Qualitätsmanagement bei der Nutzung biologischer Proben im Rahmen der epidemiologischen Multizentrenstudie IDEFICS

Dissertation

Zur Erlangung des Grades eines Doktors der Naturwissenschaften
- Dr. rer. nat.-

vorgelegt von

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Dezember 2015

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Termin des Promotionskolloquiums: 27.4.2016

“Quality is never an accident. It is always the result of intelligent effort.”

John Ruskin (1819-1900)

Zusammenfassung

Die vorliegende Dissertation beschäftigt sich mit dem Qualitätsmanagement bei der Nutzung biologischer Probenmaterialien in der Epidemiologie. Sie hatte zum Ziel, Methoden zur Qualitätssicherung bei der Probengewinnung für die IDEFICS-Studie („Identification and prevention of Dietary- and lifestyle-induced health EFfects In Children and infantS“) zu implementieren und zu evaluieren.

Bei wechselnden Bedingungen der Probennahme (Personal, Uhrzeiten, Lagerungszeiten, Umgebungstemperaturen, Transportbedingungen), vor allem in internationalen multizentrischen Studien, ist ein zentrales Qualitätsmanagement für die Nutzung biologischer Probenmaterialien zur Gewährleistung einer standardisierten Probenqualität unabdingbar. Bei Kindern ist biologisches Probenmaterial eine besonders wertvolle Ressource, die vor allem bei gesunden Kindern nicht ohne weiteres zu erhalten ist. Im Umgang damit sind also die größtmögliche Sorgfalt und ein hohes Maß an Verantwortung geboten.

Zunächst ging es um die Frage, wie eine hohe und standardisierte Probenqualität im Rahmen einer großen multizentrischen Kohortenstudie bei Kindern gewährleistet werden kann. Hierfür wurde ein Qualitätsmanagement-System für die Sammlung biologischer Proben (QMS-BS) auf Basis der ISO 9000-Normen entwickelt und in der Europäischen Multizentrenstudie IDEFICS implementiert. Die Qualität der biologischen Daten in der IDEFICS-Studie wurde anschließend anhand von Qualitätsindikatoren evaluiert, die im Rahmen des QMS-BS erhoben wurden. Für alle drei untersuchten Probentypen (Blut, Urin und Speichel) konnten potentielle Qualitätsprobleme identifiziert werden. Dabei zeigte sich die Auswirkung unterschiedlicher Probenqualität auf die Konzentration biologischer Marker. So hatten beispielsweise Kinder, die nicht nüchtern zur Blutabnahme erschienen waren, deutlich erhöhte Insulin- und Triglyzeridwerte und das nächtliche Aufsuchen der Toilette führte ebenso zu veränderten Konzentrationen der Biomarker im Urin wie die Verwendung einer Urinprobe, die erst nach dem ersten Morgenurin gesammelt wurde. Fehlende Standardisierung oder der Einschluss fehlerhafter Daten aufgrund mangelnder Dokumentation potentieller Fehlerquellen führen demzufolge zu einer höheren Streuung der Messwerte, so dass kleine Unterschiede zwischen verschiedenen Gruppen nicht mehr erkannt werden können.

Auf Basis der hohen, standardisierten Qualität der biologischen Materialien und Laboranalysen konnten die in der IDEFICS-Studie erhobenen Daten in der Folge auch zur

Generierung von pädiatrischen Referenzwerten für biologische Marker herangezogen werden. In der Literatur gab es bis dato nur Daten aus nationalen Studien, deren Studiengröße zudem für die statistische Modellierung von Referenzwerten nicht ausreichend war. Im Rahmen dieses Promotionsvorhabens wurden nun alters- und geschlechtsspezifische Referenzperzentile für Insulin, Glucose, HbA1c und HOMA-IR (Homeostasis Model Assessment for Insulin Resistance) bei normalgewichtigen, vorpubertären europäischen Kindern erstellt. Für alle vier Marker lagen die Messwerte deutlich unter den für Erwachsene bekannten Referenzbereichen und zeigten einen ansteigenden Trend über die Altersgruppen. Insulin- und HOMA-IR-Werte waren bei Mädchen in allen Altersgruppen höher als bei Jungen, Glukose-Werte waren bei Jungen etwas höher.

Eine weitere Analyse der biologischen Probendaten im Rahmen des Promotionsvorhabens beschäftigte sich prospektiv mit anthropometrischen und lebensstilbezogenen Risikofaktoren für kindliche Insulinresistenz. Insulinresistenz, d.h. das verminderte Ansprechen auf das Hormon Insulin, ist eine der wesentlichen Vorstufen des Typ 2 Diabetes. Gleichzeitig fördert sie die Entstehung von Herzkreislauf-Erkrankungen. Das möglichst frühzeitige Erkennen von Insulinresistenz und deren Prävention ist daher von entscheidender Bedeutung bei der Bekämpfung dieser Erkrankungen. Auf Grundlage der IDEFICS-Daten bestätigte sich der bekannte Einfluss von Übergewicht und Adipositas auf die Entstehung von Insulinresistenz. Es zeigte sich darüber hinaus aber auch die Wirkung des Lebensstils. So konnten Medienkonsum, Mediennutzung im Kinderzimmer und Fettkonsum als unabhängige Risikofaktoren für Insulinresistenz identifiziert werden, während objektiv gemessene körperliche Aktivität sich protektiv auf die Entwicklung von Insulinresistenz auswirkte.

Das im Rahmen dieser Promotion entwickelte und evaluierte QMS-BS konnte entscheidend zu einer hohen Qualität der biologischen Proben und Daten in der IDEFICS-Studie beitragen. Auf dieser Basis war es sowohl möglich, erstmals pädiatrische Referenzwerte für Marker der Insulinresistenz zu generieren, als auch bekannte und vermutete Determinanten kindlicher Insulinresistenz im Europäischen IDEFICS-Kollektiv zu bestätigen.

Inhalt

Originalarbeiten

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4. Peplies J, Günther K, Börnhorst C, Fraterman A, Russo P, Veidebaum T, Tornaritis M, De Henauw S, Marild S, Molnar D, Moreno LA, and Ahrens W on behalf of the IDEFICS consortium. **Determinants of insulin resistance in preadolescent children: results from the IDEFICS study.** (eingereicht bei Pediatrics)

Abbildungen

Tabellen

Abkürzungen

BIPS	Bremer Institut für Präventionsforschung und Sozialmedizin (heute Leibniz-Institut für Präventionsforschung und Epidemiologie)
BRCA1, BRCA2	Gene, die mit der Entstehung von Brustkrebs assoziiert sind
CRP	C-reaktives Protein
DNA	Desoxyribonukleinsäure
EDTA	Ethylendiamintetraazetat - Chelatbildner zur Hemmung der Blutgerinnung
EMLA	wirkstoffhaltiges Pflaster mit Lidocain und Prilocain
EU	Europäische Union
GAMLSS	Generalised Additive Models for Location Scale and Shape
HbA1c	Glykosyliertes Hämoglobin
HDL	High density lipoprotein (Lipoprotein hoher Dichte)
HOMA-IR	Homeostasis Model Assessment for Insulin Resistance
IDEFICS	Studienacronym: **I**dentification and prevention of **D**ietary- and lifestyle-induced health **EF**fects **I**n **C**hildren and infant**S**
IR	Insulinresistenz
ISO	International Organization for Standardization
CTX-Peptid	Quervernetztes C-Telopeptid des Typ I Kollagens
PAXgene	Blutröhrchen zur Stabilisierung intrazellulärer Ribonukleinsäure
RNA	Ribonukleinsäure
SOP	Standard Operation Procedure
TMF	Telematikplattform für Medizinische Forschungsnetze
QMS-BS	Qualitätsmanagmentsystem für die Sammlung biologischer Proben (Quality management system for biological samples)

1 Einleitung

Die Nutzung biologischer Probenmaterialien zur Expositionserfassung oder zur Messung von Erkrankungsmarkern ist in epidemiologischen Studien in den letzten zwei Jahrzehnten zur Routine geworden, da diese im Vergleich zu auf Fragebögen basierenden Methoden einen großen Zugewinn an Objektivität bringen. Dabei profitiert die molekulare Epidemiologie von der rasanten Entwicklung auf den Gebieten der Molekularbiologie, Zytogenetik und Biochemie, welche sowohl die Entdeckung zahlreicher neuer Biomarker, als auch eine Vielzahl neuer Technologien und Hochdurchsatzverfahren hervorgebracht haben.[1,2,3,4]

In der epidemiologischen Ursachenforschung wurde die Kausalkette vom Risikofaktor zum Endpunkt lange Zeit mit einer dazwischenliegenden „Blackbox“ dargestellt, welche die Unsicherheiten bezüglich der Pathogenese verbildlicht. In den 1990er Jahren verbreitete sich dann nach und nach die Idee, diese Blackbox durch die Betrachtung geeigneter Biomarker zu füllen (siehe Abbildung 1-1). Zu dieser Zeit war eine Unterteilung in drei wesentliche Typen von Biomarkern bereits etabliert: Marker der Exposition, Marker des Effekts und Marker der Suszeptibilität.[5]

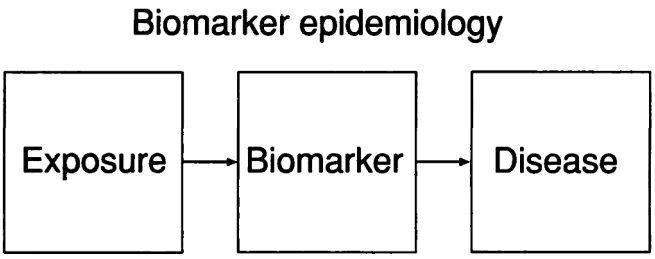

Abbildung 1-1: Biomarker als Indikatoren der Krankheitsentstehung (aus Grandjean, 1995)

Mit Markern der Exposition bezeichnet man die interne Dosis aufgenommener Schadstoffe/ Metabolite (z.B. PCB in Muttermilch, Blei in den Fingernägeln), bestimmter Nahrungsmittelbestandteile (z.B. Spurenelemente im Urin, Fette im Blut) oder die effektive Konzentration von Arzneimitteln im Blut oder Zielgewebe. Marker der Exposition dienen der exakteren Expositionsermittlung im Vergleich zu durch Befragung erhobenen Expositionen.

Marker des Effekts dienen der Absicherung von Diagnosen (z.B. Entzündungsmarker im Blut oder Beta-Amyloid im Liquor von Alzheimer-Patienten) oder stellen vorgezogene Erkrankungsendpunkte bzw. Risikoindikatoren dar (z.B. Chromosomenveränderungen, oder beeinträchtigte DNA-Reparatur für bestimmte Krebserkrankungen oder

Insulinresistenz für Typ 2 Diabetes). Mit Hilfe von Markern des Effektes kann im Idealfall eine frühzeitige Erkennung von Erkrankungen erfolgen.

Unter Markern der Suszeptibilität versteht man genetische oder erworbene Anfälligkeiten, die zu einem veränderten Metabolismus führen können. Aufgenommene Stoffe, wie z.B. Karzinogene oder Cholesterine, werden unterschiedlich verstoffwechselt, was zu einer individuell verschiedenen Konzentration dieser Stoffe im Blut und anderen Kompartimenten führt. Bei einer familiären Hypercholesterinämie beispielsweise fehlen den Betroffenen bestimmte Rezeptoren, so dass Cholesterin nicht im normalen Maß in die Zellen aufgenommen werden kann. Ein weithin bekanntes Beispiel ist die Mutation in den Brustkrebs-Genen BRCA1 oder BRCA2, die mit einer stark erhöhten Brustkrebsrate bei Frauen einhergeht. Eine erworbene Suszeptibilität kann z.B. durch eine verminderte DNA-Reparatur nach einer Exposition mit Schwermetallen entstehen.[6,7]

In der IDEFICS-Studie („Identification and prevention of Dietary- and lifestyle-induced health EFfects In Children and infantS“) wurden alle drei Arten von Biomarkern untersucht, wobei die Zuordnung nicht immer eindeutig war. Blutfette gehören beispielsweise zu den Markern der Exposition, in diesem Fall der Ernährung, können aber ebenfalls als Marker des Effektes betrachtet werden, da eine dauerhaft erhöhte Konzentration von z.B. Gesamtcholesterin ein erhöhtes Risiko für Herz-Kreislauferkrankungen darstellt. Marker der Suszeptibilität wurden in der IDEFICS-Studie in Form genetischer Risikoprofile und Genexpressionsmuster untersucht, stehen aber nicht im Fokus dieser Promotion.

Die Verwendung von Biomarkern in epidemiologischen Studien bringt eine Reihe möglicher Fehlerquellen mit sich.[8] So beinhalten epidemiologische Studien oft wechselnde Bedingungen der Probennahme und -verarbeitung[3] (Personal, Uhrzeiten, Lagerungszeiten, Umgebungstemperaturen, Transportbedingungen, Entnahme-materialien), insbesondere in internationalen multizentrischen Studien, in denen neben verschiedenen Zentren auch noch unterschiedliche Sprachen und kulturelle Gegebenheiten berücksichtigt werden müssen. Die molekularepidemiologische Praxis erfordert daher eine streng standardisierte Verarbeitung und Lagerung der biologischen Probenmaterialien.[1,9] Die konsequente Standardisierung von Zeiten und Temperaturen dient nicht zuletzt der Verringerung der intraindividuellen Variabilität der biologischen Marker. Diese tritt z.B. aufgrund zirkadianer Schwankungen auf und lässt sich, insbesondere bei nur einmaliger Probennahme, leider nur schwer abschätzen.

Die Untersuchung von Bioproben bei Kindern birgt verschiedene zusätzliche Herausforderungen: Ethische Probleme, Probleme bei der Präanalytik und das Fehlen pädiatrischer Grenzwerte für die meisten gängigen Biomarker. Die Einwilligungsquote für invasive Entnahmemethoden ist bei gesunden Kindern besonders gering. Die Präanalytik bedarf bei einer pädiatrischen Studienpopulation besonderer Aufmerksamkeit, da es für Kinder sehr viel schwieriger ist, spezifische Anforderungen der Probensammlung einzuhalten: so schaffen es Kinder zum Teil nicht, morgens vor der Blutabnahme ohne Nahrung, Saft oder Milch auszukommen. Auch die Abnahme von Morgenurin erfordert eine gewisse Disziplin beim morgendlichen Toilettengang, die Kindern sehr viel mehr abverlangt als Erwachsenen.

Die Entwicklung von Qualitätsmanagementprozeduren, wie z.B. 'Standard Operation Procedures' (SOPs) ist daher die Grundvoraussetzung zur Gewährleistung einer standardisierten Probenqualität und valider Messergebnisse geworden[10] und ist in neueren Studien mittlerweile auch etabliert.[11] Wissenschaftliche Literatur zu methodischen Aspekten und Problemen der Probennahme bzw. Präanalytik in epidemiologischen Studien ist aber leider nach wie vor selten.

2 Ziel der Dissertation

Die vorliegende Dissertation beschäftigt sich mit dem Qualitätsmanagement für biologische Probenmaterialien in der Epidemiologie. Sie hatte zum Ziel, Methoden zur Qualitätssicherung bei der Probengewinnung unter den schwierigen Rahmenbedingungen der multizentrischen IDEFICS-Studie bei präadoleszenten Kindern zu implementieren und zu evaluieren. Die gewonnenen Biomarker-Daten wurden in der Folge für die Erstellung pädiatrischer Referenzwerte und für eine ätiologische Fragestellung genutzt.

3 Einbettung der Dissertation in die IDEFICS-Studie

3.1 Notwendigkeit von Biomarkern für die IDEFICS-Studie

Bei der IDEFICS Studie handelt es sich um eine multizentrische, prospektive Kohortenstudie, welche von 2006 bis 2012 in 8 europäischen Ländern (Italien, Estland, Zypern, Belgien, Schweden, Deutschland, Ungarn und Spanien) durchgeführt wurde. Ihr wichtigstes Ziel war die Ursachenforschung bei ernährungs- und lebensstilbedingten Erkrankungen von 2-9-jährigen Kindern, mit einem Fokus auf Übergewicht und Adipositas. Darüber hinaus wurde im Rahmen der IDEFICS-Studie ein gemeindebasiertes Interventionsprogramm zur Primärprävention von Übergewicht und Adipositas bei Kindern entwickelt, implementiert und evaluiert. 23 Forschungsinstitute und klein- und mittelständische Unternehmen aus 11 verschiedenen EU-Mitgliedsstaaten nahmen an diesem von der Europäischen Union (EU) im 6. Forschungsrahmenprogramm geförderten Integrierten Projekt teil. Die Studie wurde vom Bremer Institut für Präventionsforschung und Sozialmedizin (BIPS), einer zentralen wissenschaftlichen Einrichtung der Universität Bremen koordiniert (heute Leibniz-Institut für Präventionsforschung und Epidemiologie BIPS). Ein Überblick über Design und Ablauf des ätiologischen und präventionsbezogenen Studienteils findet sich in der Literatur.[12,13]

Bei der Analyse von Biomarkern im Rahmen der beiden IDEFICS-Erhebungsphasen ging es einerseits um die Bestimmung von (vorgezogenen) Erkrankungsendpunkten (z.B. Blutglukose und Insulin für Typ 2 Diabetes, metabolisches Syndrom und Insulinresistenz) als auch um die Konzentrationsanalyse von Markern der Exposition (z.B. Spurenelemente im Urin, Vitamin D im Serum) und um die Bestimmung genetischer Risiken (ungünstige genetische Veranlagung als Marker der Suszeptibilität).

Die biologischen Marker lieferten hierbei einen wichtigen Beitrag zur Beantwortung zentraler Studienfragen der IDEFICS-Studie[14], in erster Linie für

- die Bestimmung der Prävalenz ernährungs- und lebensstilbedingter Erkrankungen (von denen beispielsweise das metabolische Syndrom unter anderem über die Marker des Fett- und Energiestoffwechsels definiert wird) und
- die Ätiologie von Adipositas und Erkrankungen (Knochengesundheit, metabolisches Syndrom, Diabetes) und die Analyse von Risikofaktoren (Ernährung, Lebensstil, psychosoziale und genetische Faktoren).

Insbesondere für die Verlaufsbeobachtung spielten die biologische Marker eine wichtige Rolle, da nur zwei Jahre zwischen den beiden Erhebungszeitpunkten der IDEFICS-Studie lagen und in dieser Zeitspanne nur von einer geringen Inzidenz bestimmter Erkrankungen wie Typ 2 Diabetes ausgegangen werden konnte. Insulinresistenz dagegen als assoziierter bzw. vorgezogener Endpunkt dieser Erkrankung war sehr viel häufiger zu erwarten. Auch bei der Evaluation der IDEFICS-Intervention kamen biologische Marker zum Einsatz. So befindet sich eine Veröffentlichung zum Einfluss der Intervention auf Marker der Insulinresistenz und andere metabolische Marker aktuell in der Begutachtung.[15]

3.2 Auswahl der Biomarker und Bioproben für die IDEFICS-Studie

Bei der Auswahl der analysierten Marker gab es zunächst eine lange, an den Forschungsfragen der Studie orientierte Wunschliste, welche neben Markern des Energiestoffwechsels (Cholesterin, Triglyzeride, HDL-Cholesterin, LDL-Cholesterin, Apolipoproteine, Glukose, HbA1c, Pro-Insulin, Insulin, Homocystein, Fettsäureprofil) und des Knochenstoffwechsels (Vitamin D, CTX-Peptide) auch diverse Ernährungsmarker (Vitamin A/ Beta-Carotin, Folsäure, Calcium, Phosphat, Magnesium, Iod, Eisen, Transferrin, Ferritin, Harnsäure, Albumin), Entzündungsmarker (CRP, Interleukin 6), endokrinologische Marker (Leptin, Adiponectin, Renin, Angiotensin, Ghrelin, Cortisol, die Peptidhormone Glucagon-like-peptide 1 und Peptid YY3), Leberenzyme (ALT, AST), einen Gerinnungsfaktor (PAI-1), die antioxidative Kapazität und ein komplettes Blutbild umfasste. Nicht im Fokus dieser Dissertation aber ein weiterer wichtiger Bestandteil der IDEFICS Studie war die Untersuchung genetischer Marker und der transkriptionellen Regulation derselben, um die Kausalzusammenhänge im Bereich der Stoffwechselwege näher zu beleuchten.

Diese Liste musste aufgrund der finanziellen Möglichkeiten, präanalytischen Anforderungen und wegen des limitierten Entnahmevolumens kindlichen Blutes im Weiteren stark gekürzt werden. Einige der Analysen wurden daher nur in Subgruppen und zunächst nur bei der Basisuntersuchung durchgeführt. Wo es möglich war, wurden die Biomarker im Urin und nicht im Blut untersucht, um die benötigte Menge kindlichen Blutes zu reduzieren bzw. die Teilnahmequote für die jeweiligen Marker zu erhöhen.

Die teilnehmenden Kinder der IDEFICS-Erhebungswellen und deren Sorgeberechtigten wurden um die Zustimmung zur Abnahme biologischer Probenmaterialien bei den Kindern gebeten: einer venösen Blutprobe im nüchternen Zustand, einer Morgenurinprobe und einer Speichelprobe.

Im Idealfall wurden zwei bis drei Blutröhrchen mithilfe eines Butterfly-Vacutainer-Blutentnahmesystems abgenommen: ein natives Röhrchen (11 ml) zur Serumgewinnung und ein mit EDTA beschichtetes Röhrchen (7 ml) zur Auftrennung von Plasma und weißen und roten Blutkörperchen. In einer Unterstichprobe wurden zusätzlich 2,5 ml Blut in einem PAXgene Röhrchen (Becton Dickinson Franklin Lakes, NJ, USA) entnommen, das mit einem RNA-stabilisierenden Zusatz für die Genexpressionsanalyse versetzt war. Alternativ konnte auch eine Kapillarblutentnahme aus der Fingerbeere erfolgen, um zumindest einige Analysen durchführen zu können.

Tabelle 3-1: Überblick über die im Rahmen der IDEFICS-Studie verwendeten Probentypen und analysierten biologischen Marker

Probentyp	Biomarker	Endpunkt
Natives Blut	Blutglukose, Gesamtcholesterin, HDL-Cholesterin, Triglyzeride	Metabolisches Syndrom, Insulinresistenz, Energiestoffwechsel
	Fettsäureprofil (Teststreifen)	Ernährungsgewohnheiten
EDTA-Plasma Rote Blutkörperchen	Fettsäureprofil (konventionelle Methode zur Validierung der Teststreifen-Methode)	Ernährungsgewohnheiten
EDTA-Vollblut	HbA1c	Diabetes, Blutzuckerlangzeitkontrolle
Serum	Insulin	Insulinresistenz
	C-reaktives Protein (CRP)	Entzündung
	Leptin Adiponectin	Energiestoffwechsel
	Vitamin D Kalzium quervernetztes C-Telopeptid des Typ I Kollagens (CTX)	Knochenstoffwechsel
RNA-stabilisiertes Vollblut (PAXgene-Röhrchen)	Genexpression bei ausgewählten Genen	Genexpression
Morgenurin	Uringlukose	Diabetes
	Albumin	Metabolisches Syndrom
	Kreatinin	Referenzmarker
	Spurenelemente (Na, K, Mg, P, Ca)	Ernährungsgewohnheiten
	Cortisol	Chronischer Stress
Speichel (Oragene DNA-Selbstentnahme Kit OG 300 oder Oragene OG 250 zur Fremdentnahme mit Hilfe von Schwämmchen)	Ausgewählte Kandidatengene (Sequenzierung der tag-Haplotypen)	Genetische Risikofaktoren

Morgenurin wurde durch die Eltern zu Hause gesammelt. Hierzu erhielten die Familien einen 125 ml Urinbecher, eine genaue Anleitung für die Urinsammlung und einen Dokumentationsbogen. Mindestens drei 6 ml-Röhrchen des Urins wurden für die Laboranalysen eingefroren.

Für die spätere DNA-Extraktion wurden Speichelproben gesammelt. Bei älteren Kindern wurde hierfür ein Selbstentnahme-Kit (Oragene OG 300) verwendet, mit dessen Hilfe eine 2 ml Speichelprobe gesammelt wurde. Kleineren Kindern wurde eine Speichelprobe mithilfe von 5 Speichel-Schwämmchen (Oragene OG 250) durch das Untersuchungspersonal entnommen (DNA Genotek Inc., Kanata, Ontario, Canada).

Eine Zusammenfassung aller im Rahmen der IDEFICS-Basiserhebung analysierten Biomarker und verwendeten Probenmaterialien ist in Tabelle 3-1 dargestellt.

3.3 Rahmenbedingungen für die biologische Probennahme bei IDEFICS

Obschon die Notwendigkeit von biologischen Markern zur Beantwortung der Studienfragen unumstritten war, wurden die Bedingungen für die Probennahme in der Vorbereitungsphase des EU-Projektes vom Konsortium kontrovers diskutiert. So gab es beispielsweise den Vorschlag, sich auf die Kapillarblutentnahme aus der Fingerbeere des Kindes zu beschränken. Bei geeigneter Vorbereitung der Hand (Anregung der Durchblutung durch Wärme) können auf diese Weise Volumina von einem oder mehreren Millilitern Blut entnommen werden.[16] Schlussendlich wurde die Entnahme von Kapillarblut aber nur als Alternative zur bevorzugten Entnahme von venösem Nüchternblut vorgesehen.

Grundsätzlich sollte Kindern ihrem niedrigen Körpergewicht entsprechend auch nur ein geringes Blutvolumen entnommen werden. Laut einem Literaturüberblick, welcher Richtlinien und Studien zur pädiatrischen Blutentnahme zusammenfasst, sollte bei kleinen Kindern nicht mehr als 1-3% des kindlichen Blutvolumens entnommen werden[17]. Den 2-9-jährigen Kindern der IDEFICS Basisuntersuchung wurde demzufolge ein maximales Blutvolumen von 1% (10-25 ml) entnommen. Wenn der erste Versuch der venösen Blutentnahme fehlschlug, durfte aus ethischen Gründen kein zweiter Entnahmeversuch erfolgen.

Die Rahmenbedingungen bei den IDEFICS-Surveys unterschieden sich grundlegend von denen eines klinischen Settings. Neben den ohnehin schwer zu standardisierenden Feldbedingungen dieser populationsbasierten und internationalen Studie (unterschiedliche Situationen der Probennahme, unterschiedliche Uhrzeiten je nach kulturell bedingt unterschiedlichen Anfangszeiten von Schulen und Kindergärten, unterschiedliche Lagerungszeiten und Transportbedingungen), die nur durch konsequente Standardisierung ausgeglichen werden konnten, waren die

Untersuchungsteams für die biologische Probennahme in allen acht Ländern auch auf die Mitarbeit der Eltern/ Sorgeberechtigten angewiesen.

Das Sammeln des ersten Morgenurins musste zwingend im häuslichen Umfeld erfolgen und dort auch von den zuständigen Erwachsenen dokumentiert werden Auf einem Dokumentationsbogen sollten die Eltern notieren, falls die Urinprobe nicht den ersten Morgenurin enthielt, für mehr als zwei Stunden ungekühlt war oder falls das Kind nachts auf die Toilette gegangen war. Die Urinprobe wurde von den Kindern oder Eltern am Entnahmetag zum lokalen Untersuchungszentrum transportiert und dort abgegeben.

Für die venöse Blutentnahme sollte eine Stunde zuvor ein Lokalanästhetikum in Form eines EMLA-Pflasters auf die Haut des Kindes appliziert werden. Die Eltern wurden vor der Blutabnahme nach Abweichungen vom vorgesehenen Nüchternstatus und ggf. zum Zeitpunkt der letzten Nahrungsaufnahme befragt.

Die Einstellung der Eltern gegenüber invasiven Entnahmetechniken war zwischen den acht Europäischen Ländern sehr verschieden, was sich in der recht unterschiedlichen Item-Responserate für die Blutabnahme widerspiegelt (siehe Abschnitt 4.1).

3.4 Probenlogisitik und Probenanalytik in der IDEFICS-Studie

Alle Materialien zur Probenentnahme und –verarbeitung waren standardisiert und wurden zentral eingekauft. Die präanalytische Probenverarbeitung fand in den lokalen Untersuchungszentren oder in lokalen Laboratorien statt, die Probenanalytik erfolgte in zentralen Laboratorien (ein nach ISO 15189 akkreditiertes Labor für die gesamte Blut- und Urinanalytik, mehrere Labore für die DNA-Analytik und je ein weiteres Labor für die RNA-Analytik und die chromatographische Analyse der Blutfettsäuren). Die Blutentnahme und Probenverarbeitung in den Untersuchungszentren wurden ausführlich dokumentiert (Stehzeiten, besonderen Vorkommnisse).

