Our study adds to the limited literature on cardiometabolic risk factors and their clustering in Nepal's local context. Further, both previous studies evaluated the composite metabolic syndrome as the outcome and as such this is the first study to assess the burden of possible combinations of risk factors comprehensively. Our study has also benefited from a large and representative sample size and use of standardized STEPS tools.

This study also has some limitations. First, due to financial constraints, the measurement of cholesterol and lipid profiles, important markers of cardiometabolic risk, was not feasible. Likewise, measurement of glycosylated haemoglobin A (HbA1c) or oral glucose tolerance tests was not possible. The latter limitation is somehow eased since, according to WHO guidelines, a single fasting plasma glucose estimation is acceptable for epidemiological purposes[28]. Additional limitation includes the study participants were nested to COBIN cohort and some of them were part of intervention study that was conducted earlier. Further, cross-sectional study design, which although appropriate for the prevalence estimates of the risk factors, inhibits us from establishing any causal inference between correlates and outcomes. Future studies with prospective design may be helpful to elucidate the longitudinal progression of the cardiometabolic risk factors, from single to consecutive concurrence.

Conclusions

Our study found a high prevalence and clustering of multiple cardiometabolic risk factors among adults in semi-urban Nepal, highlighting the growing cardiometabolic syndemics from single to multiple risk factors. The concentration of the risk factors, individually and as clusters, in specific groups such as older population, male, Janajati ethnicity, and those with a family history of diabetes posit specific higher risk groups for targeting interventions. Our findings of high burden and clustering of cardiometabolic risk factors coupled with recent GBD estimate establishing CVD as the leading cause of both mortality and morbidity in Nepal, make it clear that prevention, treatment, and control of cardiometabolic risk factors should be a public health priority in the country. Interventions tailored to reduce the burden of multiple risk factors before cardiometabolic diseases appear, such as raising awareness and conducting regular health screenings are urgently needed in a resource constrained setting like Nepal.

Methods

Study design and population. This study is embedded within a community-based management of non-communicable disease in Nepal study (COBIN)[29]. The COBIN, is an ongoing prospective cohort that aims to examine non communicable disease burden and identifying possible strategies for community-based management of major non-communicable diseases. The COBIN cohort was established in 2015 (COBIN Wave I) by selecting 2,815 random participants from a semi-urban area of Pokhara Metropolitan City of Nepal. We did a follow up study (COBIN Wave II) after one year among 2,310 participants (82%)[30]. At follow up, we added additional information on fasting blood glucose test. Ethical Review Board of Nepal Health Research Council approved this study (reference number 766; reg. no 263/2016). Respondents provided informed written consent before data collection was initiated. Participation was voluntary, and participants' identity was kept confidential. All research was reported in accordance with strengthening the reporting of observational studies in epidemiology (STROBE) statement[31].

Data collection and variables. Data collection, including the physical and biochemical measurements, took place during household visits by trained research assistants with health background. The World Health Organization (WHO) Stepwise Surveillance (STEPS) instrument was used for data collection[32]. This previously validated and publicly available tool was also used in Nepal's only nationwide surveillance of non-communicable diseases risk factors[10]. Age, education, ethnicity, occupation, height and family income were obtained from COBIN Wave I survey where as other socio-demographic, behavioural and anthropometric information, as defined below were assessed.

Cardiometabolic risk factors. The outcome of interest was three well-known risk factors for CVD[33], namely diabetes, hypertension, and overweight/obesity.

Hypertension. Three consecutive blood pressure measurements were taken using a digital blood pressure monitor after participants were seated for five minutes. The average of the last two measurements was recorded. Hypertension was defined as a systolic blood pressure of $\geq$140 mm Hg and/or diastolic blood pressure of $\geq$90 mm Hg, and/or self-reported anti-hypertensive therapy[34].

Diabetes. Fasting blood glucose level was measured by the capillary finger prick method using a standardized digital glucometer. The fasting procedure is described elsewhere[9]. Diabetes was defined following the WHO's standard definition, as blood glucose level $\geq$126 mg/dl (7.0 mmol/l) or currently on medication for raised blood glucose[35]. Additionally, participants' family history of diabetes and history of heart disease were asked as a binary response.