Die Lagerung der biologischen Proben in den Untersuchungszentren erfolgte bei -80°C (Blutproben) oder -20°C (Urinproben). Die Speichelproben wurden bis zur DNA-Extraktion bei Raumtemperatur gelagert. Extrahierte DNA wurde zentral bei -20°C aufbewahrt. Mehrfach pro Erhebungszeitraum wurden die Proben auf Trockeneis zu den zentralen Untersuchungslaboren verschickt. Da Blut als potentiell infektiöses Material und damit als Gefahrgut eingestuft wird, konnten die Proben von einigen Ländern aus nur durch Frachtflugzeuge transportiert werden. Die Einhaltung der für den gefrorenen Transport maximal möglichen Transportzeiten war in Ermangelung von Direktflügen insbesondere

für einige der weiter entfernten Untersuchungszentren eine logistische Herausforderung. Die Qualität der Proben wurde bei der Ankunft in den zentralen Laboratorien geprüft.

Für die Erfassung der Probendokumentation zu Sammlung, Verarbeitung und Lagerung aller biologischen Probenmaterialien wurde eine Bioproben-Datenbank entwickelt. Jedes Probenaliquot wurde dort mit seinem exakten Lagerort (Gefrierschrank, Fach, Box, Steckplatz) verzeichnet, so dass ein Wiederauffinden jederzeit unkompliziert gelingen konnte. Diese Bioproben-Datenbank sollte auch die Probenverschickung durch die automatisierte Erzeugung von Lieferpapieren vereinfachen. Zur Probenbeschriftung und –erfassung wurden Barcode-Etiketten mit einer unzweideutigen 10-stelligen Identifikationsnummer verwendet. Eine zentrale Bioprobenbank wurde für die nach der Probenanalytik verbleibenden Proben eingerichtet.

Vor der ersten Erhebungsphase der IDEFICS-Studie wurde im Rahmen des Qualitätsmanagements ein ausführlicher Pretest durchgeführt, bei dem die standardisierten Erhebungsprozeduren in allen Untersuchungszentren getestet wurden. Dies umfasste auch eine Testung aller Entnahmetechniken für biologische Materialien, der Verschickungs- und Laborprozeduren und der beteiligten Dokumentationssysteme auf ihre Machbarkeit, ihre Dauer, ihre Fehleranfälligkeit und die Akzeptanz durch die Studienteilnehmenden und Projektmitarbeitenden. Einige Prozeduren wurden aufgrund der Erfahrungen im Pretest modifiziert. So wurde im Bereich der Bioproben beispielsweise das Blutabnahmesystem ausgetauscht und auch einige Röhrchen und Verpackungsmaterialien durch andere Produkte ersetzt.[18]

3.5 Anwendungsfreundliche Studienkomponenten in der IDEFICS-Studie

Im Rahmen der IDEFICS-Studie kamen einige besonders anwendungsfreundliche Studienkomponenten zum Einsatz. Diese waren zwar in der Regel recht hochpreisig, dienten aber sehr erfolgreich der Standardisierung, Erhöhung der Probenqualität und/ oder der Item-Response für die entsprechenden biologischen Probentypen.

Einige der wichtigsten Biomarker des Energiestoffwechsels (Blutglukose, Gesamtcholesterin, HDL-Cholesterin, Triglyzeride) wurden unter Verwendung eines Point-of-Care-Gerätes (Cholestech LDX, Cholestech Corp., Hayward, CA, USA) direkt bei der Blutentnahme gemessen. Da hierfür ein einzelner Tropfen venöses Vollblut oder Kapillarblut genügte, konnten diese Marker zusätzlich bei den 23% der Kinder untersucht werden, von denen nur Kapillarblut entnommen wurde. Dadurch stieg der Anteil an

Point-of-Care-Messungen auf knapp 80% aller Kinder. Für die statistischen Analysen wurden die im venösen und im Kapillarblut gemessenen Daten gemeinsam ausgewertet, da die Unterschiede von vielen Autoren vor allem im Nüchternblut als vernachlässigbar beschrieben wurden.[19,20,21,22] Darüber hinaus erhöhte diese Maßnahme die Zuverlässigkeit der Blutzuckermessung, ohne dafür ein zusätzliches Glukose stabilisierendes Röhrchen verwenden zu müssen, da Glukose in anderen Röhrchen sehr schnell glykolytisch abgebaut wird[23]. Das bei IDEFICS verwendete Point-of–Care-Gerät gilt im Vergleich zu anderen Geräten seiner Art als sehr präzise[21]. Die Messwerte zeigen neben einer geringen Variabilität auch eine gute Vergleichbarkeit mit der Laboranalyse. Eine Ausnahme bilden hierbei die Glukosemesswerte, die bei Point-of-Care-Geräten generell etwas höher ausfallen als bei den entsprechenden Labormethoden[21]. Die Messwerte aus der Point-of-Care-Messung dienten außerdem für ein direktes Feedback an die beteiligten Kinder und Eltern und damit als zusätzliche Motivation, einer venösen oder Kapillarblutabnahme zuzustimmen.

Die Fettsäureanalytik erfolgte routinemäßig mit Hilfe eines innovativen Probennahmesystems, bei dem ein einzelner Tropfen Vollblut auf einem Teststreifen aus Filterpapier (Sigma–Aldrich 11312-1KT) aufgebracht und so für die spätere chromatographische Analytik haltbar gemacht wurde.[24] Da hierfür erneut sowohl venöses als auch Kapillarblut verwendet werden konnte, konnten wiederum Blutproben von 80% der Kinder auf dem Teststreifen des Probennahmesystems für die spätere Fettsäureanalytik haltbar gemacht werden. Ohne dieses innovative System[24] wäre die Fettsäureanalytik nur für die knapp 57% aller Kinder möglich gewesen, denen venöses Blut entnommen wurde.

Ein anderes Beispiel für hohe Anwendungsfreundlichkeit ist die DNA-Gewinnung aus Speichelproben. Durch diese nichtinvasive Methode konnte gewährleistet werden, dass genetische Analysen bei über 90% aller IDEFICS-Kinder möglich waren. Die Speichelproben sind zudem sehr robust und bedurften zunächst keiner weiteren Verarbeitung oder Kühlung durch die lokalen Untersuchungszentren. Die beiden bei IDEFICS verwendeten Entnahmetechniken für die Speichelproben (Selbstentnahme durch Spucken versus Entnahme mit Hilfe von Speichelschwämmchen) wurden in einer methodischen Veröffentlichung aus den Reihen des IDEFICS-Konsortiums miteinander verglichen. Die extrahierte DNA-Menge und Probenqualität war bei der Selbstentnahme etwas höher, bei der Entnahme durch Speichelschwämmchen aber ebenfalls ausreichend.

Grundsätzlich konnte durch beide Methoden hinreichend DNA von guter Qualität gewonnen werden.[25]

Auch die RNA-Extraktion mithilfe der teuren aber sehr anwendungsfreundlichen PAXgen-Röhrchen erwies sich als eine sinnvolle Studienkomponente. In einer weiteren methodischen Veröffentlichung im Rahmen der IDEFICS-Studie wurde die Nutzung von Blutzellen als Quelle von Biomarkern der Transkription beschrieben. Periphere Blutzellen dienten hierbei als Basis für die RNA-Extraktion, nachdem diese mit Hilfe der PAXgen-Röhrchen stabilisiert wurde. Die mRNA-Konzentration wurde für ausgewählte Gene gemessen, welche mit Adipositas und metabolischem Syndrom in Verbindung gebracht werden. Ein erhöhtes Expressionsniveau einzelner Gene war mit Übergewicht/ Adipositas oder metabolischen Markern (HOMA, Cholesterin, Triglyzeride) assoziiert.[26]

4 Einbezogene Veröffentlichungen

4.1 Entwicklung und Anwendung eines Qualitätsmanagementsystems bei der Entnahme und Verwendung von biologischen Proben in epidemiologischen Studien

Publikation 1: Peplies J, Fraterman A, Scott R, Russo P, Bammann K. 2010. Quality management for the collection of biological samples in multicentre studies. European Journal of Epidemiology, 25 (9):607 – 617

Die erste Veröffentlichung im Rahmen dieser Promotion beschreibt das neu entwickelte Qualitätsmanagementsystem für die Sammlung von Bioproben (QMS-BS) und die Anwendung dieses Systems in der IDEFICS-Studie. Die Entwicklung des QMS-BS orientierte sich an den Qualitätsmanagementsystemen, die durch die Internationale Organisation für Normung (International Organization for Standardization) ISO eingeführt wurden. Die ISO 9000-Normen stellen einen Rahmen dar, um ein Qualitätsmanagementsystem in einer Organisation einzuführen und zu betreiben. Bereits Ende des 20 Jahrhunderts hatte eine französische Gruppe eine Anwendung der ISO-Norm 9002 auf die arbeitsepidemiologische Forschung vorgeschlagen[27]. In der hier vorgestellten Veröffentlichung wurde diese weiter modifiziert und auf das Sammeln von biologischen Proben in multizentrischen epidemiologischen Studien übertragen.

Für jeden während der IDEFICS-Erhebungsphasen gesammelten Probentyp wurden verschiedene Instrumente der Standardisierung und Qualitätssicherung etabliert (Standardarbeitsanweisungen (SOPs), Dokumentation und Lenkung der Probengewinnung, Probenverarbeitung und Probenverschickung, zentrale Beschaffung, zentrale Probenanalytik, Qualitätskontrolle der Proben). Alle Zentren nahmen an zentralen Trainingseinheiten teil und führten nachfolgend lokale Trainingsstunden mit ihren Untersuchungsteams durch. Die Anwendung des QMS-BS bei der Probensammlung in der IDEFICS-Studie ist in Tabelle 4-1 dargestellt. Diese wurde anhand verschiedener Qualitätskriterien evaluiert: Item-Responsequoten für die verschiedenen biologischen Probenmaterialien, aufgetretene Probleme bei der Probengewinnung, Einhaltung der Vorgaben des Qualitätsmanagementsystems (Compliance) und Effizienz der eingeführten Dokumentationssysteme und Kontrollprozeduren. Insgesamt erwies sich das QMS-BS als wertvolles Instrument für das Qualitätsmanagement bei der Entnahme biologischer Proben.

Tabelle 4-1: Anwendung des QMS-BS bei der Probensammlung in der IDEFICS-Studie

QMS-BS	Nüchternblut	Morgenurin	Speichel
Spezifische Standard-arbeitsanweisungen (SOPs) für die biologische Probennahme	10 spezifische SOPs	3 spezifische SOPs Anleitung Probensammlung für Eltern	4 spezifische SOPs
Lenkung der Dokumente und Proben	Barcode IDs, Probenmanagement durch die Bioprobendatenbank		
Beschaffung und Unteraufträge	Zentrale Beschaffung aller Materialien		
	Verschiedene zentrale Labore für Blutanalytik, Analyse der Fettsäureteststreifen und RNA-Analyse	Zentrales Labor für die Urinanalytik	DNA-Extraktion in einem Zentrallabor, Genotypisierung in mehreren zentralen Laboren
Prozesslenkung: Probensammlung	Entnahme von venösem Blut, alternativ Kapillarblut Venöses Blut: 11 ml natives und 7 ml EDTA-Blut 2,5 ml in RNA-stabilisierenden Paxgene-Röhrchen	Probensammlung in Urinbecher erfolgt zuhause durch die Eltern	Oragene TM DNA Selbstentnahmekits OG 300/ OG 250 mit Schwämmchen für kleinere Kinder
Prozesslenkung: Verarbeitung in den Untersuchungszentren	Cholestech: erster Tropfen Vollblut mit Testkassette Fettsäuren: zweiter Tropfen Vollblut auf Teststreifen Blutaliquote: Auftrennung von Serum, Plasma, weißen und roten Blutzellen Paxgene-Röhrchen: keine Verarbeitung	Aliquotierung	Keine Verarbeitung
Prozesslenkung: Verschickung	Cholestech: keine Verschickung Fettsäureteststreifen: bei Raumtemperatur Blutaliquote: gefroren auf Trockeneis Paxgene-Röhrchen: gefroren auf Trockeneis	Gefroren auf Trockeneis	Bei Raumtemperatur
Prozesslenkung: Lagerung	Cholestech: keine Lagerung Fettsäureteststreifen: langfristig bei -20°C, Zwischenlagerung bei +4°C Blood aliquots: langfristig bei -80°C Paxgene-Röhrchen: bei -20°C für bis zu einem Jahr	Langfristig bei -20°C	Bei Raumtemperatur für bis zu einem Jahr/ langfristige Lagerung von Speichelproben oder extrahierter DNA bei -20°C
Prozesslenkung: Laboranalyse	Cholestech: Vor-Ort-Analyse von Glukose, Cholesterin und Triglyzeriden Fettsäureteststreifen: Lipidanalytik durch HPLC Blutaliquote: Insulin, CRP, HbA1c, Hormone des Energiestoffwechsels und Marker der Knochengesundheit Paxgene-Röhrchen: RNA-Extraktion und Genexpressionsanalyse	Spurenelemente als Marker der Ernährungsgewohnheiten, Proteine, (Kreatinin, Albumin), Kortisol	DNA-Extraktion und Genotypisierung aus Zellen der Mundschleimhaut
Qualitätssicherung biologische Proben	Qualitätskontrolle der Proben im Labor		

Die Einhaltung der Standardvorschriften war einer der im Rahmen des QMS-BS betrachteten Qualitätsindikatoren. Bei den in allen Zentren durchgeführten Standortbesuchen zeigte sich, dass die SOPs in der Regel auch wie vorgesehen umgesetzt wurden. Vereinzelt kam es allerdings zu Problemen bei Probenahme und Probenlogistik. So hatte vor allem ein Land Schwierigkeiten bei der Einhaltung der vorgesehenen Verarbeitungszeiten. In diesem Zentrum standen 11.4% der Blutproben länger als die vorgesehenen 2h vor der Zentrifugation. In allen anderen Zentren lag die Zahl mit unter 1.5% deutlich niedriger. Blutzucker, als derjenige unter den analysierten Biomarkern, der vermutlich am empfindlichsten auf verlängerte Stehzeiten reagiert, wurde allerdings direkt nach der Blutabnahme analysiert (siehe Abschnitt 3.5). Die Messwerte für Blutzucker wurden deshalb durch die verlängerten Stehzeiten nicht beeinflusst.

Bei den Urinproben war die fehlende Kühlung der Proben (länger als 2 Stunden ungekühlt) durch die Eltern das häufigste Problem (6,9% der Proben). In einem Land trat dieses Problem sogar bei etwa einem Drittel der Proben auf. Den geringsten Anteil an Qualitätseinschränkungen gab es für die Speichelproben zu verzeichnen. Hier tauchte lediglich hin und wieder das Problem eingetrockneter Proben auf (0,7% der Proben).

Für alle Arten von Bioproben wurden einige Probleme mit der Probengüte festgestellt. So gab es beispielsweise eine Reihe hämolytischer Serumproben, wobei dieses Problem der geschädigten Erythrozyten, bei denen das farbige Hämoglobin in das farblose Serum übertritt, bei der Qualitätsbeurteilung durch die Untersuchungszentren deutlich gegenüber der Qualitätsbeurteilung durch das Labor unterschätzt wurde (5,4% versus 10,3% hämolytische Serumproben). Die Hämolyse war auf eine vorzeitige und unvorsichtige Verarbeitung der nativen Blutröhrchen zurückzuführen und trat gehäuft in einem der beteiligten Länder auf (31% der Proben). Die betreffenden Proben mussten von der Insulinanalytik ausgeschlossen werden, da Insulin durch Bestandteile der beschädigten Zellwand der roten Blutkörperchen abgebaut wird[28].

Die Item-Responsequoten variierten je nach Probentyp von 56,6% für venöses Blut (79,7% für Blutproben insgesamt, d.h. inklusive Kapillarblut) und 85,6% für Urin bis hin zu 90,2% für Speichelproben. Die Beteiligung an den einzelnen Komponenten variierte außerdem stark nach Ländern. So konnte venöses Blut von 51-83% der Kinder gesammelt werden mit der Ausnahme eines Zentrums, in der sich nur 7,7% der Kinder an der venösen Blutentnahme beteiligten. Diese sehr niedrige Quote lag bei genauerer Betrachtung in der Nichtbeachtung des Studienprotokolls begründet: das Studienteam

hatte aus Gründen der lokalen Erhebungslogistik primär nur Kapillarblutproben vorgesehen und die venöse Blutabnahme nur als Zusatzkomponente angeboten, obschon dieser laut SOP der Vorzug hätte gegeben werden sollen. Für die venöse Blutabnahme sollten die Familien zudem einen weiteren Termin in einer dafür vorgesehenen Einrichtung verabreden. Es war also nicht weiter verwunderlich, dass in diesem Zentrum ein so geringer Anteil an venösem Blut gesammelt werden konnte.

Darüber hinaus bereitete vielen Untersuchungszentren die Nutzung der Bioprobendatenbank große Schwierigkeiten. Diese wurde als zu komplex empfunden, die Dokumentation der Proben erfolgte häufig erst zeitversetzt. Während der laufenden Erhebungsphase konnte die Datenbank daher in vielen Zentren nicht wie geplant zur Qualitätssicherung genutzt werden, um beispielsweise die Einhaltung der Probenlaufzeiten und Temperaturen zu kontrollieren. In einigen Fällen kam es zu so starken Verzögerungen, dass die Probenverschickung an das zentrale Untersuchungslabor ohne die automatisiert zu erstellenden Lieferscheine erfolgen musste, weil die lokalen Kühlkapazitäten erschöpft waren. Diese undokumentierten Proben konnten im Labor daher nicht routinemäßig untersucht werden und mussten dort bis zur Nachsendung der Lieferscheine zwischengelagert werden. Das zentrale Labor kam dadurch zwischenzeitlich an die Grenze seiner Lagerkapazität und musste außerdem in erheblichem Umfang Personal für das Sortieren und Identifizieren der Probenröhrchen einsetzen.

4.2 Messung der Qualität von biologischen Probenmaterialien

Publikation 2: Peplies J, Guenther K, Bammann K, Fraterman A, Russo P, Veidebaum T, Tornaritis M, Vanaelst B, Marild S, Molnar D, Moreno LA, Ahrens W. Influence of sample quality on the analyses of biological markers in the European multicentre study IDEFICS. Int J Obes (Lond). 2011 Apr; 35 Suppl 1:S104-12.

Wie im neu entwickelten Qualitätsmanagementsystem QMS-BS (siehe Abschnitt 4.1) vorgesehen, wurden verschiedene Qualitätsindikatoren bei der Gewinnung der biologischen Probenmaterialien in den IDEFICS-Untersuchungen erfasst, so z.B. der Nüchternstatus, Probleme bei der Urinprobennahme oder der Hydrationsstatus der Speichelproben nach dem Transport ins zentrale Labor. Im Rahmen der Qualitätskontrolle wurden die Laborergebnisse nach bestimmten Qualitätsindikatoren stratifiziert ausgewertet. So konnte der Einfluss bestimmter Abweichungen von den standardisierten Verfahrensanweisungen auf die mittleren Konzentrationen der biologischen Marker festgestellt werden und Proben mit bestimmten Qualitätsproblemen konnten gegebenenfalls systematisch von der Auswertung ausgeschlossen werden.

Für alle drei Probentypen (Blut, Urin und Speichel) konnten potentielle Qualitätsprobleme identifiziert werden. Erwartungsgemäß wurden für Kinder, die nicht nüchtern zur Blutentnahme gekommen waren, deutlich höhere Konzentrationen an Triglyzeriden und Insulin gemessen (siehe Tabelle 4-2). Die Messwerte für Glukose unterschieden sich dagegen nicht von denen nüchterner Kinder, vermutlich da die eingenommene Mahlzeit in der Regel bereits eine bis mehrere Stunden zurücklag, so dass der unmittelbar auf die Nahrungsaufnahme folgende Anstieg des Blutzuckers bereits in der Vergangenheit lag.

Für Urin wurden verschiedene Quellen problematischer Probenqualität dokumentiert. So führte das nächtliche Aufsuchen der Toilette ebenso zu veränderten Konzentrationen der Biomarker im Urin wie das Verpassen des ersten Morgenurins. Als unproblematisch erwies sich dagegen eine fehlende Kühlung der Urinproben (>2h) zwischen Entnahme und Erfassung im Untersuchungszentrum (siehe Tabelle 4-3). Die stratifizierte Analyse zeigte hier keine signifikanten Unterschiede in den Konzentrationen der Biomarker im Vergleich zu den konsequent kühl gehaltenen Proben.

Das einzige für Speichel dokumentierte Problem, das Eintrocknen der Proben, erwies sich als gänzlich unkritisch, da die Proben nach ihrer Rehydratation ganz normal weiter verarbeitet werden konnten.

Tabelle 4-2: Konzentration von Glukose, Triglyzeriden und Insulin nach Nüchternstatus

	Glukose (mmol/l)[#]				Triglycerides (mmol/l)[#]				Insulin (pmol/l)[##]			
	N	Mittelwert (Std.[+])	CV[§]	Regressions-koeffizient * (p-Wert) / R^2	N	Mittelwert (Std.[+])	CV[§]	Regressions-koeffizient * (p-Wert) / R^2	N	Mittelwert (Std.[+])	CV[§]	Regressions-koeffizient * (p-Wert) / R^2
Nüchtern	6488	4.57 (0.56)	0.12	Ref.	6484	0.49 (0.27)	0.55	Ref.	4711	30.15 (25.32)	0.84	Ref.
Nicht nüchtern	197	4.53 (0.54)	0.15	-0.32 (0.642) / 0.094	197	0.67 (0.29)	0.43	18.04 (<0.0001) / 0.108	136	47.40 (58.87)	1.24	2.53 (<0.0001) / 0.079

[#] Messwerte aus der Point-of-Care-Analyse von entweder venösem Blut oder Kapillarblut, [##] Gemessen in venösem Blut

* Schätzer aus der linearen Regression, adjustiert für Alter und Geschlecht.

[+] Standardabweichung, [§] Variantionskoeffizient

Tabelle 4-3: Konzentration von Kortisol, Albumin und Kalium nach Qualitätsindikator

	Cortisol (nmol/l)				Albumin (µmol/l)				Potassium (mmol/l)			
	N	Mittelwert (Std.[+])	CV[§]	Regressions-koeffizient * (p-Wert) / R^2	N	Mittelwert (Std.[+])	CV[§]	Regressions-koeffizient * (p-Wert) / R^2	N	Mittelwert (Std.[+])	CV§	Regressions-koeffizient * (p-Wert) / R^2
Keine Probleme	6555	140.87 (79.76)	0.57	Ref.	6555	17.70 (28.99)	1.64	Ref.	6550	43.81 (22.3)	0.51	Ref.
Urin ungekühlt >2h	921	142.61 (78.11)	0.55	2.69 (0.010) / 0.018	921	18.13 (19.42)	1.07	0.62 (0.378) / 0.004	918	47.72 (25.9)	0.54	4.18 (<0.0001) / 0.032
Nicht erster Morgenurin	353	178.46 (95.22)	0.53	14.92 (<0.0001) / 0.026	353	18.23 (15.07)	0.83	0.36 (0.738) / 0.003	350	64.51 (35.9)	0.56	21.26 (<0.0001) / 0.064
Kind war nachts auf der Toilette	505	201.20 (104.88)	0.52	20.53 (<0.0001) / 0.043	505	16.97 (17.97)	1.06	-0.07 (0.940) / 0.003	504	62.34 (34.0)	0.55	15.82 (<0.0001) / 0.056

* Schätzer aus der linearen Regression, adjustiert für Alter und Geschlecht.

[+] Standardabweichung, [§] Variantionskoeffizient

Es konnte gezeigt werden, dass sich die Probenqualität auf die Konzentration der biologischen Marker auswirkt. Qualitätssichernde Maßnahmen sind daher unabdingbare Voraussetzung für eine standardisierte Qualität der gesammelten Proben und resultierenden Daten.

4.3 Referenzwerte auf Basis von standardisiert erhobenen biologischen Daten

Publikation 3: Peplies J, Jiménez-Pávon D, Savva SC, Buck C, Guenther K, Fraterman A, Russo P, Iacoviello L, Veidebaum T, Tornaritis M, De Henauw S, Marild S, Molnar D, Moreno LA, and Ahrens W on behalf of the IDEFICS consortium. Percentiles of fasting serum insulin, glucose, HbA1c and HOMA-IR in pre-pubertal normal-weight European children from the IDEFICS cohort. Int J Obes (Lond). 2014 Sep;38 Suppl 2:S39-47. doi: 10.1038/ijo.2014.134.

Basierend auf den standardisiert erhobenen Biomarker-Daten konnten im Rahmen der vorliegenden Promotion erstmalig in der Literatur internationale, alters- und geschlechtsspezifische Perzentilkurven für Insulin, Glucose, HbA1c und HOMA-IR (Homeostasis Model Assessment for Insulin Resistance) modelliert werden.

Die Referenzperzentile beruhen auf den Daten von über 7000 normalgewichtigen 3-10-jährigen Kindern aus verschiedenen Teilen Europas. Die Daten beider IDEFICS-Erhebungsphasen wurden in die Studie einbezogen, Kinder unter 3 und über 10,9-Jahren wurden aufgrund zu geringer Fallzahlen ausgeschlossen. Die Beschränkung der Studienpopulation auf normalgewichtige gesunde Kinder erfolgte, da die Insulinwerte in vorangegangenen Sensitivitätsanalysen einen starken Zusammenhang mit dem BMI zeigten. Perzentilkurven der biologischen Marker wurden mithilfe der GAMLSS-Methode (General Additive Model for Location Scale and Shape)[29] als eine Funktion des Alters und stratifiziert nach Geschlecht berechnet. Für alle vier Marker lagen die Messwerte deutlich unter den für Erwachsene bekannten Referenzbereichen. Ein positiver Alterstrend konnte für alle vier Marker gezeigt werden, wobei dieser für HbA1c erst ab einem Alter von etwa 8 Jahren einsetzte. Insulin- und HOMA-IR-Werte waren bei Mädchen in allen Altersgruppen höher als bei Jungen, Glukose-Werte waren bei Jungen etwas höher (siehe Abbildung 4-1).

Andere populationsbasierte Studien, die bis dahin Grenzwerte publiziert hatten, waren in der Regel auf nationale Daten beschränkt. Die dort abgeleiteten Werte fielen zumeist deutlich niedriger aus als die der IDEFICS-Studie. Während beispielsweise das 95ste Perzentil des HOMA-IR in der IDEFICS-Studie für 9-jährige Mädchen 2,7 betrug, lag der Wert in einer kanadischen Studie[30] bei 2,1 und in einem kleinen italienischen Studienkollektiv[31] bei 2.2. Zu beachten ist dabei allerdings, dass bei vielen Studien unterschiedliche Labormethoden zur Insulinbestimmung verwendet wurden, welche zum Teil große Unterschiede in den gemessenen Insulinkonzentrationen zeigen.[32] In vielen Fällen war aber ein deutlicher Anstieg der HOMA-IR-Werte mit dem Alter der präpubertären Kinder zu sehen.[33,34]

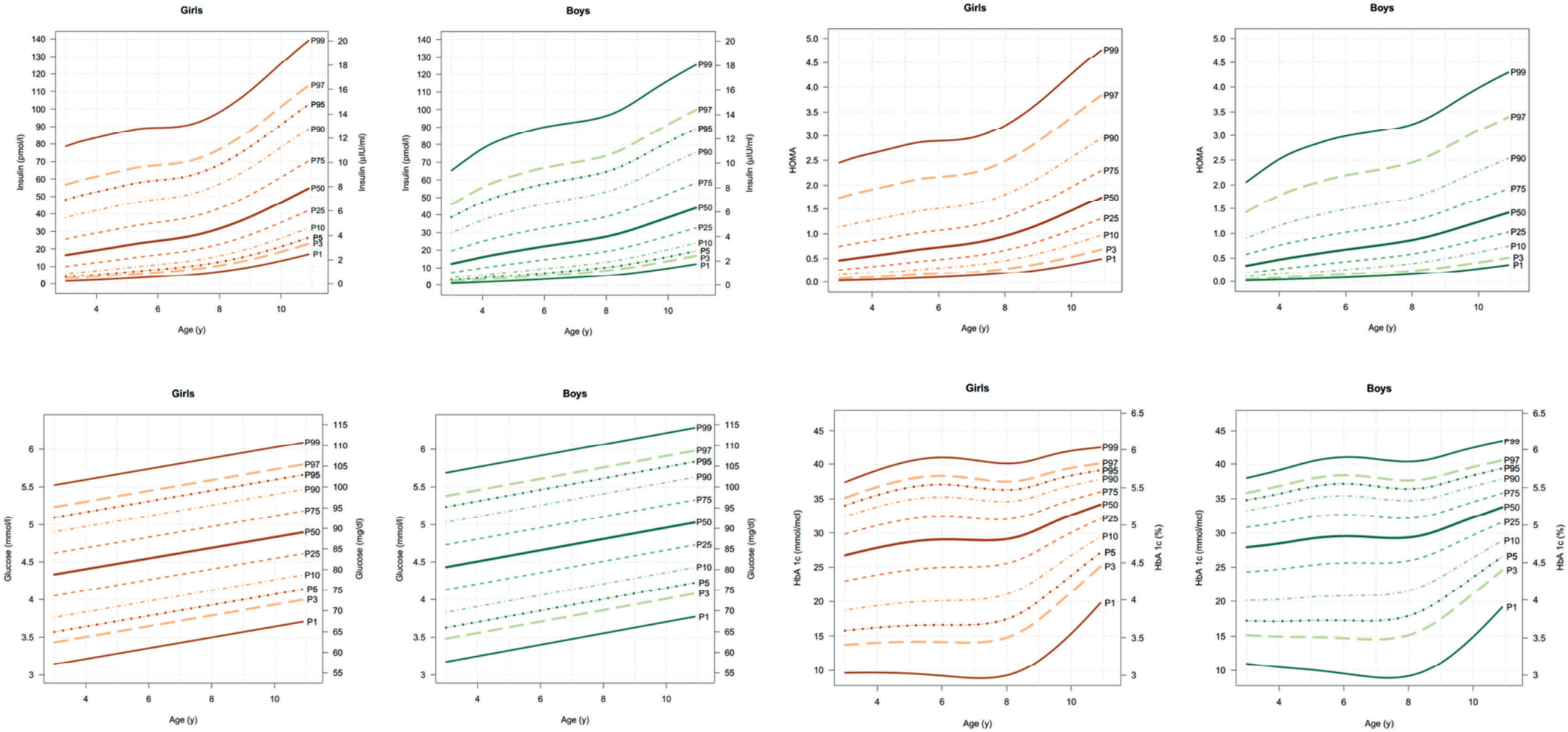

Abbildung 4-1: Perzentilkurven für Insulin, Glukose, HOMA-IR und HbA1c, basierend auf den Daten normalgewichtiger Kinder aus der IDEFICS-Studie

Viele Studien versuchen pädiatrische Grenzwerte auch mithilfe von ‚Receiver Operating Characteristic' (ROC) – Kurven zu generieren. Diese Studien beruhen aus methodischen Gründen zumeist auf adipösen Kollektiven, da diese einerseits einen höheren Anteil an Insulinresistenz aufweisen und da die von Adipositas betroffenen Kinder außerdem eher bereit sind, sich umfangreichen vergleichenden Testverfahren zu unterziehen. Die in diesen Studien ermittelten Grenzwerte für Insulinresistenz liegen in der Regel etwas höher als die 95sten Perzentilwerte aus populationsbasierten Studien. Zwei Studien[35,36] an türkischen adipösen Kindern und Jugendlichen definieren beispielsweise Grenzwerte für HOMA-IR von 3,2 und 2.7. Eine spanische Studie[37], die in einer gemischten Studienpopulation aus adipösen und normalgewichtigen Kindern durchgeführt wurde, identifizierte einen HOMA-IR-Wert von etwa ≥ 3 als besten Grenzwert. Die im Rahmen dieser Promotion gezeigten Referenzperzentile liegen also etwa mittig im Bereich der bislang postulierten Grenzwerte.

Vom IDEFICS-Konsortium wurde ein ganzer Sonderband der Fachzeitschrift "International Journal of Obesity" (IJO-Supplement) zu pädriatrischen Referenzwerten verfasst. Weitere Veröffentlichungen zu Biomarkern beschreiben Perzentilkurven für die wichtigsten Blutlipide (Gesamtcholesterin, HDL, LDL und Triglyzeride)[38], das C-reaktive Protein[39], Leptin und Adiponektin[40], CTX-Peptid[41] und für Fettsäuren im Vollblut[42]. In einer zusammenfassenden Arbeit wurde eine eigene neue Definition des metabolischen Syndroms[43] generiert. Für viele der Biomarker zeigte sich ein positiver Alterstrend (metabolisches Syndrom, Gesamtcholesterein, HDL, Leptin, CTX und einige der Fettsäuren). Für manche war dagegen ein negativer Alterstrend zu sehen (Adiponektin, LDL-Cholesterin, Gesamtcholesterin/HDl-Quotient und einige der Fettsäuren). Nur wenige der untersuchten Marker wiesen einen unveränderten Wertebereich über alle Altersklassen auf (Triglyzeride, einige Fettsäuren).

Bestehende Referenzwerte für Erwachsene liegen also für die allermeisten Biomarker oberhalb oder unterhalb der Wertebereiche, die für Kinder vor der Pubertät gemessen werden. Bei Insulin und HOMA-IR betrugen die Konzentrationen für die jüngeren Kinder beispielsweise nur etwa die Hälfte der für Erwachsene etablierten Referenzwerte. Vor diesem Hintergrund wird deutlich, dass die Verwendung eigener Referenzbereiche für Kinder in der Pädiatrie dringend notwendig ist und dass Perzentilkurven altersübergreifend einheitlichen pädiatrischen Grenzwerten in den meisten Fällen vorzuziehen sind. Die IDEFICS-Referenzwerte können hier einen wertvollen Beitrag leisten.

4.4 Risikoabschätzung auf Basis von standardisiert erhobenen biologischen Daten

Publikation 4: Peplies J, Günther K, Börnhorst C, Fraterman A, Russo P, Veidebaum T, Tornaritis M, De Henauw S, Marild S, Molnar D, Moreno LA, and Ahrens W on behalf of the IDEFICS consortium. Determinants of insulin resistance in preadolescent children: results from the IDEFICS study. (eingereicht bei Pediatrics)

Die Prävalenz kindlichen Übergewichts und kindlicher Adipositas ist in den letzten Jahren stark angestiegen. Im Laufe der letzten 30 Jahre hat das durchschnittliche Gewicht eines Kindes in den USA um mehr als 5 kg zugenommen, was dort zu einem Anteil von einem Drittel übergewichtiger und adipöser Kinder geführt hat[44]. Für Europa werden z.T. ähnliche Zahlen erreicht. Diese schwanken beispielsweise in der IDEFICS-Studie für präadoleszente Kinder je nach Land zwischen knapp 10% (Belgien) und über 40% (Italien).[43]

Insulinresistenz (IR) als eine der gängigen metabolischen Störungen im Zusammenhang mit Übergewicht und Adipositas[45,46] und als vorgezogener Endpunkt in der Entwicklung manifester kardiovaskulärer Erkrankungen und des Typ 2 Diabetes[47,48,49] ist schon vielfältig Gegenstand risikoanalytischer Betrachtungen geworden. Dabei scheint sich das Auftreten der Adipositas in einer früheren Lebensphase besonders ungünstig auf das Auftreten der assoziierten Erkrankungen auszuwirken. So führte eine Gewichtszunahme im frühen Erwachsenenleben in einer großen populationsbasierten Studie zu einem höheren Risiko für Typ 2 Diabetes und einem früheren Einsetzen der Erkrankung als dieselbe Gewichtszunahme im späteren Erwachsenenleben.[50] Auch bei Kindern und Jugendlichen wird die steigende Prävalenz von Übergewicht und Adipositas von einer zunehmenden Prävalenz von metabolischem Syndrom[51,52,53], Typ 2 Diabetes [54,55,56] und kardiovaskulären Erkrankungen[54] begleitet. Jüngere Studien liefern sogar Hinweise darauf, dass bereits im Kindes- und Jugendalter erste Zeichen kardiovaskulärer Dysfunktionen (Fettablagerungen am Herzen endotheliale Fehlfunktion, Versteifung von Ventrikel und Arterien) auftreten können[57]. Das möglichst frühzeitige Erkennen von Insulinresistenz, noch bevor sich klinische Symptome manifestieren, ist daher von entscheidender Bedeutung bei der Prävention dieser Erkrankungen.

Die Goldstandardmethode zur Messung der Insulinresistenz, die „hyperinsulinämische, euglykämische Clamp-Technik“, ist invasiv und außerdem sehr zeit- und arbeitsaufwändig und daher für die epidemiologische Forschung insbesondere bei Kindern ungeeignet. Matthews et al. zeigten aber bereits 1985, dass die Abschätzung von Insulinresistenz

durch HOMA-IR gut mit den Messwerten der Clamp-Technik korreliert[58]. Auch in jüngeren Jahren konnte sich HOMA-IR gegenüber anderen Biomarkern und Indices wie QUICKI (Quantitative Insulin Sensitivity Check Index) oder FGIR (Fasting Glucose Insulin Ratio) als Index zur Quantifizierung der Insulinresistenz bei Kindern durchsetzen[35] und wurde daher in dieser Arbeit als Surrogatmarker zur Bestimmung der Insulinresistenz verwendet.

Für das metabolische Syndrom gibt es leider gibt es nach wie vor keine allgemein anerkannte pädiatrische Definition. Bisherige Versuche, von denen einer der jüngeren auf den Daten der IDEFICS-Kohorte beruht, beziehen aber alle einen gestörten Insulin- und/ oder Glukosestoffwechsel in ihre Definition ein.[43,59,60] Ebenso gibt es bei Kindern immer noch nur wenige Studien zu den Risikofaktoren für Insulinresistenz, vor allem fehlen Daten aus Längsschnittstudien und von jüngeren Kindern[61]. Hier liefert die vierte Publikation im Rahmen der vorliegenden Promotion einen wichtigen Beitrag.

Publikation 4 zeigt die Prävalenzraten kindlicher Insulinresistenz in den verschiedenen Europäischen Ländern der IDEFICS-Studie. Anthropometrische Risikofaktoren und Faktoren des Lebensstils zum Zeitpunkt der Basiserhebung wurden zum Auftreten einer Insulinresistenz bei der Folgeerhebung in Beziehung gesetzt. Anstelle eines festen Grenzwertes für HOMA-IR wurde das 95ste alters- und geschlechtsabhängige Perzentil für HOMA-IR aus Publikation 3 der vorliegenden Promotion eingesetzt.

Die Prävalenzraten von Insulinresistenz zeigen in der vorliegenden Arbeit einen steigenden Trend mit der BMI-Kategorie[62], von 2,2% bei dünnen Kinder, über 10,9% bei normalgewichtigen und 26,5% bei übergewichtigen bis zu bemerkenswerten 66,7% bei adipösen Kindern. Dabei wurde die niedrigste Gesamtprävalenzrate mit 7,6% aller Kinder in Belgien beobachtet. Die höchste Gesamtprävalenzrate zeigte sich mit 32,1% in Italien. Die Länderunterschiede laufen in etwa parallel zu den länderspezifischen Raten von Übergewicht und Adipositas (Belgien 9,2%, Italien 51,5%), mit der Ausnahme von Spanien, wo 26,1% der Kinder übergewichtig oder adipös waren, aber nur 13,6% der Kinder nach unserer Definition eine Insulinresistenz aufwiesen. Die gezeigten Prävalenzraten stimmen recht gut mit den in der Literatur gefundenen überein, wobei diese in der Regel von älteren Studienpopulationen abgeleitet wurden und auf etwas anderen Definitionen der IR beruhen[51,63].

In der prospektiven multivariaten Analyse zeigten neben Übergewicht und Adipositas auch Geschlecht und Bereiche des Lebensstils (gemessener Mangel an körperlicher

Aktivität, Fettkonsum, Medienkonsum und Mediennutzung im Kinderzimmer) einen Zusammenhang mit der Entstehung von Insulinresistenz (Tabelle 4-4).

Tabelle 4-4: Determinanten der Insulinresistenz (HOMA-IR≥ p95*) – Ergebnisse der multivariaten logistischen Regression, unter der Annahme eines Random-Effect-Models für das Land

N=1083	Modell 1** mit BMI (z-transformiert) OR	95% CI	Modell 2** mit Taille (z-transformiert) OR	95% CI
BMI Z-Wert (Cole) (pro Einheit Abweichung vom Mittelwert)	**2.6**	**2.1-3.1**		
Taille Z-Wert (Cole) (pro Einheit Abweichung vom Mittelwert)			**2.2**	**1.9-2.6**
Mäßige bis starke körperliche Aktivität				
1. Quartil (≤ 27 Min./Tag)	Ref.		Ref.	
2. Quartil (27 - ≤ 38.7 Min./Tag)	0.9	0.6-1.5	1.1	0.7-1.7
3. Quartil (38.7 - ≤ 54.6 Min./Tag)	**0.5**	**0.3-0.9**	**0.5**	**0.3-0.9**
4. Quartil (≥ 54.6 Min./Tag)	0.7	0.5-1.1	0.7	0.5-1.1
Sex (weiblich versus männlich)	**2.2**	**1.5-3.1**	**2.5**	**1.8-3.6**
Alter (pro Jahr)	1.0	0.9-1.2	1.0	0.9-1.1
ISCED				
Niedrig (1-2)	1.2	0.7-2.2	1.3	0.7-2.3
Mittel (3-4)	1.2	0.9-1.8	1.2	0.8-1.8
Hoch (5-6)	Ref.	Ref.	Ref.	Ref.
Zeit mit audio-visuellen Medien (h/d) (pro zusätzlicher Stunde)	**1.2**	**1.0-1.4**	**1.2**	**1.0-1.4**
Fettkonsum (Propensity Score##) (pro Einheit Abweichung vom Mittelwert)	**1.2**	**1.0-1.4**	**1.0**	**1.0-1.4**

* Alters- und geschlechtsspezifisches 95. Perzentil (p95) aus Peplies et al. 2014 [64]
** Analysen für alle Parameter in der Spalte adjustiert
Konsum von Nahrungsmitteln mit hohem Fettgehalt im Verhältnis zum Gesamtkonsum an Nahrungsmitteln, erfasst im Fragebogen zur Verzehrshäufigkeit. Der Propensity Score wurde in einer modifizierten Form in das statistische Modell einbezogen: er wurde durch 10 geteilt, um besser lesbare Effektschätzer zu erhalten – eine Einheit im multivariaten Modell entspricht demzufolge 10 Einheiten des Originalscores.

Ein Wechsel aus den Gewichtskategorien „übergewichtig“ oder „adipös“ zu „normalgewichtig“ oder „dünn“ zwischen Basiserhebung und Folgeuntersuchung war außerdem mit einer Verringerung der HOMA-IR-Werte verbunden (siehe Tabelle 4-5).

Tabelle 4-5: Veränderung der HOMA-IR-Werte in Abhängigkeit der Gewichtsveränderung innerhalb von zwei Jahren

BMI* Basiserhebung	BMI* Folgeuntersuchung	N	Veränderung des HOMA-IR in 2 Jahren delta z-IR** (Std#)
Übergewichtig/adipös	Dünn/normalgewichtig	75	-0.19 (1.1)
Dünn/normalgewichtig	Dünn/normalgewichtig	2539	0.20 (1.2)
Übergewichtig/adipös	Übergewichtig/adipös	513	0.46 (1.1)
Dünn/normalgewichtig	Übergewichtig/adipös	221	0.64 (1.2)
All		3348	0.26 (1.2)

* Gewichtsstatus nach erweiterten IOTF-Kriterien (Cole, 2012)
** delta z-IR = z-Score IR (T1) - z-Score IR (T0)
# Standardabweichung

Die vorliegende ist eine der ersten prospektiven Untersuchungen in einer präadoleszenten Studienpopulation. Sie bestätigt die Hypothese, dass Übergewicht und ein hoher Taillenumfang mit der späteren Entwicklung von IR assoziiert sind, identifiziert aber auch Indikatoren eines bewegungsarmen Lebensstils als unabhängige Risikofaktoren für IR. Die therapeutische Wirkung körperlicher Aktivität, die bereits in der Literatur beschrieben wurde[65], konnte hier bestätigt werden. In einer Metaanalyse zum Einfluss der kardiorespiratorischen Fitness und des Gewichtsstatus auf die Gesamtmortalität zeigte sich, dass körperlich fitte übergewichtige und adipöse Menschen ein ähnliches Mortalitätsrisiko hatten wie fitte normalgewichtige Individuen, wohingegen unfitte Menschen, unabhängig von ihrem Gewichtsstatus, ein etwa doppelt so hohes Risiko hatten, zeitnah zu versterben[66].

Auch andere Arbeiten aus den Reihen des IDEFICS-Konsortiums befassen sich mit der Abschätzung kardiovaskulärer Risiken. In einer Analyse wurde das Ernährungsverhalten über die per Fragebogen retrospektiv erhobenen Verzehrshäufigkeiten mit einer kardiovaskulären Risikoeinstufung korreliert, in welche neben dem systolischen Blutdruck und der Hautfaltendicke auch die kardiorespiratorische Fitness und verschiedene Biomarker (Triglyzeride, HOMA-IR, Cholesterin/HDL-Quotient) einbezogen wurden. Ein hoher Konsum an zuckerhaltigen Getränken und eine geringe Zufuhr an Nüssen und Samen, Süßigkeiten, Frühstückscerealien, Marmelade, Honig und Nussnougatcremes waren mit einem erhöhten kardiovaskulären Erkrankungsrisiko assoziiert.[67]

Eine weitere Arbeit betrachtet die kardiovaskulären Risikofaktoren, die mit dem Verzicht von Schulkindern auf ein häusliches Frühstück einhergehen. Schuljungen, die nie oder selten zu Hause frühstückten, waren häufiger übergewichtig oder adipös als solche, die täglich zuhause Frühstück aßen. Sie hatten außerdem eine erhöhte Wahrscheinlichkeit für einen niedrigen (also ungünstigen) HDL-Cholesterinwerte und eine hohe Hautfaltendicke (Summenwert). Mädchen im Grundschulalter, die nie oder selten zu Hause frühstückten, hatten mit höherer Wahrscheinlichkeit einen hohen Taillenumfang, niedrige HDL-Cholesterinwerte, hohe Triglyzerid-Werte und einen hohen Cholesterin/HDL-Quotient.[68]

5 Diskussion

Auf Grundlage der einbezogenen Veröffentlichungen konnten die Ziele der vorliegenden Dissertation erreicht werden:

- Das QMS-BS erwies sich als funktionsfähiges Instrument für die Entnahme und Verarbeitung biologischer Probenmaterialien in der IDEFICS-Studie und konnte entscheidend zu einer hohen Qualität der biologischen Proben und Daten beitragen.
- Proben mit entsprechenden Qualitätsproblemen konnten gezielt von bestimmten Auswertungen ausgeschlossen werden.
- Auf dieser Basis war es möglich, pädiatrische Referenzwerte zu generieren und die biologischen Marker in die epidemiologische Risikoabschätzung einzubeziehen.

Während Referenzwerte bei Erwachsenen für viele Erkrankungen, medizinische Kenngrößen und biologische Marker schon lange etabliert sind, fehlen diese Orientierungshilfen in der pädiatrischen Praxis in den meisten Bereichen noch heute. Referenzwerte liefern wichtige Hinweise auf das Vorliegen manifester Gesundheitsstörungen oder auch vorgezogener Krankheitsendpunkte und stellen somit eine wichtige Voraussetzung für Therapie und Prävention dar. Die im Rahmen der Promotion berechneten Referenzwerte für Insulin, Glucose, HbA1c und HOMA-IR können in der kinderärztlichen Routine dazu beitragen, Kinder zu identifizieren, die ein erhöhtes Risiko für kardiovaskuläre Erkrankungen oder Typ 2 Diabetes haben. Da Insulinresistenz in den meisten Fällen als reversibel gilt[69,70], kann eine frühzeitige und gezielte Intervention, wenn sie Erfolg hat, viele Krankheitsfälle verhindern.

In diesem Zusammenhang ist auch die praktische Bedeutung der longitudinalen Risikoabschätzung zu sehen. Da sich körperliche Aktivität neben der Gewichtsreduktion als ein unabhängiger protektiver Faktor für Insulinresistenz zeigte, sollte die Förderung körperlicher Aktivität zukünftig noch stärker im Fokus von Therapie und Intervention stehen, insbesondere auch bei Kindern. Auch beim Vorhandensein von Übergewicht und Adipositas trägt körperliche Fitness entscheidend zur metabolischen Gesundheit der betroffenen bei[71,72] und reduziert das Risiko für Herz-Kreislauf-Erkrankungen und Typ 2 Diabetes[73].

Das im Rahmen der vorliegenden Promotion entwickelte Qualitätsmanagementsystem stellt in der Literatur einen der ersten umfassenden Vorschläge zum

Qualitätsmanagement für Bioproben dar. Wenig später publizierte auch die IARC einen ausführlichen Vorschlag für ein System mit Standardprozeduren und verschiedenen Kontrollmechanismen[2]. Eine Veröffentlichung jüngeren Datums beschreibt die Prozeduren und Probentypen der TEDDY (The Environmental Determinants of Diabetes in the Young) – Studie[74]. Bei der brasilianischen ELSA-Studie erfolgte die Probenverarbeitung wie bei der IDEFICS-Studie in dezentralen Laboren, die Probenanalytik aber in einem Zentrallabor[75].

Davor gab es nur gelegentlich methodische Arbeiten aus dem Bereich der Bioproben. Eines der frühesten Beispiele in der englischsprachigen Literatur aus dem Jahr 2008 ist die Beschreibung der Verarbeitung und Lagerung der biologischen Probenmaterialien für die UK Biobank, welche eine sehr umfangreiche Probensammlung auf nationaler Ebene betreibt[76]. In einer deutschen Veröffentlichung wurde über einige Module der KIGGS-Studie („Studie zur Gesundheit von Kindern und Jugendlichen in Deutschland“) berichtet.[77,78] Diese deutschlandweite pädiatrische Studie zeigt sehr ähnliche Responseraten für die verschiedenen Typen biologischer Materialien wie die IDEFICS-Studie (85% für Urinproben, knapp 53% für die venöse Blutabnahme).

In der deutschsprachigen Literatur befasst sich seit einigen Jahren vor allem die Schriftenreihe der „Telematikplattform für Medizinische Forschungsnetze“ (TMF) mit den Herausforderungen der medizinischen Forschung und der Standardisierung von Methoden. Band 5 der Schriftenreihe hatte beispielsweise die Qualitätssicherung bei Biomaterialbanken zum Thema.[9] In einem aktuellen Projekt der TMF werden Qualitätssicherungsstandards in Kohortenstudien erarbeitet.[79] In den letzten Jahren ist es zunehmend üblich geworden, auch über Design- und Logistik-Aspekte großer epidemiologischer Studien zu berichten. Die neue, große deutsche Gesundheitsstudie, die „Nationale Kohorte“, befindet sich zwar nach wie vor in der Anfangsphase, hat aber bereits über Erfahrungen aus dem Pretest und Maßnahmen der Standardisierung und Qualitätssicherung, unter anderem im Bereich Bioproben, berichtet.[80]

Bei der IDEFICS-Studie konnte das Qualitätsmanagementsystem QMS-BS davon profitieren, dass Qualitätssicherung bereits im Design des EU-Projekts berücksichtigt worden war und dort ohnehin einen hohen Stellenwert hatte. So gab es einen eigenen Arbeitsbereich für Standardisierung & Qualitätskontrolle, in welchem beispielsweise die zentralen und dezentralen Trainingseinheiten und die Standortbesuche in den Untersuchungszentren von vornherein eingeplant waren.

Problematisch war auf der anderen Seite die zeitliche Verzögerung der Kontrollsysteme, die vor allem in der verspäteten Dateneingabe in die Bioprobendatenbank begründet waren. Ein korrigierendes Eingreifen während der laufenden Erhebungsphase war so, anders als geplant, zunächst nur durch die Standortbesuche möglich, welche aber zum Teil erst einige Monate nach Beginn der Erhebung stattfanden. Die QM-Normen der ISO 9000 – Reihe, auf denen das QMS-BS beruht, schreiben eigentlich eine kontinuierliche Verbesserung der Qualität vor.[81] Die Fehleranalyse sollte zeitnah erfolgen, in einem ständigen Kreislauf aus Messung, Analyse und Verbesserung. Dieses Vorgehen wäre auch im Rahmen des QMS-BS wünschenswert gewesen. Bei zeitnaher elektronischer Übermittlung der Dokumentationsbögen hätten Abweichungen von den SOPs schon zu Beginn der Erhebungsphase erkannt und verhindert werden können. Eine Dateneingabe in dezentrale Systeme, die später zusammengeführt werden müssen, welche so auch bei der IDEFICS-Studie praktizierte stattgefunden hat, wurde in der epidemiologischen Praxis mittlerweile weitestgehend von internetbasierten Dokumentationssystemen abgelöst, die deutlich weniger fehleranfällig sind.[82] Das direkte elektronische Protokollieren von Daten (z.B. die Erfassung von Zeiten über die Log-Datei von Analysegeräten) wäre ein weiterer denkbarer Schritt, um Dokumentationsfehler zu reduzieren.

Eine Verbesserung der Item-Responseraten für einzelne biologische Probenmaterialien in der IDEFICS-Studie wäre vielleicht durch eine stärkere Motivation der beteiligten Eltern möglich gewesen. Viele Eltern sind nur zur Mitarbeit bereit, wenn sich aus der Studienteilnahme ein direkter Nutzen für das eigene Kind ergibt. Bezogen auf die venöse Blutabnahme hätte eine schnelle Rückmeldung zu den im Labor gemessenen Biomarkern einen Anreiz dargestellt. Diese Rückmeldung erfolgte in der IDEFICS-Studie aber leider erst etwa ein Jahr später und aufgrund der fehlenden Referenzwerte nur für eine reduzierte Anzahl an Biomarkern.

Bei einigen Studienkomponenten der IDEFICS-Studie war die Einhaltung der Standardprozeduren nur durch die Mitarbeit der Eltern möglich. Dieses Vorgehen ist aus Sicht des Qualitätsmanagements nicht besonders günstig, da sich die elterlichen Arbeitsschritte nur schwer standardisieren lassen (ein ‚Training' der teilnehmenden Eltern ist in einer großen populationsbasierten Studie nur schwer realisierbar) und birgt zudem die Gefahr von Selektion, weil die Einhaltung der ausgegebenen Arbeitsanweisungen möglicherweise vom Bildungsstand der Eltern abhängt. Prozeduren, die in epidemiologischen Studien durch Teilnehmende oder Eltern durchgeführt werden müssen, sollten also leicht verständlich und möglichst einfach in der Umsetzung sein. Es

empfiehlt sich darüber hinaus, insbesondere bei populationsbasierten Studien an Kindern, möglichst robuste Biomarker zu verwenden, deren Halbwertszeiten im biologischen Probenmaterial bei den gewählten Standardprozeduren deutlich unterschritten werden. Bei sehr stabilen Biomarkern, die keinen Tagesschwankungen unterliegen, könnte auf eine Nüchternblutabnahme verzichtet werden, so dass dann weder die Uhrzeit der Blutentnahme noch die vorhergegangene Nahrungsaufnahme standardisiert werden müssten.

Bei allen Bemühungen, die Zeiten der Probennahme für die Blut- und Urinproben in der IDEFICS-Studie zu standardisieren, um den Einfluss von Tagesschwankungen auszuklammern, konnten bestimmte Unterschiede zwischen den Ländern nicht ausgeglichen werden. So erfolgte die Probennahme in allen Ländern in der Regel morgens zu Beginn des Schul- oder Kindergartentages, die Anfangszeiten dieser Einrichtungen variieren aber zwischen den teilnehmenden Ländern um 1-2 Stunden. Ohnehin ist der Lebensrhythmus in Nord- und Südeuropa sehr unterschiedlich. So unterscheiden sich beispielsweise die durchschnittlichen Zeiten für das Abendessen und das Zubettgehen der Kinder um mehrere Stunden.

Die Lagerung der biologischen Probenmaterialien erfolgte in der zentralen IDEFICS-Bioprobenbank am BIPS. Diese befand sich aber zur Zeit der Einlagerung erst im Aufbau und war nur mit begrenzten finanziellen Mitteln ausgestattet. Die Entscheidung fiel daher auf eine Bioprobenbank mit einem niedrigen Automatisierungsgrad, der entsprechend mit einem hohen manuellen Aufwand einhergeht. Ein automatisiertes -80°C- oder Flüssigstickstoff-Lager mit der entsprechenden Lagerrobotik hätte - bei hohen Zusatzkosten - einen Zugewinn an Sicherheit und Temperaturkontrolle bedeutet. Bei der Aliquotierung der biologischen Probenmaterialien in der IDEFICS-Studie wurde zudem nicht an die leichte Verfügbarkeit kleinster Probenvolumina für spätere Forschungsfragen gedacht. Hierfür wäre die Verwendung eines Strohhalmsystems in flüssigem Stickstoff besser geeignet gewesen.