Overweight/obesity. Anthropometric measurements for calculating body mass index (BMI) included height measured in meter (m) using a portable standard stature scales, and weight in kilogram (kg) using digital personal scales. Participant's BMI calculated as weight/height^2, was categorized into normal vs. overweight or obese (BMI $\geq$ 25 kg/m^2)[36].

Clustering of cardiometabolic risk factors. In line with our study objective, clustering of risk factors of the metabolic syndrome was an additional outcome of interest. Based on the three risk factors, participants could be categorized into two broad combinations of risk factors, i.e., those with two risk factors (six unique combinations of having either or both of the two conditions such as hypertension and diabetes; hypertension or diabetes; hypertension and overweight/obesity; hypertension or overweight/obesity; diabetes and overweight/obesity; diabetes or overweight/obesity), and those with three risk factors (two unique combinations: all three risk factors and at least one risk factor).

Behavioral risk factors. Behavioral risk factors included current smoking, harmful alcohol use, physical inactivity, and insufficient fruits and vegetables intake, were obtained by self-report. We used the STEPS survey tool, previously validated in Nepalese context, to measure current smoking in our study[32]. Current smoking was defined as smoking at least one cigarette per day for the past 12 months. Both ex-smokers (those who smoked previously but quit subsequently) and those who never smoked cigarettes were categorized as current non-smokers. Participants were asked about the number of standard drinks consumed in the past 30 days. Among the current alcohol consumers (consumed alcohol in the past 30 days), consuming, in a single occasion per week, 8 or more standard drinks for females and 15 or more standard drinks for males was considered harmful alcohol use[37]. The Global Physical Activity Questionnaire (GPAQ)[38] was used to assess the physical activity level from participants' self-reported number of days and minutes spent on different moderate and/or vigorous activities for work, travel, and recreation. Low physical activity was defined as less than 3,000 metabolic equivalents tasks (MET) minutes of vigorous or moderate activity per week[39]. Participants reported consumption of number of servings of fruits and vegetables per week, later categorized $\geq$5 serving per week or $<$5 servings per week, following the recommended guidelines[40].

Socio-demographic factors. Age group (25–34, 35–44, 45–54, 55–64 years), sex (male/female), ethnicity (Upper caste/ Janajatis/ Dalits and ethnic minorities), and family's monthly income ($<$20,000 and $\geq$20,000 NPR) were self-reported by the participants.

Statistical analyses. All statistical analyses were performed using Stata 14.2 (StataCorp, College Station, Texas, USA). Characteristics of the study participants are expressed as mean and standard deviation (SD) for

normal continuous variables, and as proportions for categorical variables. The socio-demographic and behavioral characteristics of the study participants across the risk factors and their clustering were compared by Pearson's chi-squared test. Univariate and multivariate binary logistic regression analyses were performed to assess the covariates associated with cardiometabolic risk factors, individually and as clusters. The adjusted model included all the covariates included in this study, i.e., age group, sex, ethnicity, monthly income, smoking, physical activity, fruits/vegetables intake, harmful alcohol use, family history of diabetes, and history of heart diseases, which were selected a priori.

Ethical approval and informed consent. Ethical Review Board of Nepal Health Research Council approved this study (reference number 766; reg. no 263/2016). Respondents provided informed written consent before data collection was initiated. Participation was voluntary, and participants' identity was kept confidential. All research was reported in accordance with strengthening the reporting of observational studies in epidemiology (STROBE) statement[9,31].

Data availability

Data are available upon reasonable request made in writing to the study team.

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Author contributions
B.G. and S.R.M. conceived the idea for this research publication. B.G. performed the experiments, analysed the data, interpreted results, and wrote the first draft. S.R.M., S.G., M.R.H.H., K.J.S., K.S., P.B.S., D.N. and P.K. contributed in revision of the manuscript. All authors have read and approved the final version for publication.

Competing interests
The authors declare no competing interests.

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Supplementary paper III

Vitalograph copd-6 mini-spirometer as more than a screening device: Validation in a healthy Ugandan population

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The supplementary paper does not form part of the dissertation.

Supplementary paper III has not been published yet and has therefore been removed from this version of the document.