6 Ausblick

In der epidemiologischen Forschung steht die Erkennung von Krankheitsursachen mit dem langfristigen Ziel der Krankheitsvermeidung im Vordergrund. Dies ist auch von volkswirtschaftlichem Interesse, da die großen Zivilisationskrankheiten die Gesundheitssysteme immer stärker belasten. Die Vermeidung von Krankheiten ist in der Regel deutlich kostengünstiger als die Behandlung chronischer Erkrankungen, wenn sich diese erst klinisch manifestiert haben. Bei der Erforschung der Krankheitsursachen hat die Forschungsförderung in den letzten Jahren zunehmend auf große prospektive Kohortenstudien gesetzt. Kritiker solcher epidemiologischer Großprojekte wie der „Nationalen Kohorte“ sehen allerdings die Gefahr, dass das Sammeln von Proben und Daten zum Selbstzweck erhoben wird und fürchten insbesondere auch um den Schutz der persönlichen Daten bei solchen groß angelegten Datensammlungen[83]. Zukünftige Studien in der molekularen Epidemiologie stehen daher vor der Herausforderung, mit den sich ständig verbessernden technischen Möglichkeiten verantwortungsvoll umzugehen, um ethischen und datenschutzrechtlichen Bedenken entgegenzuwirken, insbesondere bei pädiatrischen Studienpopulationen.

Im Anbetracht der Fülle an zum Teil widersprüchlichen Veröffentlichungen zu bestimmten Fragestellungen gewinnen gepoolte Analysen in der Epidemiologie immer weiter an Bedeutung.[84] Die Standardisierung von verwendeten Methoden und die genaue Dokumentation aller Prozesse ist eine grundlegende Voraussetzung, um die Zusammenfassbarkeit verschiedener Studien zu ermitteln. Während es in der Natur wissenschaftlicher Kreativität liegt, bei der Auswahl von Methoden und Versuchsanordnungen neue Wege zu gehen und von bewährten Verfahren abzuweichen, benötigt das globale Netzwerk der Wissenschaft auf der anderen Seite eine kontinuierliche Harmonisierung von Methoden. Epidemiologische Studien, die ohne die entsprechenden Qualitätsstandards arbeiten, können allenfalls erste Hinweise auf bestimmte Zusammenhänge geben, da nicht ausgeschlossen werden kann, dass ihre Ergebnisse rein zufällig sind oder stark durch die Versuchsanordnung beeinflusst wurden. Große populationsbasierte Studien sollten heute deshalb nicht mehr auf Standardisierung und Qualitätssicherung verzichten, um reproduzierbare und mit anderen vergleichbare Studienergebnisse zu erzielen.

Die zunehmende Einbeziehung vorgezogener Erkrankungsendpunkte in die epidemiologische Forschung und eine sich stetig vergrößernde Palette an Risikofaktoren

für die Gesundheit des Einzelnen, machen auch ein Nachdenken über das ‚Recht auf Nichtwissen' immer wieder erforderlich, da das Wissen über die Risikopotentiale der eigenen Lebensführung auch eine dem Präventionsgedanken entgegenstehende negative Wirkung auf Denken Gefühle und Lebensplanung haben könnte. Passend dazu soll der englische Schriftsteller Aldous Huxley (1894-1963) bereits im letzten Jahrhundert gesagt haben: „Die medizinische Forschung hat so enorme Fortschritte gemacht, dass es überhaupt keine gesunden Menschen mehr gibt." Studienteilnehmende sollten vor diesem Hintergrund umfassend über die Inhalte potentieller Studienrückmeldungen informiert werden, um eine aufgeklärte Entscheidung zur Rückmeldung ihrer einzelnen Ergebnisse treffen zu können.

In unserer Zivilisationsgesellschaft hat sich eine Vielzahl von Erkrankungen entwickelt, die eng mit unserem sitzenden und hochtechnischen Lebensstil zusammenhängen, welcher zunehmend auch schon Kinder im Grund- und Vorschulalter betrifft. Es liegt also in unserer Verantwortung als Wissenschaftlerinnen und Wissenschaftler, den Ursachen dieser Erkrankungen nachzugehen und eine frühe Erkennung derselben zu ermöglichen. Dabei sollte ein verantwortlicher Umgang mit den menschlichen Ressourcen und technischen Möglichkeiten genauso selbstverständlich sein, wie die ‚Open Access'-Bereitstellung der anonymisierten Daten und Proben. Ein wichtiger Fokus der Forschungsförderung sollte in den nächsten Jahren aber auch weiterhin die Entwicklung gezielter Interventionen sein, ohne deren Erfolg die Erkenntnisse der ätiologischen Forschung keinen gesellschaftlichen Nutzen entfalten können.

7 Literatur

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8 Danksagung

Ich danke:

- Wolfgang Ahrens, der es mir ermöglicht hat, in der IDEFICS-Studie mitzuarbeiten. Er hat mich als Berater und Betreuer der Doktorarbeit immer wieder mit neuen Ideen für die Ausgestaltung der Arbeit versorgt.

- dem IDEFICS-Konsortium, das in all den Jahren zu einer großen Familie zusammengewachsen ist, insbesondere meinen Koautoren, die mir durch zahlreiche Diskussionen bei der Erstellung der Publikationen geholfen haben, aber auch den Survey- und Laborteams, die sich redlich bemüht haben, all unsere Vorgaben und Arbeitsanweisungen umzusetzen.

- dem Bremer IDEFICS-Team, allen voran Karin Bammann, von der ich sehr viel über fast alle Belange des wissenschaftlichen Lebens gelernt habe, aber auch Kathrin Günther und Claudia Börnhorst, die jederzeit bereit waren, mich bei biologischen oder statistischen Fragestellungen zu beraten.

- den vielen anderen Kolleginnen und Kollegen im BIPS, ohne deren Unterstützung und Ermunterung diese Dissertation vielleicht nicht zustande gekommen wäre: Antje Timmer, Christoph Buck, Timm Intemann, Anne Lanfer, Sabrina Hense, Diana Herrmann, Marc Suling, Antje Hebestreit, Ina Alvarez, Eva Gahler, Heike Gerds, Heike Jess und vielen anderen….

- den Kindern und Eltern der IDEFICS-Studie für ihre Teilnahme und Mitarbeit.

- meiner Familie und meinen Freundinnen für den fortwährenden moralischen Beistand.

- meinem Mann und meinen beiden Töchtern, weil sie mich auch in den Phasen hoher Arbeitsbelastung liebevoll ertragen, aufgeheitert und abgelenkt haben, für die eine oder andere Nackenmassage und dafür, dass mich ihre Anwesenheit in jeder Minute daran erinnert hat, was im Leben wirklich von Bedeutung ist.

Publikation 1

Eur J Epidemiol (2010) 25:607–617
DOI 10.1007/s10654-010-9481-1

METHODS

Quality management for the collection of biological samples in multicentre studies

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Received: 3 September 2009 / Accepted: 11 June 2010 / Published online: 22 June 2010

Abstract Large scale international multicentre studies require sophisticated quality management for the collection, processing and logistics of biological samples to ensure a maximum degree of standardisation across different environmental conditions and settings. This paper describes a quality management system for the collection of biological samples (QMS-BS) which was applied during IDEFICS, a large European multicentre study. The application was evaluated by several criteria like response rates for the different types of biological samples, measures of sample quality, compliance with the QMS-BS and efficiency of the document and sample control and of the quality assurance system. Response rates varied from 56.6% for venous blood collection to 90.1% for saliva collection. All sample types were associated with problems of sample quality (e.g. haemolysis of blood samples, lack of cooling for urine samples or desiccation of saliva samples). Overall compliance with the QMS-BS was good, with some exceptions mainly related to sample control. In conclusion the QMS-BS is a valuable tool for the management of biological sample collection in epidemiological multicentre studies.

Keywords Quality management · Survey · Multicentre study · Cohort study · Biological specimen bank · Sample database

This article is written on behalf of the IDEFICS consortium.

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Abbreviations

ADR	Agreement concerning the International Carriage of Dangerous Goods by Road
DNA	Deoxyribonucleic acid
FA	Fatty acid
GEP	Good epidemiological practice
IATA	International air transport association
ICAO	International civil aviation organization
IDEFICS	Identification and prevention of dietary—and lifestyle—induced health Effects in Children and infantS
IEA	International epidemiological association
INRS	Institut National de Recherche et de Securite
ISO	International organization for standardization
QMS	Quality management system
QMS-BS	Quality management system for biological samples
RFID	Radio-frequency identification
RNA	Ribonucleic acid
SOP	Standard operation procedure

Introduction

In epidemiological studies, biological samples have become an indispensable source of information [1]. Different sample

types are used to measure a broad range of biomarkers, for example hormones, lipids, glucose, protein, bulk and trace elements or genetic factors. In epidemiology these are used to determine exposure, susceptibility or effects [2].

Several factors influence the observed concentration of a biomarker in the human body, some are inherent like age or sex, but others are controllable. These can affect the concentration of a biomarker either before, during or after sample collection. Before sample collection, fasting status, medication, drug intake, physical activity and diurnal and seasonal variation play an important role. For example glucose and fatty acids are known to be closely related to food intake and therefore fasting measurements are mostly used in epidemiological studies. The levels of most biomarkers vary during the day; some can even change considerably within minutes. Salivary morning cortisol for instance was found to change by approx. 10% within 30 min in adolescents [3]. Deacon et al. [4] reported the effects of posture on melatonin concentrations in plasma and saliva. After sample collection, other variables affect the stability of a biomarker in vitro, e.g. temperature, time until processing or freezing and additives like anticoagulants or stabilising agents. As one example, stability tests for fatty acids showed that these are very unstable at room temperature without any treatment [5]. All of these factors are potential sources of bias, and thus require to be standardised. The most important influences on biological samples are summarised in Table 1. A comprehensive summary of factors influencing the quality of biological samples in molecular epidemiological studies is given in a review by Holland et al. [6].

The standardised collection of biological samples in epidemiological studies is a challenging task, especially in international multicentre studies. Environmental conditions, such as ambient temperature, distance between field study centres and laboratories, may largely vary thus affecting the quality of the biological samples. Nevertheless, if data ought to be suitable for joint data analysis, standardised collection, processing, shipment, storage and analyses of biological samples is indispensable. A reliable quality management system can ensure standardisation across different environmental conditions and settings.

The International Organization for Standardization (ISO) created their quality management system (QMS) standards in 1987. These are applicable in different types of industries, for different types of activities or processes, e.g. design, production or service delivery. The ISO QMS standards certify processes, not the product itself. The standards are regularly reviewed by the ISO, the last revision was done in 2008 and the series was called ISO 9001:2008 series [7].

Since the 1990s, the International Epidemiological Association (IEA) and many national associations have discussed quality criteria for epidemiological research. They have agreed on guidelines for Good Epidemiological Practice (GEP) which can be found on their respective websites [8]. All of these guidelines promote the idea of quality assurance in epidemiological studies, which was elaborated by Rajaraman and Samet [9]. A working group of the Department of Epidemiology at the INRS in France successfully implemented the ISO 9002 system in their department [10]. Their certified quality system includes procedures specific to the conduct of epidemiological studies in occupational epidemiology but detailed aspects of biomarker collection are not addressed by their quality system. An overview of quality aspects in molecular epidemiology was provided by Holland et al. [11].

Considering the work of Moulin et al. [10] and Holland et al. [11], we present a newly developed quality management system designed for the collection of biological samples in epidemiological studies (QMS-BS). The applicability of the QMS-BS was evaluated in the context of the IDEFICS study, a large European multi-centre study on childhood obesity, where several types of biological samples were collected. To evaluate the application of the QMS-BS, the following criteria were applied: sample

Table 1 Major sources of bias for biomarkers before, during and after sample collection

Before sample collection	During sample collection	After sample collection
Fasting status (food, drinking, medication, smoking)	Posture of study subject	Centrifugation conditions
Timing of collection (diurnal variation, seasonal variation)	Sample type (e.g. venous blood vs. capillary blood)	Haemolysis of blood samples
Recent exercise	Collection materials (e.g. tourniquet, collection cups)	Storage temperature
State of health (e.g. infections, fever, lipaemia, pregnancy)	Use of additives (e.g. anticoagulating agents, stabilising agents)	UV light (e.g. direct sunlight)
	Insufficient volume in tube	Time before processing/freezing
	Order of draw	Shipping conditions (temperature and time)
	Sterility	

response rates, measures of sample quality, compliance with the QMS-BS and efficiency of the document and sample control and of the quality assurance system.

Methods

Quality management system for biological samples (QMS-BS)

The conduct of an epidemiological study is suitable for the implementation of an ISO standard as shown by Moulin et al. [10]. Table 2 shows the embedding of the newly developed quality management system for the collection of biological samples (QMS-BS) into the ISO system, in analogy to the quality system for occupational epidemiology by Moulin. The key elements of the QMS-BS are described below.

Standard operating procedures (SOPs)

In clinical research, SOPs are defined as "detailed written instructions to achieve uniformity of the performance of a specific function" [12]. The QMS-BS foresees SOPs that are study-specific and have to be defined for each type of biological sample on the following aspects:

- *Collection*: Consent, time of collection, materials, devices, temperatures, and prerequisites like e.g. fasting status
- *Processing*: Laboratory devices, calibration procedures, time and temperature ranges allowed for processing steps
- *Shipping*: Conditions, intervals
- *Storage*: Temperatures, sorting conditions

Table 2 Quality management system for the collection of biological samples (QMS-BS), adapted from the quality system for occupational epidemiology (Moulin 1998)

ISO 9002 elements	QMS-BS
Production procedures	Standard procedures (SOPs) specific to the collection of biological samples
Document and data control	Document and sample control
Purchasing	Purchases and subcontracting
Process control	Process control for biological samples:
	Collection
	Processing
	Shipping
	Storage
	Laboratory analyses
Inspection and testing	Quality assurance for sample collection

ISO 15189:2007 specifies in detail the QMS requirements for medical laboratories and ensures both regular internal and external quality audits and participation in interlaboratory comparisons [13]. Since ISO 15189:2007 accredited laboratories have SOPs for all their procedures, the QMS-BS does not include SOPs on laboratory analyses.

For the QMS-BS an SOP template was developed (see Fig. 1) which also includes a document log to record changes to previous versions. SOPs have to be accessible to study personnel at any time during their work. In a multicentre study, if the study personnel do not have sufficient skill of English language, all SOPs must be translated to the native languages. Possible errors introduced by translation are minimised by back translation.

Document and sample control

Identification and tracing of biological samples requires appropriate labelling of each sample aliquot. ID-numbers given to study subjects and samples should be pseudonymised in a way that does not allow for the identification of the donor but still clearly defines the sample and enables its retrieval at any time.

Depending on the storage needs, ID-labels should be frost-resistant for down to −80°C or even suitable for storage in liquid nitrogen. An SOP has to address how labels are correctly attached to the biosample tubes, as samples with missing ID-labels will usually be excluded from the study. Coding of IDs in barcodes minimises reading mistakes and simplifies tracing of samples with the help of scanners. There are 1D, 2D or 3D barcode systems and radio-frequency identification (RFID) tags. The choice should depend on the amount of information that needs to be coded and the space available on the ID-stickers. 3D-labels are smallest and contain most information. RFID tags have read and write capabilities and can store up to 2 KB. If biological specimens are stored in a biobank for later analysis, a biosample database should be used for efficient retrieval and documentation of storage conditions. If all samples are used up for laboratory analysis, it might be more economic to use simple delivery notes.

Purchases and subcontracting

Uniform sampling equipment and consumables are important means of standardisation in a multicentre study. Use of standard materials can be assured by central purchasing, but additional management and shipping costs have to be accounted for. Alternatively, all materials have to be clearly defined for local purchase. In this case, feedback on the realisation of the requirements should be collected prior to study commencement.

Version	Date	Comment	Author

1 Person responsible for SOP

_____

2 Name of the procedure

_____

3 Short description of the procedure

_____

_____

4 Scope of application (e.g.project or institute)

_____

5 Glossary (technical terms and abbreviations)

_____

_____

6 Detailed description of procedure (on additional pages)

- What is analysed/ examined?
- Executing person (doctor, interviewer, nurse…?)
- Prerequisites (storage, stability, criteria for exclusion, examination only at special time of day?, etc.)
- General principle of analysis/ examination
- Equipment (including calibration instructions) and resources needed for the analysis/ examination
- Consumables needed for the analysis/ examination
- **Conduct of analysis/ examination step by step**.
- How and where are the analysis/ examination and its results documented?
- How are possible problems dealt with (FAQs)?
- Detection limits, precision, validity, reliability…

7 Annex (related SOPs, standards, laws, instruction manuals which are attached)

_____

_____

Fig. 1 SOP template developed for the QMS-BS

Several components of biological sample collection can be subject to subcontract, either if they cannot be accomplished by own personnel or as a means of standardisation to improve quality. Possible candidates for subcontracting are sample collection, processing or sample analysis. It has to be kept in mind that study elements which are subcontracted are usually more difficult to control by the study management concerning adherence to defined SOPs.

Process control

Before the beginning of the study, biological samples of interest must be identified along with all the steps for their collection, processing, storage, shipment and analyses. In a multicentre study there are two main options for sample processing: it can either be done locally, followed by freezing and shipping on dry ice or centrally, after shipping

on wet ice. To evaluate the best option several parameters have to be weighed against each other: shipping times and costs (if processing is done centrally, daily shipping is necessary) and the higher need for quality assurance if several local laboratories are involved. Either way a lot of conditions need to be defined including minimum and maximum resting times before centrifugation, analysis or freezing and temperatures at which samples should be kept at each stage.

Provisions should be taken for each sample type regarding packaging, shipping intervals and delivery documentation. Shipment of biological samples requires a tracking system to follow packages and a notification by the sender before shipping to ensure that the recipient can accept the package and properly store the samples upon receipt. In the European Community, human biosamples are not generally considered as potentially infectious material any more [14, 15]. Dry ice used for shipping however, is subject to the dangerous goods regulations of the International Air Transport Association (IATA), which implies e.g. that only cargo planes are used for transportation.

SOPs for long-term storage of biological samples define storage temperatures, a system for sample retrieval, a surveillance system to detect equipment failures, an emergency plan, regular quality checks, and sample inventories.

Many laboratories have a QMS accredited according to ISO 15189. It is advisable to choose an accredited central laboratory to guarantee for uniform sample analysis. If shipment of samples from the survey centres to a central laboratory cannot be achieved within 48 h, transportation, even on dry ice, is not a safe option and decentral sample analysis should be preferred. Depending on the stability of the markers of interest there might be a need to analyse certain parameters directly upon collection (point-of-care analysis).

Quality assurance

According to ISO standards a set of activities should be introduced to ensure that the defined SOPs are implemented appropriately: This includes training activities and pretesting of all procedures. Internal and external quality audits should be conducted to verify adherence to the defined SOPs. All means and results of quality assurance have to be documented and should be comprehensible for the study personnel.

Application of the QMS-BS in the IDEFICS-study

IDEFICS is an Integrated Project in the 6th Framework Programme of the European Commission tackling the "Identification and prevention of dietary- and lifestyle-induced health effects in children and infants". A total of 16,188 pre-school and primary school children from eight European countries were examined during the baseline survey in 2007/2008. One major objective of the IDEFICS study was to assess the distribution of diet- and lifestyle-related health problems and to understand the causal pathways leading there. The main emphasis of the study was put on three disorders: obesity/overweight, insulin resistance and impaired bone health. Each of these health problems is associated with a set of biological markers or is even partly defined by them, as it is the case for insulin resistance. The background of the study, its research goals and instruments have been described elsewhere in detail [16, 17].

Sample collection in the IDEFICS study was conducted according to the newly introduced QMS-BS (see Table 3). Children in the baseline survey were asked to donate fasting venous blood (native and EDTA blood), morning urine and saliva samples. If venous blood could not be obtained, capillary blood was taken where possible.

A set of SOPs was developed and translated to all survey languages describing the collection, processing, storage and shipping of all types of biological samples in detail.

Barcoded labels were used for sample tracking. Each biological sample was labelled with an unambiguous 10 digit identification number (ID) with the last two digits clearly defining the type of aliquot. A biosample database was developed to record detailed information on the pre-analytical conditions (collection, processing and storage) of each sample (e.g. cryotube or vial) and its storage location (down to the position in the cryobox) so that retrieval of samples for further use or withdrawal of samples can be done easily. The database facilitates shipping of samples to the central laboratories by an automated generation of delivery notes. A central biobank of remaining samples was built up for long-term storage.

All sampling kits and processing material were purchased centrally. Biochemical analyses were carried out in a central laboratory accredited according to ISO 15189. DNA extraction, genotyping, RNA extraction and gene expression analysis were conducted at different central laboratories.

As blood lipids and blood glucose were markers of primary interest to assess the risk of study participants for metabolic syndrome, these were assessed on site by point-of-care analysis with the Cholestech LDX analyser [18]. The decision was made to use this relatively expensive method because it only uses one drop of venous or capillary blood; thus children who only agreed to give capillary blood could be included to maximize response rates for these key measures. The immediate feedback on these core variables also served as an incentive for the parents. Another drop of blood was sufficient for the fatty acid (FA) analysis of circulating lipids and was collected via a simple kit (Sigma–Aldrich cod. 11312-1KT). This method, developed by Marangoni et al. [19], avoids the complex

Table 3 Application of the QMS-BS to biological sample collection in IDEFICS

QMS-BS	Fasting blood	Morning urine	Saliva
Standard procedures (SOPs) specific to the collection of biological samples	10 specific SOPs	3 specific SOPs Information sheet on collection for parents	4 specific SOPs
Document and sample control	Barcode IDs	Barcode IDs	Barcode IDs
	Sample management by biosample database	Sample management by biosample database	Sample management by biosample database
Purchases and subcontracting	Central purchase of all collection and processing material	Central purchase of collection kits and tubes	Central purchase of DNA kits
	Central laboratory for blood analyses	Central laboratory	DNA extraction at central laboratory
	Central laboratory for analyses of FA test strips		Genotyping at the central laboratories (central for each gene)
	Central laboratory for RNA analyses		
Process control: sample collection	Collection of venous blood; alternatively capillary blood	Collection is done by parents at home	Oragene™ DNA Self-collection kits OG 300 or OG 250 with sponges for smaller children
	For venous blood: 11 ml native and 7 ml EDTA blood 2,5 ml into Paxgene tubes with RNA stabilising agent		
Process control: processing at survey centre	*Cholestech:* first drop of whole blood applied to test cassette	Aliquoting	N/A
	FA test strip: second drop of whole blood applied to strip		
	Blood aliquots: separation of serum, plasma, WBC, RBC		
	Paxgene tubes: N/A		
Process control: shipping	*Cholestech:* N/A	Frozen on dry ice	At room temperature
	FA test strip: at room temperature		
	Blood aliquots: frozen on dry ice		
	Paxgene tubes: frozen on dry ice		
Process control: storage	*Cholestech:* N/A	Long term storage at −20°C	At room temperature for up to a year/ long term storage of saliva or DNA at −20°C
	FA test strips: long term at −20°C, short term at +4°C		
	Blood aliquots: long term at −80°C		
	Paxgene tubes: at −20°C for up to a year		
Process control: laboratory analyses	*Cholestech:* on site analysis of glucose, cholesterols and triglycerides	Minerals as markers of dietary habits, proteins (creatinine, albumin), cortisol	DNA extraction and genotyping from mouth mucosal cells
	FA test strips: composition of circulating lipids by HPLC		
	Blood aliquots: insulin, CRP, HbA1c, selected hormones of energy metabolism and markers of bone health		
	Paxgene tubes: RNA extraction and gene expression analysis		
Quality assurance for sample collection	Quality check of samples at the laboratory	Quality check of samples at the laboratory	Quality check on DNA yield and purity

procedures for collection, storage, shipment, and sample preparation involved with the conventional method of FA analysis.

Apart from point-of-care analyses, all biomarkers were analysed by central laboratories. Markers analysed in blood samples comprised insulin, CRP and HbA1c, as well as

Table 4 Overview of biological markers analysed in the IDEFICS-study

Sample type	Biological marker	Exposure
Native blood	Point-of care analysis:	Metabolic syndrome, Insulin resistance
	Blood glucose, total cholesterol, HDL cholesterol Triglycerides	
	Fatty acid profiles (collection kit)	Dietary patterns
EDTA plasma RBC	Fatty acid profiles (conventional methodology for validating the collection kit)	Dietary patterns
EDTA blood	HbA1c	Diabetes
Serum	Insulin	Insulin resistance
	C-reactive protein (CRP)	Inflammation
	Leptin, Adiponectin	Markers of energy metabolism
	Vit D	Bone metabolism
	Ca	
	NTX-peptide	
Whole blood collected in RNA-stabilising PAXgene tubes	Quantity of RNA for selected genes	Gene expression
Morning urine	Urinary glucose	Diabetes
	Urinary albumin	Metabolic syndrome
	Urinary creatinine	Reference marker
	Minerals (Na, K, Mg, P, Ca)	Dietary patterns
	Cortisol	Chronic stress
Saliva	Selected candidate genes (sequencing of tag-haplotypes)	Genetic risk factors

hormones of energy/fat metabolism and markers of bone metabolism. Markers analysed in morning urine included glucose, albumin, and creatinine, as well as several minerals and cortisol. An overview of all biological markers assessed during the IDEFICS study is given in Table 4.

PAX gene tubes containing an RNA stabilising agent were used to collect blood for gene expression analysis in a subsample of children [20]. In order to maximise response rates, sample collection of morning urine was performed at home by the parents, who received a collection kit and a detailed instruction sheet and DNA was obtained non-invasively from saliva samples. Collection procedures for saliva differed by the children's ability to spit the required amount of saliva: Oragene™ DNA collection kit OG 250 with saliva sponges were used for younger children who were usually not yet able to spit, Oragene™ DNA Self-Collection Kit OG 300 were used otherwise. The kits are user-friendly and provide a high amount of good quality DNA [21]. Samples of all types were processed at the local survey centres and shipped to central laboratories at regular intervals.

All procedures were instructed at a central training which was mandatory for all survey centres. The whole set of instruments was tested during a pretest (Suling, in preparation). During the survey, a central telephone hotline was established for all questions regarding the biological samples. Survey site visits were conducted by a central quality control unit where the practical field work was inspected and deviations from SOPs were corrected directly if possible, or else remedial actions were initiated.

Results

Evaluation of applied QMS-BS during the IDEFICS baseline survey

Response rates for different biological sample types in the eight survey centres are listed in Table 5. Response rates varied for different types of biological samples with highest response rates for non-invasive sample types. Urine samples were obtained from 85.6% of the children in total, with the lowest response of 67.9% in centre 3. Saliva samples were collected from 90.2% of the children; again centre 3 had the lowest response rate of 76.7%. Two of the centres (centre 7 and 8) nearly reached completeness for this sample type. For blood sampling, it was attempted to collect venous blood; only children that were not willing to give venous blood were asked for capillary blood. In total, a remarkable 79.7% of the children gave capillary or venous blood and were thus eligible for point-of-care analysis. Centre 4 obtained a substantially lower rate than

Table 5 Response rates for biological samples during the baseline survey of IDEFICS

Study centre	Total blood	Venous blood	Capillary blood	Urine	Saliva	Subjects included*
Centre 1	1,812	1,296	516	1,946	1,986	2,250
	80.53%	57.60%	22.93%	86.49%	88.27%	100.00%
Centre 2	1,325	882	443	1,419	1,552	1,719
	77.08%	51.31%	25.77%	82.55%	90.29%	100.00%
Centre 3	1,729	184	1,545	1,615	1,825	2,380
	72.65%	7.73%	64.92%	67.86%	76.68%	100.00%
Centre 4	1,184	996	188	1,498	1,640	1,923
	61.57%	51.79%	9.78%	77.90%	85.28%	100.00%
Centre 5	1,540	1,058	482	1,596	1,638	1,810
	85.08%	58.45%	26.63%	88.18%	90.50%	100.00%
Centre 6	1,609	1,413	196	1,858	1,975	2,066
	77.88%	68.39%	9.49%	89.93%	95.60%	100.00%
Centre 7	2,407	2,133	274	2,550	2,534	2,567
	93.77%	83.09%	10.67%	99.34%	98.71%	100.00%
Centre 8	1,327	1,221	106	1,405	1,481	1,507
	88.06%	81.02%	7.03%	93.23%	98.27%	100.00%
Total	12,933	9,183	3,750	13,887	14,631	16,222
	79.73%	56.61%	23.12%	85.61%	90.19%	100.00%

*A study subject was included if at least data on age, sex, height and weight was collected

the other centres (61.6%). This centre also had some initial problems in obtaining fasting blood (7.3% of non-fasting blood samples compared to an average of 1.4%). Venous blood could be collected from on average 56.6% of children across all countries. Response rates for venous blood ranged from 51 to 83% between the centres with the exception of centre 3 where venous blood was only collected from 7.7% of the children. The site visit showed that the low response rate at centre 3 resulted from non-compliance with the study protocol: due to the specific situation in the study region children were primarily asked for capillary blood at the survey site and then given the additional opportunity for later venipuncture at another facility.

As part of process control various measures of sample quality were recorded accompanying the different steps of sample collection and a quality check was performed for each sample in the laboratory upon arrival (see Table 6). Collection of morning urine was documented by the parents on a collection sheet; this included recording of potential problems with sample quality. About 2.9% of the parents reported that the sample was not the first morning urine, 3.9% that the children went to the toilet at night and 6.9% stated that the urine was left uncooled for several hours. These rates varied among the centres: for 45% of the urine samples of centre 4 at least one of these problems was reported compared to only 0.3% of centre 3. The quality check of urine samples at the central laboratory on the other hand generally showed no problems (data not shown).

Venous blood samples were repeatedly associated with several problems as noted both by the study centres in the biosample database and by the central laboratory. According to the study centres an average of 5.4% of the samples were haemolytic with a maximum prevalence of 25.1% in centre 1. The central laboratory on the other hand categorised 10.3% of samples as haemolytic, where again centre 1 was of main concern with 30.8% of samples classified as haemolytic. High amounts of haemolytic samples were also reported for centre 2, 4 and 8 (13.8, 8.2 and 12.1%, respectively). It can thus be concluded that the assessment of haemolytic samples by the study centres was not a suitable tool to detect problematic samples for laboratory analyses. Coagulation of EDTA samples occurred in 0.7% of the checked samples, most of which came from centre 4. All centres had occasional problems to reach the required filling quantity for the aliquots. In total 4% of the samples were considered to be 'short samples' by the central laboratory.

Saliva samples were least error-prone of all sample types; the small fraction of dry samples (0.7%) which arrived at the central laboratory (probably due to leakage) were re-hydrated and still extracted with a lower, but yet sufficient, yield of DNA.

Compliance with the QMS-BS varied between survey centres and components. All survey centres participated in the central training and conducted subsequent local trainings as foreseen in the quality assurance system. The survey centres implemented all standardised procedures

Table 6 Measures of samples quality during IDEFICS baseline survey

Study centre	1	2	3	4	5	6	7	8	Total
Morning urine									
Samples collected	1,946	1,419	1,615	1,498	1,596	1,858	2,550	1,405	13,887
Problems with sample quality									
Any problem	499	12	5	679	60	176	37	221	1,689
% of urine samples	25.64	0.85	0.31	45.33	3.76	9.47	1.45	15.73	12.16
Problems with collection									
Not first morning urine	145	6	2	60	37	44	30	80	404
% of urine samples	7.45	0.42	0.12	4.01	2.32	2.37	1.18	5.69	2.91
Child went to the toilet at night	95	2	2	202	15	123	2	97	538
% of urine samples	4.88	0.14	0.12	13.48	0.94	6.62	0.08	6.90	3.87
Problems with processing									
Urine uncooled for several hours	326	4	1	522	8	12	8	77	958
% of urine samples	16.75	0.28	0.06	34.85	0.50	0.65	0.31	5.48	6.90
Problems with storage	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Venous blood									
Samples collected	1,296	882	184	996	1,058	1,413	2,133	1,221	9,183
Problems with collection									
Child not fasting for >8 h	2	13	0	71	33	18	17	29	112
% of blood samples	0.15	1.47	0.00	7.13	3.12	1.27	0.80	2.38	1.22
Problems with processing									
Samples haemolytic	399	122	3	82	61	35	94	148	944
% of blood samples	30.79	13.83	1.63	8.23	5.77	2.48	4.41	12.12	10.28
Samples coagulated	0	0	0	41	5	1	12	6	65
% of blood samples	0.00	0.00	0.00	4.12	0.47	0.07	0.56	0.49	0.71
Short samples	75	1	17	64	47	105	29	34	372
% of blood samples	5.79	0.11	9.24	6.43	4.44	7.43	1.36	2.78	4.05
Permitted times exceeded	1	4	0	0	121	1	6	4	137
% of blood samples	0.08	0.45	0.00	0.00	11.44x	0.07	0.28	0.33	1.49
Problems with storage	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Saliva									
Samples collected	1,986	1,552	1,825	1,640	1,638	1,975	2,534	1,481	14,631
Problems with collection	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Problems with processing	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Problems with storage									
Dry samples	14	9	16	7	14	6	33	6	105
% of saliva samples	0.70	0.58	0.88	0.43	0.85	0.30	1.30	0.41	0.72

according to SOPs as verified by the external quality audits during site visits. The only exception was the SOP for blood collection, were one centre (centre 3) did not follow the instructions to take venous blood preferentially as mentioned above. For some other components compliance was lower. Most notably, centres had difficulties using the biosample database. The software was considered to be too complex and data entry too time-consuming. This caused a delay in data entry which in turn resulted in problems in the central laboratory, where biochemical analyses could not be conducted for samples delivered without a corresponding database. Moreover, due to the delay it was not possible to carry out direct quality control of sample processing with the biosample database as initially planned. Variables recorded in the database like time intervals, e.g. between collection of blood samples and their centrifugation represent important measures of quality assurance. As depicted in Table 6 especially one centre had problems to manage sample processing within the requested time span. 11.4% of the samples in centre 5 were centrifuged later than foreseen in the SOP, for all other centres this rate was below 1.5%.

Discussion

This paper introduces a quality management system for the collection of biological samples in epidemiological studies (QMS-BS). The QMS-BS was applied to a large multicentre study, the IDEFICS-study with the goal to collect biological samples of a standardised quality across all study centres and to build up a large biobank of blood, saliva and urine samples from children from different European regions. Evaluation showed that high average response rates were reached for all sample types. Similar rates were achieved during the German KIGGS study, a health study on children and adolescents, where a response rate of 85% was reached for urine samples in the 'Iodine Module' of the study [22] and 52.6% for an environmental survey module which included venous blood sampling amongst others [23]. Measures of sample quality revealed several problems which mainly occurred during sample collection and processing. Central purchase of consumables guaranteed comparability but was accompanied by high shipping costs; depending on the monetary value of the respective consumables up to 10% were added to their costs. Overall compliance with the QMS-BS was good in the study centres, although some exceptions were noted. These were related primarily to sample control. Study centres complained about the complexity of the biosample database, leading to a considerable delay in data entry. The database could thus not be used for quality control during the ongoing survey, only retrospectively. Nevertheless biobanking and sample retrieval would not have been possible without the biosample database.

Generally, not all differences between centres could be avoided by the QMS-BS. In particular, blood response rates and sample quality differed substantially between centres. Different response rates for invasive sample types may be related to national characteristics, whereas differences in sample quality were probably rather based on different survey logistics, e.g. allocated manpower or geographical distances between sample collection and processing. The influence of sample quality on the analyses of biological makers in the IDEFICS-study will be the topic of future investigations.

It should be noted that the introduction of a quality management system is not able to solve all problems during sample collection in epidemiological studies. Another key aspect is the complexity of the applied procedures. Decisions made for user-friendly solutions within IDEFICS were generally expensive but very successful like e.g. point-of-care analysis of the main biological markers for metabolic syndrome (glucose, total cholesterol, HDL, triglycerides), DNA colletion with saliva kits that didn't need to be processed or cooled, and FA test strips that only needed one drop of blood for the analysis of complete fatty acid profiles. These solutions generally reached high response rates and offered a standardised quality. For example, because point-of-care analysis was employed for blood glucose and lipid analyses, response rates for the respective markers were increased by about 23% (representing the children which only agreed to the collection of capillary blood).

Results of the evaluation will contribute to further optimisation of biological sample collection for the IDEFICS follow-up survey that starts in autumn 2009. The biosample database has been substantially simplified and a barcode scanner and laptop will be used for storage documentation. Moreover, procedures for serum collection will be changed to reduce the amount of haemolytic samples which was most likely caused by insufficient clotting times in combination with the completely native blood tubes that were used to allow for the removal of two drops of venous blood for point-of-care analysis and FA analysis before clotting started. To enhance compliance with the QMS-BS in the IDEFICS follow-up survey different options are currently discussed. Krockenberger et al. [24] investigated the adherence to SOPs in the context of clinical trials. They found that, for questions about the daily work, the staff was more likely to ask a colleague rather than to read the corresponding SOP (18.4 vs. 13.8%). The authors suggest a computer-based information retrieval system for SOPs to increase the ease-of-use and usefulness of SOPs wich might also be an interesting option for the QMS-BS. Other options are centralised re-training sessions and/or special emphasis on selected aspects of biological samples collection during the external site visits.

Practical aspects of field work and sample collection are often neglected in scientific exchange. The QMS-BS fills this gap and represents a systematic approach to sample collection for application in epidemiological studies. Over all its application in IDEFICS helped to obtain a high quality standard for the biological samples collection in this European multicentre study.

Acknowledgments This work was done as part of the IDEFICS Study (www.idefics.eu). We gratefully acknowledge the financial support of the European Community within the Sixth RTD Framework Programme Contract No. 016181 (FOOD). The authors are indebted to the IDEFICS survey centres for their work in gathering the data. We also thank Wolfgang Ahrens for helpful suggestions on the manuscript.

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Publikation 2

International Journal of Obesity (2011) 35, S104–S112
 0307-0565/11
www.nature.com/ijo

ORIGINAL ARTICLE

Influence of sample collection and preanalytical sample processing on the analyses of biological markers in the European multicentre study IDEFICS

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Objective: To evaluate the influence of a standardised sampling protocol and process quality across the different IDEFICS (Identification and prevention of dietary- and lifestyle-induced health effects in children and infants) centres on the results of the biochemical measurements.
Design: Baseline survey within the community-based intervention study.
Subjects: A total of 16 224 children, aged 2–8 years, enrolled in the IDEFICS baseline survey in 8 European countries. Venous or capillary blood samples were collected from 12 430 children, urine samples from 13 890 children and saliva samples from 14 019 children.
Methods: A set of quality indicators was recorded for the biological blood, urine and saliva samples collected during the IDEFICS study. Results of blood and urine measurements were analysed and stratified by selected quality indicators.
Results: Concentrations of biological markers in blood and urine measured during the IDEFICS baseline survey are associated with several quality indicators assessed in this study. Between-country variations of these biomarkers are described. It was confirmed that fasting has a big influence on the concentration of certain biomarkers. Biomarkers in morning urine samples may be erroneous if the study subjects void during the night or if samples are not taken from the very first morning urine.
Conclusions: The analysed data underline that a standardised sampling protocol is of major importance, especially in multicentre studies, but non-compliance is ever present in spite of well-defined standard operation procedures. Deviations from the protocol should therefore always be documented to avoid error pertaining to the concentration of biological markers.
International Journal of Obesity (2011) **35,** S104–S112; doi:10.1038/ijo.2011.41

Keywords: biological sample; biomarker; sample quality; fasting status; morning urine; multicentre study

Introduction

Biological samples have become an essential source of information for studying childhood obesity and the risk of metabolic disorders.[1] Different types of samples are used to measure a broad range of biomarkers, such as glucose, lipids, hormones or genetic factors, to identify children at risk. A definition of the metabolic syndrome for children (>10 years of age) and adolescents was recently suggested using the well-established biochemical risk markers.[2]

The standardised collection of biological samples in epidemiological studies is a challenging task, especially in international multicentre studies. Environmental conditions, such as ambient temperature or distances between field study centres and laboratories, may vary and consequently affect the quality of the biological samples. National characteristics such as typical mealtimes or sleeping times or duration may also have an effect on the respective biological markers analysed in the different national study populations.

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In general, the observed concentration of a biomarker in the human body is influenced by several factors; some are inherent, such as age or sex, whereas others are controllable. These sources of error may affect the level of a biomarker before, during or after sample collection. Before sample collection, preanalytical conditions, such as fasting status, medication, drug use, physical activity and diurnal and seasonal variation, may have an important role. Major influences during sample collection include body posture of the studied participant or the collection materials used. After sample collection, the stability of a biomarker *ex vivo* is potentially influenced by environmental factors such as temperature or ultraviolet light, treatment conditions (such as time until processing/freezing or use of additives) and storage/shipping conditions. Holland *et al*.[3] gave a summary of the factors influencing the quality of biological samples in molecular epidemiological studies. To ensure standardisation across the different environmental conditions and settings within the IDEFICS (Identification and prevention of dietary- and lifestyle-induced health effects in children and infants) study, a set of standard operating procedures (SOPs) was developed. This included SOPs on the collection of biological samples, as well as on procedures for initial processing, shipment, storage and documentation.

The aim of the present study was to assess the quality of biological samples across the IDEFICS survey centres, considering quality indicators (for example, fasting status or occurrences before urine collection) recorded during the baseline survey of this European multicentre study.[4]

Materials and methods

Study design

IDEFICS is an integrated project within the Sixth Framework Programme of the European Commission. During the baseline survey in 2007/2008, 16 224 preschool and primary school children from eight European countries were examined, including the collection of biological samples. The subsequent community-based intervention was followed by a second survey, which continued until May 2010. The background of the study, its research goals and instruments have been described elsewhere in detail.[5,6]

Biological samples in IDEFICS

Children participating in the IDEFICS baseline survey were asked to donate fasting venous blood, morning urine and saliva samples. EMLA patches (AstraZeneca, London, UK), which contain local anaesthetics, were offered to the children who agreed to donate venous blood to allow for painless blood withdrawal.[7] If venous blood could not be obtained, capillary blood was taken where possible. The set of blood samples generally included two tubes of venous blood: one plain tube for serum collection and one EDTA-coated tube for glycated haemoglobin analysis and separation into plasma and red and white blood cells. Additionally, a PAXgene tube (Becton Dickinson Franklin Lakes, NJ, USA) containing an RNA-stabilising agent was drawn from a subsample of children for gene expression analysis.[8] Morning urine was collected by the parents, who received a collection kit along with a detailed instruction sheet. Saliva was collected for DNA extraction. The collection procedure for saliva differed by the children's ability to spit the required amount of saliva: Oragene DNA collection kit OG 250 with saliva sponges was used for younger children who were usually not yet able to spit, while Oragene DNA self-collection kit OG 300 was used for older children (DNA Genotek Inc., Kanata, Ontario, Canada). The kits were user friendly and provided a high amount of good-quality DNA.[9] Preanalytical sample processing of blood and urine samples was done at the local survey centres or at local laboratories; sample analysis was done at a central laboratory. All blood samples were stored at −80 °C and all urine samples at −20 °C. If a centre could not guarantee for storage at −80 °C, they were instructed to ship the blood samples to the biobank/central laboratory at monthly intervals. Saliva was stored at room temperature before DNA extraction. Extracted DNA and the remaining saliva were stored at −20 °C. A central biobank of remaining samples was built up for long-term storage.

Blood glucose, cholesterol, high-density lipoprotein cholesterol and triglycerides were assessed on site at each study centre by point-of-care analysis using the Cholestech LDX analyser (Cholestech, Hayward, CA, USA)[10] either in venous or in capillary blood. This procedure was chosen because only one drop of blood was needed for the analyses. Other biomarkers analysed in blood samples comprised insulin, C-reactive protein and glycated haemoglobin, as well as the adipokines leptin and adiponectin. Calcium, cross-linked collagen N-telopeptides and vitamin D were included as biomarkers of bone metabolism. The fatty acid composition of circulating lipids was analysed from one drop of venous or capillary blood by a new method developed by Marangoni *et al*.[11] A large set of biomarkers were also analysed in urine samples, as a higher response was expected from the study participants for this non-invasive sample type compared with venous blood. These comprised glucose, albumin and creatinine, as well as biomarkers of dietary habits (sodium, calcium, phosphate, magnesium and potassium) and cortisol as a biomarker of chronic stress, which has been proposed to be relevant for the development of overweight/obesity.[12]

Quality indicators

To evaluate the standardised quality of collected biological samples, a number of quality indicators were recorded during the different steps of sample collection. Table 1 shows possible consequences of deviations from the protocol. Collection of urine samples and documentation on a urine collection sheet were done by the parents, who

Table 1 Quality indicators for blood, urine and saliva samples collected within the IDEFICS study

	Source of information	*Expected problem*	*Stratified analysis*
Venous blood			
Child not fasting for >8 h	Recorded by survey staff (information reported by parent or child)	Overestimation of blood glucose and triglyceride concentrations	Yes
Samples haemolytic	Recorded by laboratory staff	Degradation of insulin	No
Samples coagulated	Recorded by laboratory staff	Analysis of sample not reliable	No
Short samples	Recorded by laboratory staff	Analysis of sample not reliable	No
Permitted times exceeded	Recorded by survey staff	Risk of degradation of biomarkers	No[a]
Capillary blood			
Child not fasting for >8 h	Recorded by survey staff (information reported by parent or child)	Overestimation of blood glucose and triglycerides	Yes
Morning urine			
Not the first morning urine	Reported by parents	Results not reliable for biomarkers with diurnal rhythm	Yes
Child went to the toilet at night	Reported by parents	Results not reliable for biomarkers with diurnal rhythm	Yes
Urine uncooled for several hours	Reported by parents	Risk of degradation of biomarkers	Yes
Saliva			
Dry samples	Recorded by laboratory staff	Reduced yield of DNA	No[b]

Abbreviation: IDEFICS, Identification and prevention of dietary- and lifestyle-induced health effects in children and infants. [a]Exceeding of permitted times occurred only in one country in more than 0.5% and was thus not included in the stratified analysis. [b]Details on the quality of collected saliva samples and extracted DNA are described by Koni *et al.*[13]

recorded the following occurrences: 'sample not first morning urine', 'sample uncooled for more than 2 h' and 'child went to toilet at night'. For the collection of blood, fasting was requested in the respective SOP and information on fasting status was reported by parents or children to the field staff during examination. Other quality indicators were documented during the quality check performed in the central laboratory (condition of blood samples upon arrival at laboratory) or based on variables recorded by the survey staff (for example, time interval between collection of blood samples and their centrifugation). Saliva samples were checked on their hydration status before DNA extraction. A small fraction of samples (0.7%) was found to be dried up, but could all be rehydrated and still extracted with a sufficient yield of DNA. Details on the quality of collected saliva samples and extracted DNA have been described by Koni *et al.*[13] A comprehensive overview of the quality management applied in the IDEFICS study has been provided elsewhere in detail.[14] Feedback on sample quality was given to the centres throughout the survey to continuously improve sample collection.

Analyses by quality indicators were restricted to urine parameters and to fasting status for blood samples. Analyses of venous and capillary blood samples were combined, as biomarker concentrations have been shown to be consistent for both types of blood, as reported, for instance, by Park *et al*. for glucose.[15] Coagulated or small volume blood samples could not be analysed. Haemolytic samples were excluded from analyses of insulin because an insulin-degrading enzyme is released from lysed red blood cells.[16] Exceeding the length of time permitted for certain steps of the SOP (for example, time between collection and centrifugation of blood samples) only occurred in one country in more than 0.5% of samples, and these data were not included in the stratified analysis.

Statistical procedures

Concentrations of biomarker analyses in blood and urine are presented as mean, standard deviation and coefficient of variation, stratified by a quality indicator.

To investigate whether a particular quality indicator had a significant effect on the concentration of a biomarker, linear regression analyses, adjusted for age and sex, were conducted. All analyses were carried out in the total sample and were stratified by country. Two-tailed *P*-values of regression parameter estimates and R^2 of the regression model were calculated. All analyses were performed using SAS statistical software version 8.2 (SAS Institute Inc., Cary, NC, USA).

For each sample type, samples of some study centres were excluded from statistical analysis if the number of samples affected by certain quality problems was too small (<10 subjects). This was the case for Cyprus, Hungary and Italy regarding fasting status for blood collection, and Cyprus, Hungary and Estonia regarding parental documentation of urine collection.

Results

Response of participants

The proportion of children who provided the required information and samples (response proportion) varied for different types of biological samples.[4,14] Urine samples were

obtained from 85.6% of the children. Blood samples were collected from 79.7% of the children in total (capillary or venous blood) and were thus eligible for point-of-care analysis. Venous blood could be collected from 56.6% of the children across all countries.

Quality indicators

Assessment of quality indicators showed that any problems with the collection of urine were documented by the parents for 12% of the samples. For 2.9% of the samples, parents reported that the collected urine was not the first morning urine; for 3.9% they reported that the children went to the toilet at night; and for 6.9% they stated that the urine was left uncooled for more than 2 h. These proportions varied substantially between centres: for 45% of the urine samples in Belgium at least one of these problems was reported compared with only 0.3% in Cyprus.

Table 2 Distribution of quality indicators by age and sex (in percent of each group)

	Girls		*Boys*	
	2–5 years	*6–9 years*	*2–5 years*	*6–9 years*
Child not fasting (%)	3.70	2.87	4.22	2.67
Urine uncooled for >2 h (%)	5.68	5.60	6.49	5.61
Not the first morning urine (%)	1.64	2.89	2.21	2.85
Child went to the toilet at night (%)	4.15	1.69	5.71	2.13

Venous blood samples were reported to be non-fasting in 1% of the children. According to the central laboratory, 9.3% of the blood samples were haemolysed, with a maximum of 30.8% of the samples classified as haemolysed in Italy. High proportions of haemolysed samples were also reported for Estonia, Spain and Belgium (13.8, 12.1 and 8.2%, respectively). Coagulation of EDTA samples occurred in 0.7% of samples, most of which came from Belgium. All centres had occasional problems in reaching the required filling quantity for the aliquots. In total, 4% of the samples were considered to be 'too small a volume' by the central laboratory. One centre in particular exceeded the requested time span for sample processing: 11.4% of the samples in Sweden were centrifuged later than foreseen in the SOP; for all other centres, this proportion was below 1%.

The age and sex distribution of the four quality indicators included in the stratified analyses is depicted in Table 2. Non-fasting samples were taken from younger girls and boys more often. This age group also voided during the night more often, whereas a higher proportion of older girls and boys delivered a sample that was not taken from the first morning urine. Overall, the quality features were quite evenly distributed between both sexes.

Biomarkers in serum after stratification

Serum concentrations of glucose, triglycerides and insulin are depicted in Table 3. Among the biomarkers analysed

Table 3 Concentrations of glucose, triglycerides and insulin, stratified by country and fasting status

Country[a]	*Point of care (venous and capillary blood)*								*Venous blood*			
	Glucose (mmol l^{-1}l)				*Triglycerides (mmol l^{-1}l)*				*Insulin (pmol l^{-1}l)*			
	N	*Mean (s.d.)*	*CV*	*Regression estimate (P-value)/R^2*[b]	N	*Mean (s.d.)*	*CV*	*Regression estimate (P-value)*[b]	N	*Mean (s.d.)*	*CV*	*Regression estimate (P-value)*[b]
Estonia												
Fasting	1216	4.71 (0.56)	0.12	Reference	1215	0.30 (0.12)	0.42	Reference	732	35.20 (36.50)	1.04	Reference
Non-fasting	28	4.46 (0.64)	0.14	−5.92 (0.002)/0.066	28	0.34 (0.18)	0.54	3.79 (0.071)/0.009	18	84.04 (119.90)	1.43	6.15 (<0.0001)/0.068
Belgium												
Fasting	916	4.40 (0.55)	0.13	Reference	917	0.64 (0.24)	0.38	Reference	803	24.82 (25.18)	1.01	Reference
Non-fasting	105	4.67 (0.73)	0.16	5.43 (<0.0001)/0.073	105	0.81 (0.44)	0.55	14.03 (<0.0001)/0.044	72	44.80 (45.02)	1.00	2.83 (<0.0001)/0.068
Sweden												
Fasting	1516	4.43 (0.47)	0.11	Reference	1518	0.29 (0.14)	0.49	Reference	961	25.83 (18.25)	0.71	Reference
Non-fasting	15	4.38 (0.58)	0.13	−0.98 (0.639)/0.109	15	0.26 (0.06)	0.23	−2.03 (0.525)/0.010	10	35.85 (17.24)	0.48	1.53 (0.042)/0.129
Germany												
Fasting	1576	4.78 (0.49)	0.10	Reference	1573	0.67 (0.29)	0.43	Reference	1331	34.63 (23.52)	0.68	Reference
Non-fasting	13	4.59 (0.61)	0.13	−2.09 (0.377)/0.090	13	0.96 (0.46)	0.47	25.28 (0.0003)/0.017	10	41.12 (17.75)	0.43	1.43 (0.136)/0.139
Spain												
Fasting	1264	4.44 (0.50)	0.11	Reference	1261	0.59 (0.19)	0.33	Reference	884	28.57 (21.07)	0.74	Reference
Non-fasting	36	4.25 (0.56)	0.13	−1.60 (0.267)/0.140	36	0.58 (0.20)	0.35	−0.55 (0.847)/0.011	26	35.93 (41.99)	1.17	1.52 (0.010)/0.067
All												
Fasting	6488	4.57 (0.56)	0.12	Reference	6484	0.49 (0.27)	0.55	Reference	4711	30.15 (25.32)	0.84	Reference
Non-fasting	197	4.53 (0.54)	0.15	−0.32 (0.642)/0.094	197	0.67 (0.29)	0.43	18.04 (<0.0001)/0.108	136	47.40 (58.87)	1.24	2.53 (<0.0001)/0.079

Abbreviation: CV, coefficient of variation. [a]Cyprus, Hungary and Italy were excluded from statistical analysis because the number of non-fasting children was below 10 in these countries. [b]Estimates from linear regression analysis, adjusted for age and sex.

within the IDEFICS baseline survey, these were expected to be influenced most by food intake. Stratification by fasting status showed that for the large majority of children the SOP was followed and fasting samples were collected. Only 197 out of the 6685 venous and capillary blood samples included in the analysis were non-fasting.

Fasting glucose concentrations were similar in participants from Belgium, Sweden and Spain (around 4.4 mmol l^{-1}) and slightly higher in Germany and Estonia (4.8 ± 0.5 and 4.7 ± 0.6 mmol l^{-1}, respectively). Fasting glucose concentrations in the three countries that were not included in the stratified analysis were also in a comparable range, that is, 4.7 ± 0.6 mmol l^{-1} in Cyprus, 4.8 ± 0.6 mmol l^{-1} in Hungary and 4.9 ± 0.4 mol l^{-1} in Italy. Fasting serum triglyceride concentrations differed substantially between centres. Swedish, Estonian and Hungarian children had mean triglyceride concentrations of around 0.3 mmol l^{-1}, whereas mean values between 4.9 ± 0.3 and 0.82 ± 0.6 mmol l^{-1} were reached in children from Belgium, Germany, Spain, Cyprus and Italy. Overall, reported fasting status did not have a significant influence on the glucose concentrations. Serum triglyceride concentrations were higher when children were not fasting in four of the five countries; for two of these countries the differences were statistically significant. Fasting insulin concentrations ranged from 24.8 ± 25.2 pmol l^{-1} in Belgium to 35.2 ± 36.5 pmol l^{-1} in Estonia, and were higher for non-fasting children in all countries. Differences in insulin concentrations between fasting and non-fasting conditions reached statistical significance in all countries except for Germany, where the number of non-fasting children was very low.

Biomarker concentrations are depicted in Figure 1 for all children depending on their fasting status. There was no effect for glucose, whereas significant differences were identified for triglycerides and insulin across all countries.

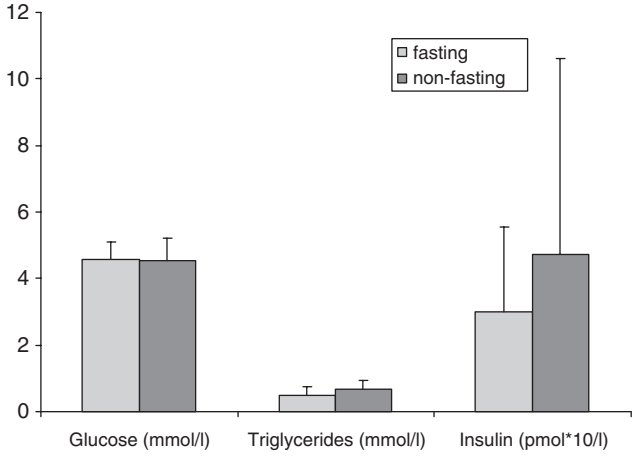

Figure 1 Biomarkers (mean + s.d.) measured in the blood of children with different fasting status.

Biomarkers in urine after stratification

Table 4 shows the results of the laboratory analysis for selected biomarkers analysed in urine (cortisol, albumin and potassium), stratified by the quality indicators recorded by the parents. For 6555 of the analysed samples no problems with urine quality were reported, 921 samples were left uncooled for more than 2 h, 353 samples were not taken from the first morning urine and 505 urine samples were collected from children who had voided during the night before urine collection.

Mean urinary cortisol concentrations varied from 119.4 ± 49.4 nmol l^{-1} in Italy to 183.8 ± 196.2 nmol l^{-1} in Estonia when no problems were reported. Urine samples from children who had voided during the night had significantly higher cortisol concentrations than those from children with no reported problems for urine collection. Higher cortisol concentrations were also found when urine samples were not taken from the first morning urine. The differences were statistically significant for all countries except for Spain. Lack of cooling for more than 2 h did not have a clear influence on urinary cortisol concentration. A statistically significant difference was only seen in Italy, where urinary cortisol concentrations in uncooled samples were slightly higher than in samples with no reported problems.

Urinary concentrations of albumin ranged from 15.6 ± 8.0 to 19.3 ± 47.93 μmol l^{-1} in samples without problems. Mean urinary concentrations of potassium were lowest in Hungary (36.2 ± 21.2 mmol l^{-1}) and highest in Belgium (50.0 ± 22.6 mmol l^{-1}) when no problems with urine quality were indicated. Lack of cooling for more than 2 h did not lead to a statistically significant difference for this biomarker in most countries except for Italy. Mean urinary potassium concentrations were significantly higher in all countries except for Spain, when the urine sample was not derived from first morning urine or when the child had voided during the night.

Mean concentrations of urinary biomarkers are depicted in Figure 2 for all children along with the quality indicated for their urine sample. The figure shows that voiding at night and collection of a sample excreted later than the first void urine strongly affected the concentrations of cortisol and potassium.

Discussion

High average response proportions were reached for all sample types (blood, urine and saliva) collected during the IDEFCIS study. Similar proportions were achieved during the German Health Interview and Examination Survey for Children and Adolescents, where a response rate of 85% was reached for urine samples[17] and 52.6% for venous blood samples.[18] Differences in the proportions of certain quality problems across the centres are likely to have been caused by

Table 4 Concentrations of cortisol, albumin and potassium, stratified by country and quality indicators

Country[a]	*Cortisol (nmol l^{-1})*				*Albumin (μmol l^{-1})*				*Potassium (mmol/l)*			
	N	*Mean (s.d.)*	*CV*	*Regression estimate (P-value)/R^{2b}*	N	*Mean (s.d.)*	*CV*	*Regression estimate (P-value)/R^{2b}*	N	*Mean (s.d.)*	*CV*	*Regression estimate (P-value)/R^{2b}*
Italy												
No problem	1414	119.43 (49.40)	0.41	Reference	1414	16.86 (10.29)	0.61	Reference	1411	40.03 (19.2)	0.48	Reference
Urine uncooled for >2 h	323	125.86 (55.75)	0.44	2.29 (0.043)/0.004	323	18.42 (13.48)	0.73	1.07 (0.022)/0.012	320	44.15 (26.9)	0.61	4.18 (0.001)/0.019
Not the first morning urine	141	176.64 (68.45)	0.39	20.63 (<0.0001)/0.096	141	20.65 (20.72)	1.00	2.57 (0.0003)/0.018	138	68.96 (36.9)	0.54	29.03 (<0.0001)/0.139
Child went to the toilet at night	89	163.28 (67.34)	0.41	15.07 (<0.0001)/0.027	89	16.42 (8.23)	0.50	−0.15 (0.864)/0.010	88	52.92 (28.8)	0.54	9.95 (<0.0001)/0.021
Belgium												
No problem	820	147.22 (72.86)	0.49	Reference	820	18.45 (22.46)	1.22	Reference	820	50.03 (22.6)	0.45	Reference
Urine uncooled for >2 h	506	154.67 (90.53)	0.59	2.29 (0.160)/0.017	506	18.29 (23.77)	1.30	−0.03 (0.972)/0.005	506	50.72 (25.1)	0.49	0.11 (0.930)/0.050
Not the first morning urine	58	194.64 (105.43)	0.54	16.88 (<0.0001)/0.037	58	17.83 (10.72)	0.60	−0.38 (0.852)/0.004	58	69.43 (37.5)	0.54	19.01 (<0.0001)/0.092
Child went to the toilet at night	195	223.01 (102.67)	0.46	25.58 (<0.0001)/0.098	195	17.03 (22.32)	1.31	−0.58 (0.644)/0.004	195	70.63 (35.0)	0.50	18.13 (<0.0001)/0.113
Sweden												
No problem	1524	153.01 (104.88)	0.69	Reference	1524	16.10 (17.68)	1.10	Reference	1524	43.03 (22.1)	0.51	Reference
Urine uncooled for >2 h	8	152.55 (65.41)	0.43	−1.37 (0.919)/0.009	8	18.39 (11.01)	0.60	1.88 (0.663)/0.004	8	54.13 (31.6)	0.58	9.63 (0.214)/0.026
Not the first morning urine	36	218.04 (109.85)	0.50	22.91 (0.0004)/0.017	36	17.01 (11.74)	0.69	0.80 (0.695)/0.004	36	66.56 (32.3)	0.49	22.73 (<0.0001)/0.045
Child went to the toilet at night	15	250.77 (137.72)	0.55	34.22 (0.0006)/0.017	15	16.17 (5.65)	0.35	0.09 (0.976)/0.003	15	75.13 (39.1)	0.52	31.18 (<0.0001)/0.045
Germany												
No problem	1637	141.78 (71.21)	0.50	Reference	1637	19.33 (47.39)	2.45	Reference	1637	44.81 (24.6)	0.55	Reference
Urine uncooled for >2 h	12	129.50 (67.07)	0.52	−3.38 (0.650)/0.010	12	16.42 (3.55)	0.22	−2.66 (0.778)/0.005	12	35.17 (21.9)	0.62	−8.28 (0.240)/0.025
Not the first morning urine	41	229.49 (147.11)	0.64	31.42 (<0.0001)/0.039	44	18.19 (12.03)	0.66	−0.44 (0.931)/0.005	41	85.05 (36.6)	0.43	39.59 (<0.0001)/0.081
Child went to the toilet at night	114	204.05 (129.72)	0.64	20.85 (<0.0001)/0.039	113	16.81 (7.97)	0.47	−0.95 (0.756)/0.005	113	61.73 (35.3)	0.57	14.18 (<0.0001)/0.049
Spain												
No problem	1160	146.34 (82.80)	0.57	Reference	1160	17.99 (26.23)	1.46	Reference	1158	43.61 (21.2)	0.49	Reference
Urine uncooled for >2 h	72	134.03 (56.58)	0.42	−5.26 (0.142)/0.011	72	15.91 (7.25)	0.46	−1.14 (0.596)/0.002	72	43.83 (21.9)	0.50	−1.70 (0.511)/0.051
Not the first morning urine	77	123.95 (52.99)	0.43	−5.42 (0122)/0.014	79	14.71 (1.30)	0.09	−2.78 (0.187)/0.003	77	40.92 (19.3)	0.47	0.77 (0.760)/0.051
Child went to the toilet at night	92	180.12 (83.63)	0.46	12.49 (<0.0001)/0.022	93	17.72 (24.20)	1.37	0.28 (0.881)/0.002	93	52.55 (29.0)	0.55	7.51 (0.001)/0.056
All												
No problem	6555	140.87 (79.76)	0.57	Reference	6555	17.70 (28.99)	1.64	Reference	6550	43.81 (22.3)	0.51	Reference
Urine uncooled for >2 h	921	142.61 (78.11)	0.55	2.69 (0.010)/0.018	921	18.13 (19.42)	1.07	0.62 (0.378)/0.004	918	47.72 (25.9)	0.54	4.18 (<0.0001)/0.032
Not the first morning urine	353	178.46 (95.22)	0.53	14.92 (<0.0001)/0.026	353	18.23 (15.07)	0.83	0.36 (0.738)/0.003	350	64.51 (35.9)	0.56	21.26 (<0.0001)/0.064
Child went to the toilet at night	505	201.20 (104.88)	0.52	20.53 (<0.0001)/0.043	505	16.97 (17.97)	1.06	−0.07 (0.940)/0.003	504	62.34 (34.0)	0.55	15.82 (<0.0001)/0.056

Abbreviation: CV, coefficient of variation. [a]Cyprus, Hungary and Italy were excluded from statistical analysis because the number of non-fasting children was below 10 in these countries. [b]Estimates from linear regression analysis, adjusted for age and sex.

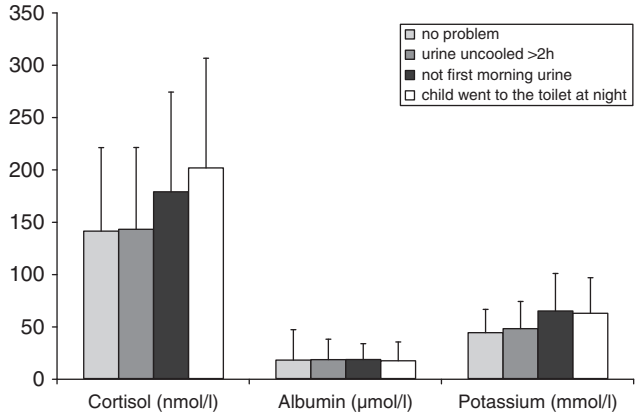

Figure 2 Biomarkers (mean + s.d.) measured in urine samples with different quality properties.

differences in survey logistics (for example, for 'urine uncooled for more than 2 h'), but also depended on the reporting behaviour of the parents. In some countries the parents were present during the examination, whereas in others the children were examined during school/pre-school hours in the absence of the parents. In this case the assessment of fasting status had to rely on the children's reporting only. Collection of morning urine was conducted and documented by the parents only; thus, a good communication structure between the survey team and the parents might have enhanced the quality of this sample type as well as the reporting of deviations from the protocol.

The number of haemolysed samples was striking, especially in some of the participating centres. This could be explained by the plain blood collection tubes without clotting activator, which were used during the baseline survey to allow for sufficient time to take out the aliquots for point-of-care analysis and the fatty acid collection kit. Some centres had problems to adhere to the prolonged resting times that were required for these tubes. Early centrifugation, that is, before clotting was completed, leads to a higher rate of haemolysis in the respective centres. Different blood collection tubes, that is, standard serum tubes with clotting activator, were used for the follow-up survey, and first exploratory analyses showed that haemolysis was no longer a problem. As a consequence, it had to be taken into account that additional personnel was needed for the immediate processing of samples after blood withdrawal.

As expected, concentrations of insulin and triglycerides were higher when children were not in the fasting state. A study on healthy women[19] also showed moderately elevated concentrations of blood triglycerides in non-fasting compared with fasting study subjects (mean values of 1.3 mmol l^{-1} for fasting versus 1.5 mmol l^{-1} for non-fasting samples). Nevertheless, when non-fasting, triglycerides were less useful as a risk marker for incident cardiovascular disease, as the association with cardiovascular disease was shown to be less pronounced on the basis of non-fasting blood samples in this prospective cohort study.[19]

There were no relevant differences in blood glucose concentrations between fasting and non-fasting samples. This could be attributed to the very strict definition of fasting status applied during this study. A child was classified as non-fasting after the consumption of a minimal amount of drink or food, for example, a sip of juice/milk or a bite of an apple or a piece of bread. Very rarely, the intake of a complete meal was reported. The absence of a difference between fasting and non-fasting samples might also be explained by the fact that food intake of non-fasting children normally took place at home before they went to school. Therefore, time intervals of probably at least 1 h lay between glucose intake and sample withdrawal, and thus glucose kinetics was likely to have passed its maximum already. The elevated concentrations of insulin in non-fasting children suggest that the information given for the fasting status was valid and that downregulation of glucose was ongoing. Demographic analysis showed that non-fasting children were only slightly younger than fasting children (5.6 versus 5.9 years of age) and distributed in a similar way across body mass index categories. Variation between countries was negligible for concentrations of glucose, stronger for those of insulin and very pronounced for concentrations of triglycerides, even though mean values of all countries and for all three biomarkers were within the range reported in other studies on children.[20,1]

Urinary concentrations of cortisol and potassium were higher when the child had voided during the night or when the sample was not taken from first morning urine. This finding can be explained by the diurnal rhythms known for these two biomarkers. Urinary potassium excretion has the lowest excretory rate during the night and shows a peak in the early morning.[21,22] A similar rhythm is known for cortisol, where the morning peak is especially high and the concentration of (salivary) cortisol was found to decline very fast, that is, by $\sim$10% within 30 min after its peak in adolescents.[23] Variation between countries on the other hand was relatively small for all urinary biomarkers.

There was no indication of degradation by lack of cooling for more than 2 h for any of the biomarkers. Delivery of morning urine to the local survey centres was conducted by parents or children without cooling. Ambient temperature usually reaches its minimum around the early morning hours, when children are on their way to school. The IDEFICS baseline survey was conducted between September and May when even in southern European countries like Italy, Spain or Cyprus ambient temperature hardly exceeds room temperature during morning hours. Before freezing, sample transportation to the local laboratory was generally carried out with the help of cooling boxes. Thus, ambient temperature is an unlikely source of error even for blood samples. After freezing, all samples were shipped on dry ice to the central laboratory.

The analysed data underline the well-known fact that fasting has a substantial influence on the concentrations of certain biomarkers. It shows that biomarkers in morning urine are error prone if study subjects void during the night or if samples are not taken from the very first morning urine. Up to now, no studies could be found in the literature that recorded voiding during the night before collection of a morning urine sample. Documentation of this confounding factor should become a standard, especially when smaller children are involved. Our results also suggest that reporting by the parents is a reliable tool for preanalytical sample control and should generally be included in epidemiological studies when samples are collected at home. In general, it can be concluded that, beyond the fact that a standardised sampling protocol is of major importance in epidemiological studies, standards for preanalytical sample management are needed. As non-compliance is ever present in spite of well-defined SOPs, deviations from the protocol should always be documented to avoid errors concerning the concentrations of biomarkers.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was done as part of the IDEFICS study (http://www.idefics.eu). We gratefully acknowledge the financial support of the European Community within the Sixth RTD Framework Programme Contract No. 016181 (FOOD). We thank the IDEFICS children and their parents for their participation.

The information in this document reflects the authors' view and is provided as is.

Statement of ethics

We certify that all applicable institutional and governmental regulations regarding the ethical use of human volunteers were followed during this research. Approval by the appropriate ethical committees was obtained by each of the eight centres engaged in the fieldwork. Participants were not subjected to any study procedure before both the children and their parents gave their consent for examinations, collection of samples, subsequent analysis and storage of personal data and collected samples. The participating children and their parents could consent to single components of the study while refraining from others. For ethical reasons, the amount of blood drawn varied according to age, weight and height of the children, and did not exceed 1% of the estimated blood volume of the child. Consequently, about 10–25 ml of blood was withdrawn from the 2- to 8-year-old children.

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Publikation 3

OPEN

International Journal of Obesity (2014) **38,** S39–S47

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ORIGINAL ARTICLE

Percentiles of fasting serum insulin, glucose, HbA1c and HOMA-IR in pre-pubertal normal weight European children from the IDEFICS cohort

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OBJECTIVES: The aim of this study is to present age- and sex-specific reference values of insulin, glucose, glycosylated haemoglobin (HbA1c) and the homeostasis model assessment to quantify insulin resistance (HOMA-IR) for pre-pubertal children.
METHODS: The reference population consists of 7074 normal weight 3- to 10.9-year-old pre-pubertal children from eight European countries who participated in at least one wave of the IDEFICS ('identification and prevention of dietary- and lifestyle-induced health effects in children and infants') surveys (2007–2010) and for whom standardised laboratory measurements were obtained. Percentile curves of insulin (measured by an electrochemiluminescence immunoassay), glucose, HbA1c and HOMA-IR were calculated as a function of age stratified by sex using the general additive model for location scale and shape (GAMLSS) method.
RESULTS: Levels of insulin, fasting glucose and HOMA-IR continuously show an increasing trend with age, whereas HbA1c shows an upward trend only beyond the age of 8 years. Insulin and HOMA-IR values are higher in girls of all age groups, whereas glucose values are slightly higher in boys. Median serum levels of insulin range from 17.4 and 13.2 pmol l^{-1} in 3– < 3.5-year-old girls and boys, respectively, to 53.5 and 43.0 pmol l^{-1} in 10.5– < 11-year-old girls and boys. Median values of glucose are 4.3 and 4.5 mmol l^{-1} in the youngest age group and 49.3 and 50.6 mmol l^{-1} in the oldest girls and boys. For HOMA-IR, median values range from 0.5 and 0.4 in 3– < 3.5-year-old girls and boys to 1.7 and 1.4 in 10.5– < 11-year-old girls and boys, respectively.
CONCLUSIONS: Our study provides the first standardised reference values for an international European children's population and provides the, up to now, largest data set of healthy pre-pubertal children to model reference percentiles for markers of insulin resistance. Our cohort shows higher values of Hb1Ac as compared with a single Swedish study while our percentiles for the other glucose metabolic markers are in good accordance with previous studies.

International Journal of Obesity (2014) **38,** S39–S47; doi:10.1038/ijo.2014.134

INTRODUCTION

Insulin resistance is one of the most common metabolic alterations related to obesity.[1,2] It represents a key element of the metabolic syndrome and an important link between obesity and other metabolic as well as cardiovascular complications.[3,4] Children with insulin resistance are also at risk for type 2 diabetes[5] and a high proportion of cases apparently remain undiagnosed initially.[6]

Although reference data for body mass index (BMI) were published by organisations like International Obesity Taskforce, World Health Organization, Centers for Disease Control and Prevention and many others and these are widely used in paediatrics, there is still a lack of adequate reference data for markers of insulin resistance.[7] Fasting insulin and homeostasis model assessment to quantify insulin resistance (HOMA-IR) have been suggested among others as surrogate markers for screening purposes in adults,[8] as the gold standard method to measure insulin sensitivity (the hyperinsulinaemic euglycaemic clamp) is very labour- and time-intensive and thus not feasible in epidemiological research. Oral glucose tolerance testing, a procedure widely used to measure insulin response in clinical practice, is also not feasible in a setting-based field study like ours. Matthews *et al.*[9] showed that estimates of insulin resistance from HOMA-IR correlated well with estimates from the clamp-technique. Glycosylated haemoglobin (HbA1c) is a standard marker for glycaemic control in diabetic patients but has also been proposed as a predictive marker of insulin resistance.[8]

Population-based data on insulin resistance in children are rare, especially for pre-pubertal children and from large-scale epidemiological studies. Several authors have shown data on the distribution of insulin, glucose and HOMA-IR values in paediatric populations, and some also suggested cut-off values for insulin and HOMA-IR.[10–17] However, all of these studies were limited to national study populations and sample sizes were mostly too small for statistical modelling of reference values. None of the studies included > 1000 pre-pubertal children, except for a Mexican cross-section with about 2500 children in the age stratum of 6–10 years.[13] All authors describe a pronounced age

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dependency of insulin and HOMA-IR, and to a lesser extent also of glucose values. Studies in adolescents show a peak of insulin and HOMA-IR values in puberty and again a slight decline towards adulthood.[10,13,18] Comparability of results is limited due to the multitude of different laboratory procedures (for example, enzyme-linked immunosorbent assay, radioimmunoassay, immunoenzymometric assay, immunoelectrochemiluminometric assay) used for the determination of insulin. These different assays can show up to a twofold variation in insulin concentrations.[19]

The aim of this study is to present age- and sex-specific reference values for insulin, glucose and HOMA-IR based on a European population of normal weight pre-pubertal children from eight European countries who participated in at least one wave of the IDEFICS ('identification and prevention of dietary- and lifestyle-induced health effects in children and infants') surveys (2007–2010) and received standardised examinations and laboratory measurements.

MATERIALS AND METHODS

Study population

A population-based prospective cohort study was one of the key elements of the IDEFICS project. All children of the defined age group who lived in the selected study regions and attended one of the participating pre- or primary schools were eligible for participation. Children and parents were approached via schools and preschools to ensure inclusion of all social groups. Written consent of parents and verbal assent of children were given separately to the different modules of the examination, that is, participants were free to refrain from single components like blood drawing.

The baseline survey in the school year 2007/2008 included 16 228 preschool and primary school children aged 2–9 years from eight European countries (Belgium, Cyprus, Estonia, Germany, Hungary, Italy, Spain and Sweden). Of these, 11 292 were followed up again after 2 years. In the follow-up survey (2009–2010), 2517 new children were additionally included.

Both waves of surveys comprised anthropometrical measurements and examinations of children as well as parental self-completion questionnaires on lifestyle habits and dietary intakes of children. The physical examination programme during the IDEFICS surveys covered standard anthropometric measures, that is, height, weight and circumferences of waist, hip, upper arm and neck, as well as skinfold thicknesses, and the measurement of blood pressure and pulse rate. BMI was calculated as weight (in kg) divided by height squared (in m) and classified according to the International Obesity Taskforce criteria.[20]

Biomarkers were analysed in blood, urine and saliva samples. Standardised procedures were used by all survey centres and a quality management system was established.[21] Venous blood was collected after an overnight fast from 9185 of the baseline and 962 of the children newly recruited at follow-up. In addition, 1011 children who did not provide blood at baseline survey, provided blood at follow-up. The present analyses included children of both waves of surveys with available data for height, weight and the laboratory analyses of interest. If serum measurements of a child were available for both survey waves, preference was given to the baseline survey. Girls and boys below 3 and above 10.9 years of age were excluded from the study sample due to the small number of children in these age strata ($n < 100$) to avoid instability of the statistical model. Children were also excluded if they had a diagnosis of diabetes or if they reported to be non-fasting at the time of blood withdrawal. The present analysis is thus based on 7074 children, as depicted in Figure 1. The background of the IDEFICS study, its research goals and instruments have been described elsewhere in detail.[22]

Laboratory analyses

Children participating in the IDEFICS baseline survey were asked to provide fasting venous blood, morning urine and saliva samples. If consent was not given for venous blood withdrawal, capillary blood was taken with the consent of the parents and the children. Blood glucose was assessed on site at each study centre by point-of-care analysis using the Cholestech LDX analyser (Cholestech, Cholestech Corp., Hayward, CA, USA) either in venous or capillary blood. Precision and accuracy of this analyser were comparable to clinical diagnostic laboratory methods[23] with a slight positive bias of glucose measurement.[24] Pre-analytical sample processing of blood samples was done at the local survey centres or at local laboratories. Samples were then frozen (at − 80 °C) and shipped to a central laboratory, certified by ISO 15189:2007, for later analysis of insulin, HbA1c and other biological markers. Insulin was analysed by an electrochemiluminescence immunoassay (Roche Modular System, Mannheim, Germany), HbA1c was analysed by high-performance liquid chromatography (AUTO-GA variant). Details on the biological sampling procedures can be obtained from an earlier publication.[25] HOMA-IR was calculated as fasting insulin ($\mu IU\ ml^{-1}$) × fasting glucose ($mmol\ l^{-1}$)/22.5.

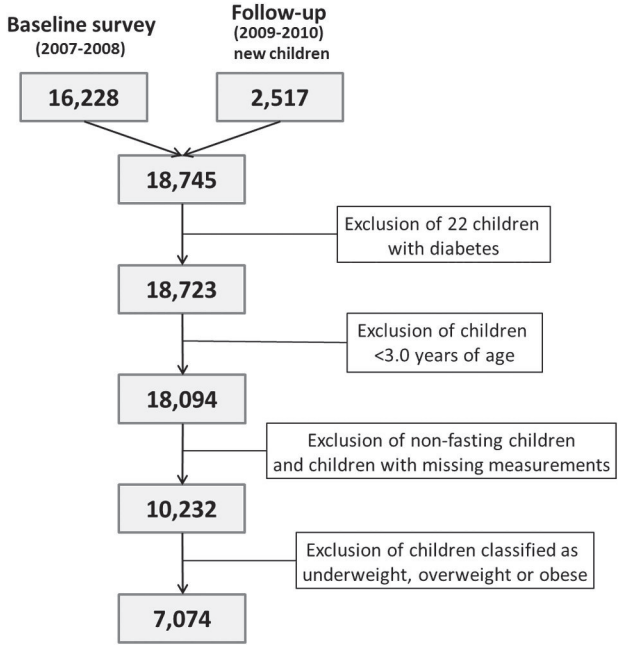

Figure 1. Flow chart of children included in the analysis.

Statistical analyses

Percentile curves of insulin, glucose and HOMA-IR were calculated as a function of the covariate age stratified by sex using general additive model for location scale and shape (GAMLSS) method as an extension of the LMS method.[26] The LMS method models three parameters: the skewness L, the median M and the coefficient of variation S. The skewness accounts for the deviation from a normal distribution using a Box–Cox transformation, the median of the outcome variable is modelled depending on one explanatory variable and the coefficient of variation accounts for the variation of data points around the mean and adjusts for non-uniform dispersion. The GAMLSS method is able to model more than one covariate and also other distributions that particularly include the kurtosis. We used the gamlss package (version 4.2–6) of the statistical software R (version 3.0.1).[27] Different distributions were fitted to the observed distribution of insulin, glucose and HOMA-IR. Moreover, the influence of age on parameters of the considered distributions were modelled either as a constant, as a linear function, or as a cubic spline of the covariates. Goodness-of-fit was assessed by the Bayesian information criterion and by Q–Q plots to select the final model including the fitted distribution of insulin, glucose and HOMA-IR and the influence of covariates on distribution parameters. Worm plots were used as a diagnostic tool to assess whether adjustment for kurtosis was required.[28] Finally, percentile curves for the 5th, 10th, 25th, 50th, 75th, 90th and 95th were calculated based on the model that showed the best fit.[27,29]

The final models for insulin and HOMA-IR for boys and girls considered a Box–Cox *t* (BCT) distribution modelling μ as a cubic spline depending on age, $\log(\sigma)$ as a linear function of age, and ν and τ as constants. With regard to glucose, a lognormal distribution was used for boys and girls considering μ as a linear function of age and $\log(\sigma)$ as a constant.

RESULTS

The reference population is composed of 3434 girls and 3640 boys. One-year age groups (3.0–10.9 years of age) include from about 250 up to 1500 children. Characteristics of this population are presented as mean values (± s.d.) or median (25th, 75th percentile) by age group in Table 1 for the study population before and after restriction to normal weight children. Anthropometrical measures and glucose concentrations show only little differences between boys and girls, but insulin concentrations are higher in girls than in boys through all age groups, which is also reflected in slightly higher HOMA-IR values.

Percentiles of insulin, glucose and HOMA-IR are presented in half-year age groups in Table 2 and Figures 2–4, respectively. Concentrations of all three biomarkers clearly show a positive trend with age, which is also observed for the variance of insulin and HOMA-IR, whereas for glucose the variance is similar in all age groups. Insulin and HOMA-IR values are higher in girls than in boys for all age groups, whereas glucose values are slightly higher in boys.

For insulin, 5th and 95th percentiles range from 4.2–49.3 and 3.5–41.0 pmol l^{-1} in 3– < 3.5-year-old girls and boys, respectively, to 25.7–100.7 and 19.4–88.2 pmol l^{-1} in 10.5– < 11-year-old girls and boys. For glucose, 5th and 95th percentiles were 3.6–5.2 and 3.7–5.3 mmol l^{-1} in 3– < 3.5-year-old girls and boys up to 4.2–5.7 and 4.3–5.9 mmol l^{-1} in 10.5– < 11-year-old girls and boys. For HOMA-IR, 5th and 95th percentiles ranged from 0.1–1.5 and 0.1–1.3 in 3– < 3.5-year-old girls and boys to 0.8–3.4 and 0.6–3.0 in 10.5– < 11-year-old girls and boys.

A table showing percentiles for children of all weight groups is provided in Supplementary Table A. Percentile values for glucose show only negligible differences as compared with the values for normal weight children. Inclusion of overweight and obese children has little impact on lower percentiles of insulin and HOMA-IR but has a very pronounced effect on the upper percentiles, especially in the older age groups. The 95th percentile of insulin in 10.5– < 11-year-old girls for instance, is 125.7 compared with 100.7 pmol l^{-1} in normal weight girls of the same age. A sensitivity analysis was conducted to illustrate the influence of BMI on age-specific values of insulin, glucose and HOMA-IR. A strong dependency on weight status was observed for insulin and HOMA-IR but not for glucose. Insulin percentile curves for children of all weight groups versus only normal weight girls and boys are depicted in Figure 5. Results of the sensitivity analyses for glucose and HOMA-IR are provided in Supplementary Figures A and B. Three different reference populations for BMI were used to test the robustness of the model.[20,30–32] Preference was given to the classification by Cole *et al.*,[20] as differences between the three reference systems were not substantial and this classification led to the smallest number of excluded children.

Percentile curves for HbA1c are provided in Supplementary Figure C. Serum concentrations show nearly no variation for younger children (median of 26.8 for girls and 27.9 mmol mol^{-1} for boys at 3 years of age to 29.0 mmol mol^{-1} for both sexes at 8 years of age) and a slight increase for the 8– < 11-year-old children (median of 34.4 mmol mol^{-1} for girls and 33.3 mmol mol^{-1} for boys at 10.9 years of age).

DISCUSSION

This study presents age- and sex-specific reference percentiles of insulin, glucose and HOMA-IR for pre-pubertal children on the basis of the IDEFICS cohort of children from eight European countries. This is the first time, reference percentiles of these biomarkers are based on a multinational children's cohort. At the same time, with over 7000 subjects, it is by far the biggest cohort ever used to generate such reference data. Nevertheless, previous studies of local populations have shown similar distributions of insulin, glucose and HOMA-IR values in children. As the age group of 9-year-olds was considered most frequently, this age group will be used for comparison in the following.

In our reference population the 5th and 95th percentile values of insulin are 15.3–81.3 pmol l^{-1} for 8.5– < 9.5-year-old girls and 12.5–74.3 pmol l^{-1} for 8.5– < 9.5-year-old boys. Insulin values were also measured in a representative sample of 2244 school children from Quebec who were 9, 13, and 16 years old in 1999 (ref. 11) (insulin measured by the ultrasensitive insulin assay on the Access immunoassay system by Beckman Coulter, Mississauga, ON, Canada), in a representative sample of 1976 healthy French individuals aged 7–20 years in 2006–2008 (ref. 12) (insulin measured with a microparticle enzymoimmuno assay on an AxSYM analyser by Abbott, Abbott Park, IL, USA) and in a random sample of 1137 healthy 9- and 15-year-old school children examined in a cross-sectional study in Sweden in 1998/99 (ref. 17) (insulin assay not reported). In the pre-pubertal group of 9-year-olds, the 5th and 95th percentiles of insulin were 11.8–59.0,[11] 13.9–83.3 (ref. 12) and 11.1–78.5 pmol l^{-1} (ref. 17) in girls and 10.4–54.2,[11] 10.4–69.5 (ref. 12) and 10.4–64.6 pmol l^{-1} in boys. Overall, insulin concentrations were showing a positive trend with age until puberty and revealed pubertal peaks, which were sharper in females than in males. These results are in good accordance with the percentile values from our study. Studies in mixed non-Caucasian populations on 6132 school children aged 6–18 years in Mexico[13] (insulin measured with a microparticle enzymoimmuno assay on an AxSYM analyser by Abbott) and 2153 normal weight children and adolescents in Chile[16] (insulin measured by chemiluminescence on the ADVIA Centaur CP Immunoassay System, Bayer HealthCare AG, Leverkusen, Germany) also showed a gradual increase of insulin values between 6 and 13 years of life. Possibly due to the fact that older children were included, insulin concentrations were higher in the Chilean population than in our study cohort: the 5th and 95th percentiles were 29.9–150.0 pmol l^{-1} for girls and 26.4–117.4 pmol l^{-1} for boys in Tanner stages I and II.[16]

For glucose, the 5th and 95th reference percentiles in our population are 4.0–5.6 mmol l^{-1} for 8.5– < 9.5-year-old girls and 4.1–5.8 mmol l^{-1} for 8.5– < 9.5-year-old boys. Remarkably, glucose curves showed a linear trend in this pre-pubertal age group. To verify linearity of this functional relationship, we also applied a cubic model to the data. As both curves looked very similar, we preferred to use the simpler model. In addition, we know from literature[12] that glucose curves only start to flatten during puberty, which may lead to a linear functional relationship for the age range that we have considered here. Glucose values were very similar in the three above-mentioned studies from Canada,[11] France[12] and Sweden.[17] For 9-year-olds, the 5th and 95th percentiles were 4.4–5.6,[11] 3.9–5.2 (ref. 12) and 4.3–5.4 mmol l^{-1} (ref. 17) in girls and 4.7–5.7,[11] 4.0–5.2 (ref. 12) and 4.4–5.5 mmol l^{-1} (ref. 17) in boys. Percentile values of glucose showed a positive trend with age, although the trend was less pronounced as compared with insulin. A distinct pubertal peak of serum glucose concentrations was only observed in females.

For HOMA-IR, the 5th and 95th percentiles in our reference population are 0.4–2.7 for 8.5– < 9.5-year-old girls and 0.4–2.5 for boys of this age. Estimates from the 1999 Quebec sample[11] (insulin measured by the ultrasensitive insulin assay on the Access immunoassay system by Beckman Coulter) showed slightly lower HOMA-IR values (5th and 95th percentile) of 0.3–2.1 and 0.3–1.9 for 9-year-old girls and boys, respectively. A small Italian cross-section of healthy children and adolescents reported values of 0.6–2.2 for pre-pubertal girls and 0.4–2.2 for the respective boys[15] (insulin measured by radioimmunoassay, Radim Kit, Rome, Italy). The above-mentioned studies in Mexico[13] (insulin measured with a microparticle enzymoimmuno assay on an AxSYM analyser by Abbott) and Chile[16] (insulin measured by chemiluminescence on the ADVIA Centaur CP Immunoassay System, Bayer

Table 1. Characteristics of study population by one-year age group, before and after restriction to normal weight children

Age	*Girls*							*Boys*						
	N	*Height (cm)*[a]	*Weight (kg)*[a]	*BMI (kg m^{-2})*[a]	*Insulin (pmol l^{-1})*[b]	*Glucose (mmol l^{-1})*[a]	*HOMA-IR*[b]	N	*Height (cm)*[a]	*Weight (kg)*[a]	*BMI (kg m^{-2})*[a]	*Insulin (pmol l^{-1})*[b]	*Glucose (mmol l^{-1})*[a]	*HOMA-IR*[b]
All children (n = *10232)*														
3– < 4	415	100.5 (±4.9)	16.0 (±2.4)	15.8 (±1.4)	18.1 (11.1, 27.8)	4.4 (±0.5)	0.5 (0.3, 0.8)	457	101.4 (±5.1)	16.3 (±2.7)	15.8 (±1.4)	15.3 (7.6, 24.3)	4.5 (±0.5)	0.4 (0.2, 0.7)
4– < 5	611	106.7 (±5.0)	17.8 (±2.7)	15.6 (±1.8)	20.8 (13.9, 31.9)	4.5 (±0.5)	0.6 (0.4, 1.0)	667	108.1 (±4.8)	18.5 (±2.8)	15.8 (±1.7)	18.8 (11.8, 28.5)	4.6 (±0.5)	0.6 (0.3, 0.9)
5– < 6	606	113.9 (±5.4)	20.6 (±3.6)	15.8 (±1.9)	25.7 (16.7, 37.5)	4.6 (±0.5)	0.7 (0.5, 1.1)	673	114.8 (±5.2)	21.0 (±3.7)	15.9 (±2.0)	22.9 (13.9, 32.6)	4.7 (±0.5)	0.7 (0.4, 1.0)
6– < 7	801	120.4 (±5.4)	23.5 (±4.5)	16.1 (±2.3)	27.8 (18.1, 41.0)	4.7 (±0.5)	0.8 (0.5, 1.2)	809	121.9 (±5.4)	24.3 (±5.1)	16.3 (±2.5)	25.7 (17.4, 37.5)	4.8 (±0.5)	0.8 (0.5, 1.2)
7– < 8	1089	126.5 (±5.7)	26.9 (±5.6)	16.7 (±2.6)	31.9 (22.2, 45.1)	4.7 (±0.5)	1.0 (0.7, 1.4)	1110	127.8 (±6.1)	27.4 (±5.8)	16.7 (±2.7)	28.5 (19.4, 41.0)	4.8 (±0.5)	0.9 (0.6, 1.3)
8– < 9	873	131.6 (±5.9)	29.9 (±6.4)	17.2 (±2.9)	37.5 (26.4, 52.8)	4.8 (±0.4)	1.1 (0.8, 1.7)	887	132.7 (±6.1)	30.4 (±6.6)	17.2 (±3.0)	31.9 (20.8, 47.2)	4.9 (±0.5)	1.0 (0.6, 1.5)
9– < 10	422	137.8 (±7.0)	33.9 (±7.9)	17.7 (±3.2)	38.2 (32.6, 65.3)	4.9 (±0.4)	1.4 (1.0, 2.2)	416	138.7 (±6.2)	35.1 (±8.2)	18.1 (±3.4)	41.7 (29.9, 58.3)	5.0 (±0.5)	1.3 (0.9, 1.9)
10– < 11	207	143.0 (±7.0)	37.8 (±8.8)	18.3 (±3.4)	54.2 (41.0, 79.9)	5.0 (±0.5)	1.7 (1.3, 2.6)	189	142.2 (±6.4)	38.1 (±9.2)	18.7 (±3.5)	44.4 (32.6, 70.1)	5.0 (±0.5)	1.5 (1.0, 2.2)
All	5024	122.0 (±12.9)	25.1 (±7.9)	16.5 (±2.6)	30.6 (19.4, 45.1)	4.7 (±0.5)	0.9 (0.6, 1.4)	5208	122.6 (±12.8)	25.5 (±8.1)	16.6 (±2.7)	23.4 (16.7, 40.3)	4.8 (±0.5)	0.8 (0.5, 1.3)

Age	*Girls*							*Boys*						
	N	*Height (cm)*[a]	*Weight (kg)*[a]	*BMI (kg m^{-2})*[a]	*Insulin (pmol l^{-1})*[b,c]	*Glucose (mmol l^{-1})*[a]	*HOMA-IR*[b]	N	*Height* (cm)[a]	*Weight*(kg)[a]	*BMI (kg m^{-2})*[a]	*Insulin (pmol l^{-1})*[b]	*Glucose (mmol l^{-1})*[a]	*HOMA-IR*[b]
Normal weight children according to Cole et al.[20] (n = *7074)*														
3– < 4	318	100.2 (±4.9)	15.9 (±1.8)	15.8 (±0.9)	18.8 (11.1, 27.8)	4.4 (±0.6)	0.5 (0.3, 0.8)	329	101.3 (±4.7)	16.4 (±1.8)	15.9 (±0.8)	13.9 (7.6, 25.0)	4.5 (±0.5)	0.4 (0.2, 0.7)
4– < 5	433	106.4 (±4.8)	17.6 (±2.0)	15.5 (±0.8)	20.1 (13.9, 29.2)	4.5 (±0.4)	0.6 (0.4, 0.9)	497	107.9 (±4.8)	18.3 (±2.0)	15.7 (±0.8)	18.8 (11.8, 27.1)	4.6 (±0.5)	0.5 (0.3, 0.8)
5– < 6	422	113.4 (±5.3)	19.8 (±2.2)	15.3 (±0.9)	24.3 (16.0, 35.4)	4.6 (±0.5)	0.7 (0.4, 1.1)	491	114.5 (±5.0)	20.5 (±2.2)	15.6 (±0.8)	20.8 (13.2, 30.6)	4.7 (±0.5)	0.6 (0.4, 0.9)
6– < 7	558	120.1 (±5.3)	22.3 (±2.5)	15.4 (±1.0)	24.3 (17.4, 36.1)	4.6 (±0.5)	0.7 (0.5, 1.1)	561	121.3 (±5.1)	23.0 (±2.5)	15.6 (±1.0)	23.6 (16.7, 34.7)	4.8 (±0.5)	0.7 (0.5, 1.1)
7– < 8	738	126.1 (±5.6)	25.2 (±3.1)	15.8 (±1.1)	29.2 (20.8, 39.6)	4.7 (±0.5)	0.9 (0.6, 1.2)	763	127.3 (±5.7)	25.7 (±3.1)	15.8 (±1.0)	26.4 (18.1, 37.5)	4.8 (±0.5)	0.8 (0.5, 1.2)
8– < 9	558	131.1 (±5.8)	27.8 (±3.5)	16.2 (±1.3)	34.7 (25.0, 45.8)	4.7 (±0.4)	1.1 (0.7, 1.5)	619	132.2 (±6.1)	28.3 (±3.5)	16.2 (±1.2)	29.2 (19.4, 40.3)	4.9 (±0.5)	0.9 (0.6, 1.3)
9– < 10	278	137.1 (±6.9)	31.1 (±4.2)	16.5 (±1.3)	41.0 (30.6, 55.6)	4.9 (±0.4)	1.3 (0.9, 1.8)	255	137.6 (±6.0)	31.5 (±4.2)	16.6 (±1.3)	36.8 (28.5, 47.2)	5.0 (±0.5)	1.2 (0.9, 1.6)
10– < 11	129	142.7 (±6.7)	35.3 (±5.3)	17.2 (±1.5)	50.7 (39.6, 65.3)	5.0 (±0.5)	1.6 (1.3, 2.1)	125	140.5 (±6.1)	33.8 (±5.3)	17.1 (±1.4)	40.3 (29.2, 53.5)	5.0 (±0.5)	1.3 (0.9, 1.7)
All	3434	121.0 (±12.9)	23.5 (±5.8)	15.8 (±1.2)	27.8 (18.8, 40.3)	4.6 (±0.5)	0.8 (0.5, 1.3)	3640	121.7 (±12.5)	23.9 (±5.8)	15.9 (±1.1)	24.3 (15.3, 36.1)	4.8 (±0.5)	0.8 (0.5, 1.1)

Abbreviations: BMI, body mass index; HOMA-IR, homeostasis model assessment to quantify insulin resistance. [a]Data of parameters with normal distribution are presented as mean values (± s.d.). [b]Data of parameters with skewed distributions are presented as median (25th, 75th percentile). [c]Measured by an electrochemiluminescence immunoassay.

Table 2. Percentiles of insulin (pmol l^{-1}), glucose (mmol l^{-1}) and HOMA-IR in normal weight children calculated with GAMLSS

Age[a]	*Percentiles for girls*											*Percentiles for boys*										
	1	*3*	*5*	*10*	*25*	*50*	*75*	*90*	*95*	*97*	*99*	*1*	*3*	*5*	*10*	*25*	*50*	*75*	*90*	*95*	*97*	*99*
Insulin (pmol l^{-1})[b]																						
3– < 3.5	1.74	3.34	4.41	6.40	10.69	17.23	26.59	39.05	49.52	58.12	80.10	1.30	2.46	3.25	4.73	8.03	13.28	21.07	31.78	40.99	48.66	68.60
3.5– < 4	2.09	3.87	5.05	7.20	11.76	18.62	28.31	41.07	51.70	60.41	82.53	1.63	2.99	3.91	5.61	9.35	15.19	23.75	35.40	45.34	53.59	74.90
4– < 4.5	2.48	4.44	5.72	8.02	12.85	19.99	29.95	42.93	53.68	62.43	84.57	2.00	3.56	4.60	6.51	10.63	16.99	26.20	38.61	49.12	57.79	80.09
4.5– < 5	2.91	5.07	6.45	8.92	14.00	21.42	31.64	44.84	55.68	64.48	86.61	2.37	4.12	5.27	7.36	11.81	18.57	28.26	41.17	52.02	60.94	83.76
5– < 5.5	3.40	5.75	7.23	9.85	15.17	22.85	33.29	46.65	57.56	66.36	88.41	2.78	4.71	5.96	8.21	12.96	20.07	30.14	43.43	54.52	63.60	86.70
5.5– < 6	3.90	6.41	7.98	10.72	16.24	24.07	34.62	47.99	58.83	67.54	89.25	3.22	5.33	6.68	9.09	14.10	21.53	31.92	45.50	56.76	65.94	89.16
6– < 6.5	4.41	7.08	8.73	11.57	17.22	25.16	35.73	48.99	59.68	68.25	89.47	3.68	5.96	7.40	9.95	15.20	22.87	33.49	47.24	58.57	67.76	90.90
6.5– < 7	4.99	7.83	9.56	12.52	18.34	26.41	37.04	50.28	60.88	69.33	90.19	4.16	6.61	8.14	10.82	16.27	24.15	34.94	48.79	60.13	69.29	92.24
7– < 7.5	5.70	8.76	10.59	13.71	19.77	28.09	38.94	52.33	62.98	71.44	92.24	4.69	7.31	8.92	11.73	17.38	25.47	36.42	50.35	61.68	70.80	93.55
7.5– < 8	6.60	9.92	11.90	15.23	21.64	30.34	41.58	55.34	66.23	74.84	95.90	5.29	8.08	9.79	12.75	18.62	26.93	38.08	52.15	63.52	72.64	95.26
8– < 8.5	7.69	11.34	13.50	17.10	23.95	33.15	44.94	59.25	70.50	79.38	100.98	6.00	9.02	10.85	13.97	20.14	28.77	40.24	54.59	66.12	75.33	98.07
8.5– < 9	8.99	13.02	15.38	19.28	26.65	36.45	48.88	63.86	75.57	84.78	107.09	6.90	10.20	12.17	15.54	22.10	31.20	43.17	58.04	69.91	79.36	102.59
9– < 9.5	10.53	14.99	17.57	21.83	29.78	40.25	53.43	69.19	81.44	91.04	114.20	7.97	11.59	13.74	17.38	24.42	34.07	46.66	62.18	74.50	84.26	108.18
9.5– < 10	12.30	17.24	20.06	24.69	33.28	44.48	58.46	75.05	87.88	97.90	121.98	9.15	13.11	15.44	19.36	26.87	37.08	50.29	66.43	79.18	89.25	113.80
10– < 10.5	14.26	19.68	22.76	27.77	36.99	48.90	63.66	81.04	94.42	104.83	129.75	10.41	14.71	17.22	21.40	29.37	40.09	53.85	70.55	83.66	93.98	119.04
10.5– < 11	16.37	22.27	25.59	30.97	40.78	53.37	68.82	86.91	100.77	111.51	137.13	11.77	16.39	19.07	23.52	31.92	43.12	57.38	74.56	87.98	98.51	123.97
Glucose (mmoll^{-1})																						
3– < 3.5	3.19	3.48	3.62	3.82	4.11	4.39	4.68	4.97	5.16	5.30	5.59	3.22	3.53	3.68	3.89	4.19	4.49	4.79	5.10	5.30	5.45	5.76
3.5– < 4	3.23	3.52	3.66	3.85	4.14	4.43	4.72	5.00	5.20	5.34	5.63	3.26	3.57	3.72	3.92	4.23	4.53	4.83	5.14	5.34	5.49	5.80
4– < 4.5	3.26	3.56	3.69	3.89	4.18	4.46	4.75	5.04	5.23	5.37	5.67	3.30	3.61	3.76	3.96	4.27	4.57	4.87	5.17	5.38	5.53	5.84
4.5– < 5	3.30	3.59	3.73	3.93	4.21	4.50	4.79	5.07	5.27	5.41	5.70	3.34	3.65	3.79	4.00	4.30	4.61	4.91	5.21	5.42	5.56	5.87
5– < 5.5	3.33	3.63	3.77	3.96	4.25	4.54	4.82	5.11	5.31	5.45	5.74	3.38	3.69	3.83	4.04	4.34	4.64	4.95	5.25	5.46	5.60	5.91
5.5– < 6	3.37	3.66	3.80	4.00	4.28	4.57	4.86	5.15	5.34	5.48	5.77	3.41	3.72	3.87	4.08	4.38	4.68	4.99	5.29	5.49	5.64	5.95
6– < 6.5	3.41	3.70	3.84	4.03	4.32	4.61	4.90	5.18	5.38	5.52	5.81	3.45	3.76	3.91	4.11	4.42	4.72	5.02	5.33	5.53	5.68	5.99
6.5– < 7	3.44	3.73	3.87	4.07	4.36	4.64	4.93	5.22	5.41	5.55	5.85	3.49	3.80	3.95	4.15	4.46	4.76	5.06	5.36	5.57	5.72	6.03
7– < 7.5	3.48	3.77	3.91	4.11	4.39	4.68	4.97	5.25	5.45	5.59	5.88	3.53	3.84	3.98	4.19	4.49	4.80	5.10	5.40	5.61	5.76	6.06
7.5– < 8	3.51	3.81	3.95	4.14	4.43	4.72	5.00	5.29	5.49	5.62	5.92	3.57	3.88	4.02	4.23	4.53	4.83	5.14	5.44	5.65	5.79	6.10
8– < 8.5	3.55	3.84	3.98	4.18	4.46	4.75	5.04	5.33	5.52	5.66	5.95	3.61	3.91	4.06	4.27	4.57	4.87	5.18	5.48	5.69	5.83	6.14
8.5– < 9	3.59	3.88	4.02	4.21	4.50	4.79	5.07	5.36	5.56	5.70	5.99	3.64	3.95	4.10	4.31	4.61	4.91	5.21	5.52	5.72	5.87	6.18
9– < 9.5	3.62	3.91	4.05	4.25	4.54	4.82	5.11	5.40	5.59	5.73	6.03	3.68	3.99	4.14	4.34	4.65	4.95	5.25	5.56	5.76	5.91	6.22
9.5– < 10	3.66	3.95	4.09	4.28	4.57	4.86	5.15	5.43	5.63	5.77	6.06	3.72	4.03	4.18	4.38	4.68	4.99	5.29	5.59	5.80	5.95	6.25
10– < 10.5	3.69	3.99	4.13	4.32	4.61	4.90	5.18	5.47	5.67	5.80	6.10	3.76	4.07	4.21	4.42	4.72	5.03	5.33	5.63	5.84	5.98	6.29
10.5– < 11	3.73	4.02	4.16	4.36	4.64	4.93	5.22	5.51	5.70	5.84	6.13	3.80	4.10	4.25	4.46	4.76	5.06	5.37	5.67	5.88	6.02	6.33
HOMA-IR[c]																						
3– < 3.5	0.04	0.08	0.11	0.16	0.29	0.48	0.78	1.17	1.51	1.79	2.51	0.03	0.06	0.08	0.12	0.22	0.38	0.62	0.97	1.27	1.52	2.18
3.5– < 4	0.05	0.10	0.13	0.19	0.32	0.53	0.83	1.24	1.59	1.88	2.61	0.04	0.07	0.10	0.15	0.26	0.44	0.71	1.10	1.43	1.71	2.42
4– < 4.5	0.06	0.11	0.15	0.21	0.35	0.57	0.89	1.31	1.66	1.95	2.69	0.05	0.09	0.12	0.17	0.30	0.50	0.80	1.21	1.57	1.86	2.61
4.5– < 5	0.07	0.13	0.17	0.24	0.39	0.62	0.95	1.38	1.74	2.03	2.77	0.06	0.11	0.14	0.20	0.33	0.55	0.87	1.30	1.67	1.97	2.75
5– < 5.5	0.08	0.15	0.19	0.27	0.43	0.67	1.00	1.44	1.81	2.10	2.85	0.07	0.12	0.16	0.23	0.37	0.60	0.93	1.38	1.76	2.07	2.86
5.5– < 6	0.10	0.17	0.21	0.29	0.46	0.71	1.05	1.49	1.86	2.15	2.89	0.08	0.14	0.18	0.25	0.41	0.65	0.99	1.46	1.84	2.16	2.95
6– < 6.5	0.11	0.19	0.23	0.32	0.49	0.74	1.09	1.53	1.89	2.18	2.90	0.10	0.16	0.20	0.28	0.44	0.69	1.05	1.52	1.91	2.22	3.02
6.5– < 7	0.13	0.21	0.26	0.35	0.53	0.78	1.13	1.57	1.93	2.22	2.93	0.11	0.18	0.23	0.31	0.48	0.74	1.10	1.57	1.96	2.28	3.07
7– < 7.5	0.15	0.24	0.29	0.38	0.57	0.84	1.19	1.64	2.00	2.29	3.00	0.13	0.20	0.25	0.34	0.52	0.78	1.15	1.63	2.02	2.34	3.13
7.5– < 8	0.17	0.27	0.33	0.43	0.63	0.91	1.28	1.75	2.12	2.41	3.14	0.14	0.23	0.28	0.37	0.56	0.83	1.21	1.70	2.09	2.41	3.20
8– < 8.5	0.21	0.31	0.38	0.49	0.71	1.01	1.40	1.89	2.27	2.58	3.33	0.17	0.26	0.31	0.41	0.61	0.90	1.29	1.79	2.19	2.51	3.31
8.5– < 9	0.24	0.36	0.44	0.56	0.79	1.12	1.54	2.05	2.46	2.78	3.56	0.19	0.29	0.35	0.46	0.67	0.98	1.39	1.92	2.33	2.66	3.48
9– < 9.5	0.29	0.43	0.51	0.64	0.90	1.25	1.70	2.24	2.67	3.01	3.83	0.23	0.34	0.40	0.52	0.75	1.08	1.52	2.06	2.50	2.84	3.68
9.5– < 10	0.34	0.50	0.59	0.73	1.02	1.39	1.87	2.46	2.91	3.26	4.12	0.26	0.39	0.46	0.59	0.83	1.18	1.64	2.21	2.66	3.02	3.89
10– < 10.5	0.41	0.57	0.67	0.83	1.14	1.55	2.06	2.67	3.15	3.52	4.41	0.30	0.44	0.52	0.65	0.92	1.28	1.77	2.36	2.82	3.19	4.07
10.5– < 11	0.47	0.66	0.76	0.94	1.27	1.70	2.24	2.88	3.38	3.77	4.69	0.35	0.49	0.58	0.72	1.00	1.39	1.89	2.50	2.97	3.35	4.25

Abbreviations: GAMLSS, general additive model for location scale and shape; HOMA-IR, homeostasis model assessment to quantify insulin resistance. [a]Percentile values were modelled for midpoints of age groups. [b]Measured by an electrochemiluminescence immunoassay. [c]HOMA-IR was calculated as fasting insulin (μIU ml^{-1}) × fasting glucose (mmol l^{-1})/22.5.

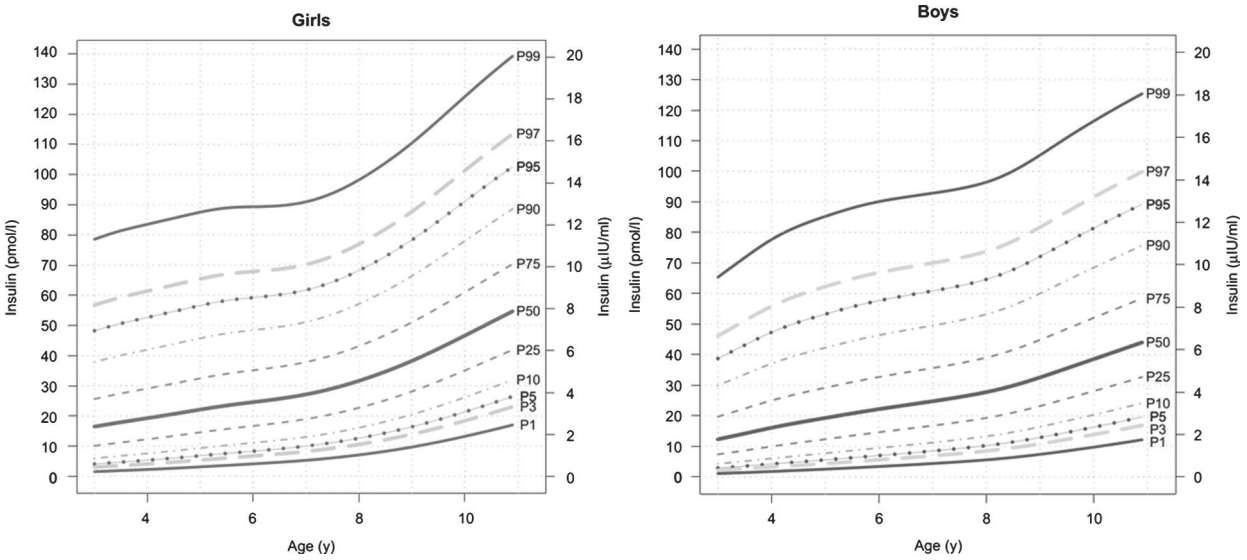

Figure 2. Percentiles of fasting serum insulin (measured by an electrochemiluminescence immunoassay) from normal weight children of the IDEFICS cohort (2007–2010).

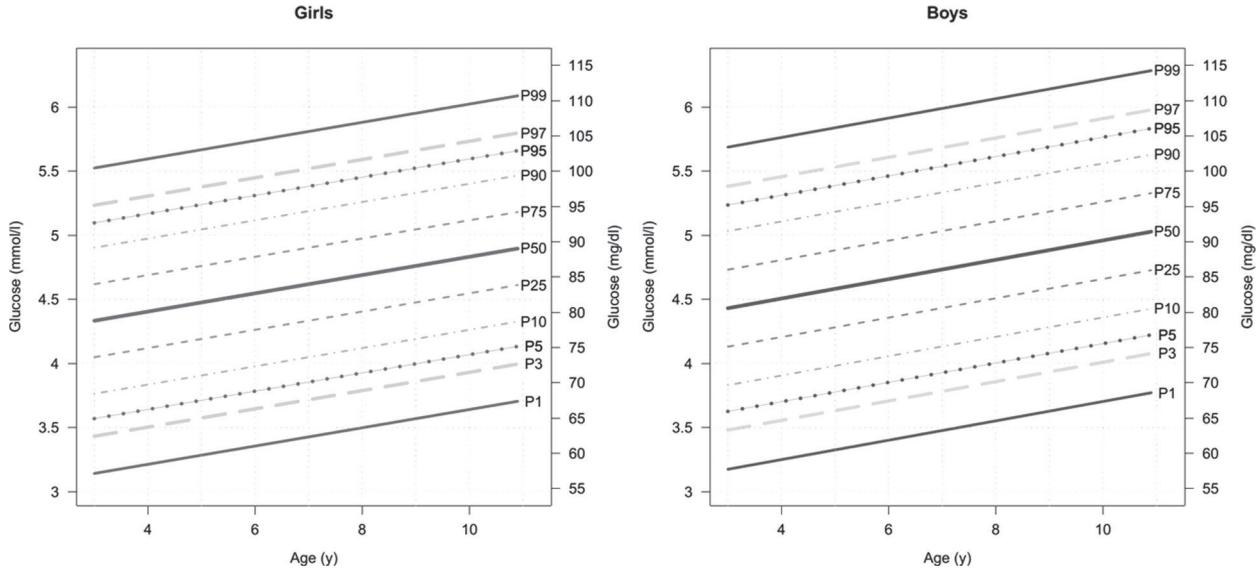

Figure 3. Percentiles of fasting blood glucose (measured by point-of-care analysis) from normal weight children of the IDEFICS cohort (2007–2010).

HealthCare AG) also showed an increase of HOMA-IR from the age of 6 to the age 13 years. Similar to insulin, percentile values were obviously higher for the combined group of Tanner stages I and II, as compared with our study population, that is, HOMA-IR values were 0.9–4.9 for girls and 0.8–3.9 for boys. A HOMA-IR value of 2.0 was suggested as a cut-off point for pre-pubertal children in a population-based small Italian study[14] (insulin measured by an immunoenzymometric assay, AIA-Pack IRI, Tosoh, Tokyo, Japan). Cut-off values were also derived from oral glucose tolerance testing in studies with obese children. Two Turkish studies on 148 and 82 obese paediatric patients, proposed cut-off points of HOMA-IR $\geqslant 2.7$ for both sexes[33] (insulin was measured using the IMMULITE immunoassay, IMMULITE Diagnostic Products Corporation, Los Angeles, CA, USA) or HOMA-IR $\geqslant 2.2$ for girls and $\geqslant 2.7$ for boys[34] (insulin measured by an immunoradiometric assay kit, INS-Irma Biosource, Nivelles, Belgium).

HbA1c as a standard marker for glycaemic control in diabetes has rather little relevance in healthy children. However, as it is frequently measured in population-based studies, we included it in our analysis and we present the results in Supplementary Figure C. To our knowledge, there is only one recent study that aimed to define paediatric reference values for HbA1c.[35] This Swedish cohort investigated healthy children from 6 months to 18 years of age and suggested cut-off values of 16.9–27.9 mmol mol^{-1} (2.5th and 97.5th percentile), which are lower than the 5th and 95th percentiles observed in our study population, that is, 15.8–34.4 and 16.9–34.4 mmol mol^{-1} for 3-year-old girls and boys, and up to 26.8–38.8 and 15.8–39.9 mmol mol^{-1} for 10.9-year-old girls and boys.

Overall, the distributions of insulin, glucose and HOMA-IR shown in previous studies are in good accordance with the reference percentiles in the present analysis. For the subgroup of 9-year-old girls and boys, the 5th and 95th percentile values are very similar to cohorts from European countries or Canada. Concentrations were generally higher in countries with mixed populations including people with indigenous ancestry or when

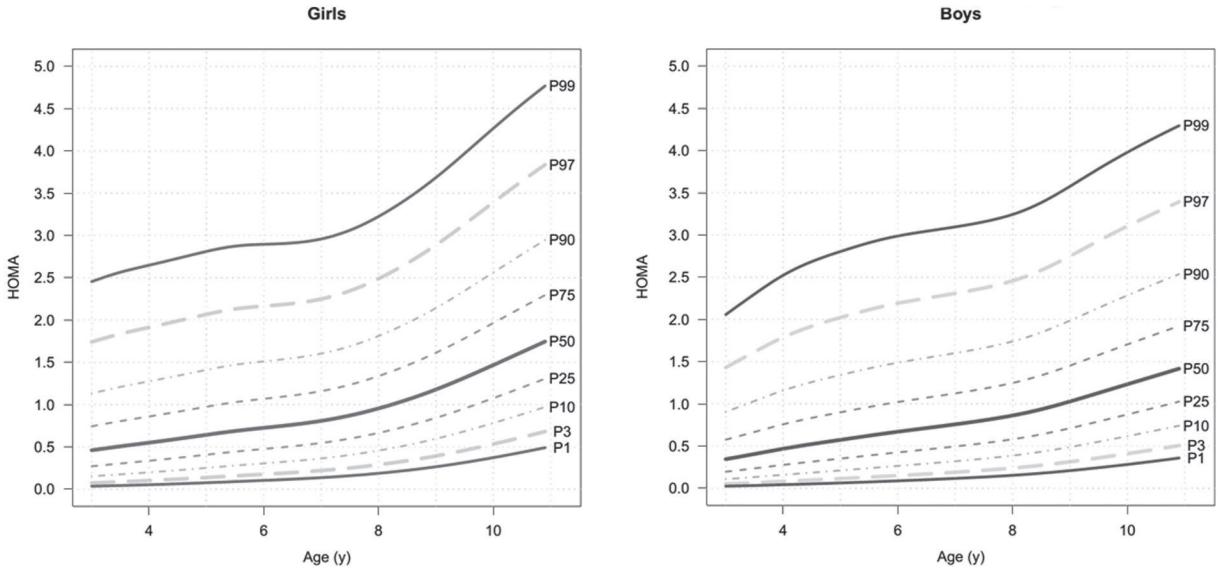

Figure 4. Percentiles of HOMA-IR from normal weight children of the IDEFICS cohort (2007–2010). Insulin was measured by an electrochemiluminescence immunoassay, glucose was measured by point-of-care analysis.

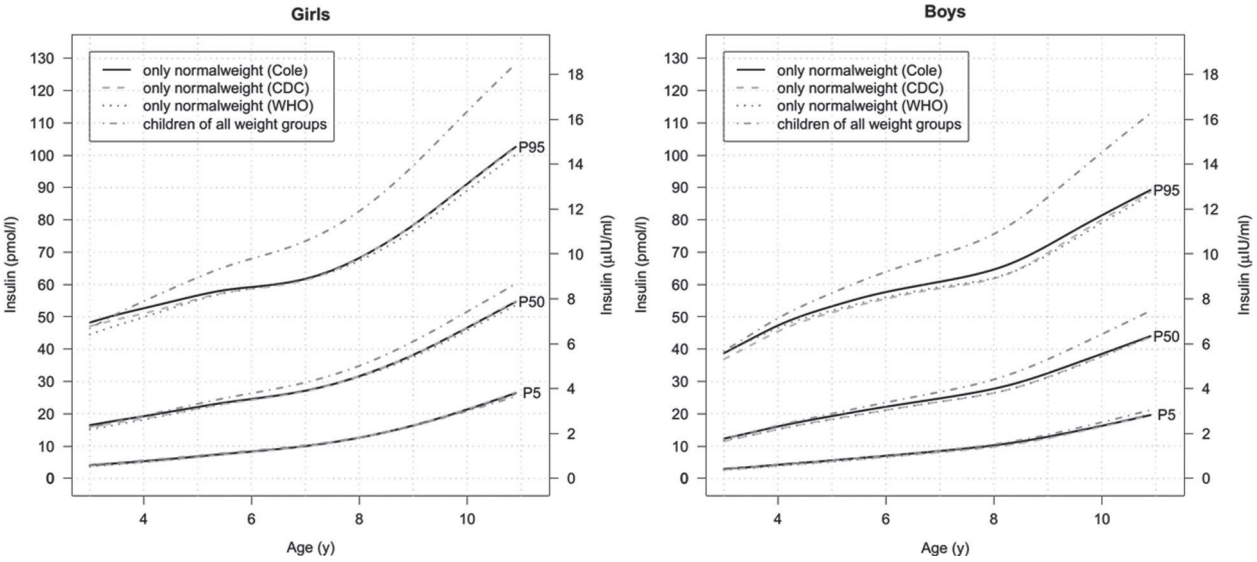

Figure 5. Sensitivity analysis showing percentiles of fasting serum insulin according to different definitions of normal weight and for the whole study group including children of all weight groups (classification of normal weight according to Cole *et al.*/International Obesity Taskforce,[20] Centers for Disease Control and Prevention[30] and World Health Organization[31,32]).

adolescents were included (for example, combined analysis for Tanner stages I and II), which corresponds well with the pubertal peak described for all of the considered markers. Differences in insulin and HOMA-IR levels could also be due to the variety of different insulin assays used in the referenced studies.

In our study, only normal weight children were included into the modelling of percentile curves as the insulin level is known to be strongly influenced by BMI.[36] Despite the strong influence of BMI on markers of insulin resistance, the relationship between BMI and HOMA-IR has been shown to be much weaker in children than in adults.[37] Within the growing group of children with overweight and obesity, paediatricians are faced with the challenge of identifying individuals at the greatest risk of comorbidity. It has been pointed out by other authors that interventions to halt weight gain and promote weight loss in children are of limited success and demand significant resources and continuous follow-up and monitoring.[38] This puts the caregiver in the dilemma of where to allocate the limited available resources and who among the obese children will benefit most from an intervention.[1] The provision of paediatric reference curves for insulin resistance is thus long overdue.

The main strengths of the present study are the large sample size and the standardised assessment of anthropometrical and laboratory measurements. The statistical modelling was done according to the most advanced methodology, which has also been used by the most recent studies in this field.[29,39,40] Some limitations of the study should also be accounted for: even though the study was designed to reach all eligible children in the selected study regions via their settings, the overall participation rate was just above 50%,[22] and a non-response bias, for example, towards higher social status may well be present. Nevertheless, the influence of social status on insulin resistance is likely to be mediated by weight status, which is one of the reasons why we restricted our analyses to normal weight children. Another

problem might be caused by the fact that pubertal stages were not assessed in the IDEFICS surveys. Even though children of our age range are considered to be pre-pubertal, we cannot exclude that a few of them might already have developed signs of beginning puberty that might better be excluded from the reference population.

In summary, the reference percentiles presented here are to our knowledge the first to be based on a multinational children's population that is also the largest cohort that was up to now used to model reference percentiles for markers of insulin resistance (insulin measured by electrochemiluminescence immunoassay). These reference values may help to identify children with insulin resistance who have an elevated risk for cardiovascular disease and type 2 diabetes mellitus. These reference values will thus allow for a more focused and earlier behavioural or therapeutic intervention. As insulin resistance has been shown to be reversible in most cases by healthy eating and physical activity,[41,42] these reference values may hopefully contribute to reduce the burden of disease in the long run.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was done as part of the IDEFICS study (www.idefics.eu). We gratefully acknowledge the financial support of the European Community within the Sixth RTD Framework Programme Contract No. 016181 (FOOD). We express our sincerest gratitude to Timm Intemann for his enduring help with the GAMLSS modelling and to Ina Alvarez for technical support. We also wish to thank the IDEFICS children and their parents for their participation. The authors are also grateful to the Volkswagen Foundation that financially supported the production of this supplement.

STATEMENT OF ETHICS

We certify that all applicable institutional and governmental regulations concerning the ethical use of human volunteers were followed during this research. Approval by the appropriate Ethical Committees was obtained by each of the eight centres involved in the field work. Study subjects were not submitted to any study procedure before both the children and their parents had given their consent to examinations, collection of samples, subsequent analyses and storage of personal data and collected samples. Study subjects and their parents could consent to single components of the study while refraining from others. For ethical reasons the amount of blood drawn varied according to age, weight and height of the children and did not exceed 1% of the estimated blood volume of the child. Consequently, a volume of 10–25 ml (depending on the weight of the child) was not exceeded during blood withdrawal from the 2–11-year-old children.

DISCLAIMER

The information in this document reflects the author's view and is provided as is.

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Supplementary Information accompanies this paper on International Journal of Obesity website (http://www.nature.com/ijo)

Publikation 4

Determinants of insulin resistance in preadolescent children: results from the IDEFICS study

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Short title

Determinants of insulin resistance in children

Funding source

This work was done as part of the IDEFICS study (www.idefics.eu). We gratefully acknowledge the financial support of the European Community within the Sixth RTD Framework Programme Contract No. 016181 (FOOD).

Potential Conflicts of Interest

The authors have no conflicts of interest relevant to this article to disclose.

Financial Disclosure

The authors have no financial relationships relevant to this article to disclose.

Abbreviations

BIA	bioelectrical impedance analysis
BMI	body mass index
HOMA-IR	homeostasis model assessment to quantify insulin resistance
IDEFICS	Project acronym: "Identification and prevention of dietary- and lifestyle-induced health effects in children and infants"
IR	insulin resistance
ISCED	International Standard Classification of Education
MS	metabolic syndrome
MVPA	time spent in moderate to vigorous physical activity
OR	odds ratio
p95	95^{th} percentile
PA	physical activity
SACINA	Self-administered children and infants nutrition assessment, a standardized 24-h recall method

What's known on this subject?

Overweight and obesity are associated with insulin resistance in older children and adolescents, but prospective data and data from preadolescent children are still rare.

What this study adds

Overweight/ obesity and a sedentary lifestyle are prospectively associated with insulin resistance in preadolescent children. Longitudinal data shows that physical activity improves insulin resistance already in these young children.

Contributors' Statement:

Ms Peplies conceptualized the study, contributed to the design of the sample collection procedures, conducted the analyses, interpreted the data, drafted the initial manuscript, revised the manuscript, and approved the final manuscript as submitted.

Dr Börnhorst gave statistical support and advice on data analyses, critically reviewed the manuscript, and approved the final manuscript as submitted.

Prof Dr. Ahrens coordinated the IDEFICS study, coordinated and supervised data collection at one of eight survey centres, supervised data analyses, critically reviewed the manuscript and approved the final manuscript as submitted.

Dr. Fraterman supervised biological sample analyses, contributed to the design of the sample collection procedures, and approved the final manuscript as submitted.

Dr. Günther gave advice on data analyses, reviewed the manuscript, and approved the final manuscript as submitted.

Dr. Russo contributed to the design of the data collection instruments, coordinated and supervised data collection at one of eight survey centres, reviewed the manuscript, and approved the final manuscript as submitted.

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Abstract

Objective

This study investigates prospective associations of anthropometrical and lifestyle indices with insulin resistance (IR) in European children from the IDEFICS cohort. It also shows country-specific prevalence rates of IR.

Methods

This longitudinal study included 3348 preadolescent children aged 3 to 10.9 years from 8 European countries. The outcome measure in the present analysis is HOMA-IR (homeostasis model assessment) as a common proxy indicator to quantify IR at follow-up and in its longitudinal development. Anthropometrical measures and lifestyle indices were considered as determinants of IR. Prospective associations between IR at follow-up and anthropometrical and lifestyle indices were estimated by logistic regression models.

Results

Country-specific prevalence rates of IR showed a positive trend with BMI category. Prospective multivariate analyses showed the strongest positive associations of IR with BMI z-score and z-score of waist circumference but also for sex, audio-visual media time and an inverse association of objectively determined physical activity (PA). A favourable change in IR was associated with a favourable longitudinal development of weight status.

Conclusions

This study is, to our knowledge, the first prospective study on IR in a preadolescent children's population. It supports the common hypothesis that overweight and obesity are the main determinants of IR. Our data also indicate that physical inactivity and a sedentary lifestyle are likewise associated with the development of IR, independent of weight status. The promotion of PA should thus be considered as an equal option to dietary intervention for the treatment of insulin resistance in the paediatric practice.

Introduction

Insulin resistance (IR), a reduced physiological response of the peripheral tissues to normal levels of insulin, is a growing concern in childhood obesity. It is also a central aspect of the metabolic syndrome (MS) and most likely a link between obesity and type 2 diabetes[1–5]. Fasting insulin and HOMA-IR have been suggested among others as surrogate markers for screening purposes in adults[6], as the gold standard method to measure insulin sensitivity, the hyperinsulinemic euglycemic clamp, is invasive and very labour- and time-intensive and thus not feasible in epidemiological research. Estimates of IR from HOMA-IR (homeostasis model assessment to quantify IR) have been shown to correlate well with estimates from the clamp-technique[7], especially in healthy populations[8]. Given the constantly growing prevalence of metabolic disorders in children and adolescents[9], it appears to be more and more important to identify children at risk before clinical symptoms occur.

Several risk factors for IR have been suggested, e.g. weight status [10] and measures of central and peripheral adiposity[11,12], dietary factors like intake of total fat or saturated fat[13,14], physical inactivity[15], low or high birth weight for gestational age[9,16] and maternal factors like gestational diabetes[17] or unbalanced maternal nutrition[18]. Nevertheless, in children, population-based epidemiological data on the determinants of IR are still rare and mostly available from cross-sectional studies. A recent review[19] on the clustering of obesogenic behaviours in children or adolescents also concluded that further research is needed particularly in younger children and from longitudinal studies.

The importance of metabolic health in the presence of obesity has been of growing research interest in the last years. Metabolically healthy obesity is apparently associated with lower levels of adiposity and a high level of PA[23,24]. Increased PA and cardiorespiratory fitness are also believed to attenuate the risk of cardiovascular disease, type 2 diabetes, and metabolic syndrome, independent of weight status[25]. A meta-analysis on the influence of cardiorespiratory fitness and weight status on mortality from all causes showed that overweight and obese fit individuals had similar mortality risks as normal weight fit individuals. Compared to normal weight fit individuals, unfit individuals had twice the risk of mortality regardless of BMI[26].

The present study aims to describe country-specific prevalence rates of insulin resistance in the IDEFICS (“Identification and prevention of dietary- and lifestyle-induced health effects in children and infants”) cohort of European children. It also looks into the metabolic status in relation to the longitudinal development of the weight status between the two IDEFICS

surveys. This study investigates the prospective associations between anthropometrical and lifestyle indices with insulin resistance in preadolescent European children.

Methods

Study design

IDEFICS is an Integrated Project within the 6th Framework Programme of the European Commission. The baseline survey in 2007/2008 included 16,228 pre-school and primary school children aged 2 to 9 years from eight European countries. The survey comprised anthropometrical measurements and examinations of children as well as parental self-completion questionnaires. Biomarkers were analysed in blood, urine and saliva samples. Standardized procedures were used by all survey centres. Venous blood was collected from 9,185 of the IDEFICS children. The majority of children were re-examined after two years during a follow-up survey (n= 11,292 plus 2,517 newly recruited children). The background of the study, its research goals and instruments have been described elsewhere in detail [27].

Study sample

After exclusion of non-fasting children, children with diagnosed diabetes, children who had missing data for waist circumference or one of the laboratory analyses of interest in one of the surveys, and children from Cyprus (due to the very small sample size that fulfilled the requirements), 3,348 children remained for the present analysis. For the prospective analyses, children with insulin resistance at baseline were also excluded, which reduced the sample to 3125 children. Due to missing information for some of the potential risk factors or confounders, certain analyses were conducted in smaller samples (numbers are indicated in the tables). A selection effect is very unlikely as characteristics of subgroups (900 children with data on sleep duration or 1163 children with accelerometer data) only differed by the first or second decimal place (data not shown).

Statement of Ethics

Ethical approval was obtained from the relevant local or national ethics committees by each of the eight study centers.

Questionnaire data

Data on education, lifestyle habits and dietary intakes of children was retrieved from parental self-completion questionnaires. Parental education was coded country-by-country according

to the International Standard Classification of Education ISCED[32]. Media use was described by the time spent with audiovisual media (hours/week) and the number of audiovisual media devices (TV, Computer, Internet connection, DVD player, Playstation, Game console) located in the child's bedroom. For dietary assessment, propensity scores for sugar and fat consumption of children were developed from a parental food frequency questionnaire[33,34]. A continuous index was developed, using the total weekly frequency for high-sugar or high-fat items divided by the individual's total consumed food frequencies.

Measurement data

Body mass index (BMI) was calculated as weight (in kg) divided by height squared (in m). Sex-specific BMI categories were interpolated for continuous age according to the extended IOTF criteria[28]. Information on sleep duration was collected in the context of a parental 24-h recall. Parents were asked about their children's get up and bed time (hour/minute) of the previous day. Sleep duration on weekday nights was calculated resulting in a continuous estimate of sleep hours per night as described previously.[29 30] Activity data (time-varying accelerations) of a subset of participating children was recorded by uniaxial accelerometers (ActiGraph®, LLC, Pesacola, FL, USA). The activity monitors were set to record PA in a 60 second epoch. Time spent performing either moderate or vigorous intensity of PA (average minutes over all valid recording days) was calculated according to the cut points proposed by Evenson[35].

Blood samples

Details on the biological sampling procedures can be obtained from a previous publication[31]. Blood glucose was assessed on site at each study centre by point-of-care analysis using the Cholestech LDX analyser (Cholestech®) either in venous or capillary blood. Insulin was determined by electrochemiluminescence immunoassay in the central laboratory. HOMA-IR was calculated as fasting insulin (µIU/ml) x fasting glucose (mg/dl)/405.

Statistical analyses

Data of parameters with normal distribution are presented as mean values (± standard deviation); data of parameters with skewed distributions are presented as median (25th, 75th percentile). Normality of distribution was assessed by the Kolmogorov-Smirnov test. Prevalence of IR was calculated using the age- and sex-specific 95th percentile (p95) of HOMA-IR derived from normal weight IDEFICS children as cut off value for each half year age group[37]. Furthermore, an age- and sex-specific z-score was calculated for HOMA based on this healthy paediatric population. Delta z-IR was calculated as the difference between the z-scores of

HOMA-IR-values at T1 and T0 to depict the development of HOMA-IR between the two surveys. Logistic regression analyses was limited to children without IR at T0, i.e. to all children with a HOMA-IR<p95in their corresponding half year age groups (cut-off ranged from 1.5-2.9 for 3-9 year old girls and 1.3-2.7 for the respective boys)[37]. An indicator variable for presence of HOMA-IR at follow-up (HOMA-IR above or below p95) was defined as dependent variable for the logistic regression analyses. Univariate logistic regression analyses were conducted for potential risk factors for all children and children with normal weight only. Multivariate mixed logistic models were calculated separately for the exposures BMI z-score and z-score of waist circumference to avoid collinearity. Covariables were added to the model if they were significant in the univariate analysis for normal weight children. A random country effect was included in the model to account for the clustered study design. 'Even though no significant effect was seen for the nutritional covariables, fat consumption score (as continuous marker) was additionally included into the model. ORs and 95% confidence limits were calculated for all children and for boys and girls separately. All analyses were performed using SAS® statistical software version 9.3 (SAS Institute, Inc., Cary, NC).

Results

Characteristics of the study population at baseline (T0) and during the follow-up survey (T1) are presented in Table 1. Girls and boys exhibited almost no differences as to age and anthropometrical measures, with the exception of skinfolds (sum of two skinfolds was higher in girls) and percentage of fat free mass calculated from BIA, which was higher in boys. There were small sex differences for the biochemical markers: insulin values were higher in girls and glucose levels were slightly higher in boys. Also sleep duration and media consumption were a little higher in boys, and time spent in MVPA was substantially higher in boys.

Prevalence rates of IR at follow-up were determined for children in the different BMI categories and are shown in Table 2, stratified by country. The lowest prevalence of IR was observed in Belgium (7.6%) and Sweden (11.1%), the highest in Italy with 32.1%. IR prevalence clearly showed an increasing trend with BMI, from an overall 2.2% among thin (underweight) children and 10.9% in normal weight, via 26.5% in overweight, reaching a remarkable 66.7% in obese children. The percentage of children in our cohort who were overweight or obese according to IOTF criteria[28] differed substantially between countries, with the lowest values in Belgium (9.2%) and Sweden (11.9%) and highest percentage (~50%) in Italy. Country-specific prevalence rates of IR ran about in parallel with rates of overweight and obesity, with the exception of Spain, where 26.1% of children were either overweight or obese but only 13.6% of the children showed IR according to our definition.

Longitudinal data were analysed for time varying exposure of weight status on IR (Table 3). IR was considered as time-varying outcome (delta z IR), which was negative (a lower HOMA-IR value at T1) for children with weight loss between the surveys and highest (with a mean delta z of 0.64) for children with substantial weight gain between T0 and T1. Both, children with a low BMI (thin or normal weight) at both measurements and children with a high BMI (overweight or obese) in both surveys also showed increased values of HOMA-IR in T1, with a higher increase for the overweight or obese children.

Possible baseline determinants of IR at follow-up were analysed in univariate logistic regression models (Table 4). Crude odds ratios are shown for all children and for normal weight children only. IR at T1 was positively associated with female sex, increasing age, being from Italy, Hungary, Germany, Estonia and Spain when compared to Belgium which had lowest overall IR prevalence, low SES (maximum parental ISCED of 3 or lower), overweight and obesity, waist circumference, sleep duration ≤ 9 hours/night, media consumption (more than 7 hours/week of audio-visual media time) and number of audio-visual media in bedroom (any media). No associations were observed for the consumption of sugar or fat. MVPA at baseline (upper two quartiles) showed a protective effect on the development of IR two years later. This remained unchanged when the analysis was limited to 5-8 year old children, the age range that the applied cut-off-points by Evenson[35] were calibrated for (data not shown). When only children with normal weight at baseline were considered, an association with IR at follow-up was still evident for sex, being from Hungary, Italy, Germany or Estonia compared to Belgium, low SES, media consumption and audio-visual media in bedroom and MVPA (3rd quartile). We also looked at different anthropometric markers (skinfolds, fat free mass from BIA, weight-to-height-ratio, waist-to-height-ratio) but there were only little differences in the associations of these markers with HOMA-IR (data not shown) and the strongest associations were seen for BMI and waist circumference.

Results from multiple logistic regression analysis are presented in Table 5. Sex (being female), BMI (z-score) and waist circumference (z-score) at baseline were the strongest predictors of HOMA-IR at T1. MVPA showed the same pattern of association as in the univariate analysis: results were strongest for the 3rd quartile. A small but significant increase of risk was also seen for audio-visual media time and fat consumption score. ISCED, age and country showed no influence on HOMA-IR in the multivariate model.

Discussion

This study describes country-specific prevalence rates of insulin resistance in the IDEFICS cohort, showing an increasing trend of IR prevalence with BMI category. It indicates that a favourable longitudinal development of weight status leads to a favourable change in metabolic status. The study shows prospective associations between both, weight status and physical inactivity with insulin resistance in preadolescent European children.

Prevalence proportions of IR found in our study (10.9% in normal weight, 26.5% in overweight, 66.7% in obese children) were in agreement with those reported in the literature, even though comparability is limited, because previous studies were based on older children and used different definitions of IR. [10, 38,22]

In our study, weight status and waist circumference at baseline appeared as the main risk factors for IR at follow-up, but sex and lifestyle indices (objectively determined PA, fat consumption score, audio-visual media time and media in bedroom) were also associated with incidence of IR. The age effect which was seen in the univariate analysis can probably be attributed in large part to the increase of overweight and obesity with age as it completely disappeared in the multivariate analysis. The higher risk of IR in girls compared to boys also persisted when older children (> 7 years at baseline) were excluded from the analysis to avoid possible influences of early puberty. Sex differences in HOMA-values, i.e. higher values in girls, were already found in the youngest children in the IDEFICS cohort.[37] This relationship is reversed at some stage in adolescents, possibly due to the protective effect of oestrogen.[39] Fat consumption, as expressed by a propensity score[33,34], was also connected with IR risk in the multivariate model. The PA level of children in our study was rather low and only a small proportion of children (24.2% of the boys and 9.7% of the girls) reached the daily activity level recommended by the World Health Organization (≥ 60 min/ day)[40]. Considering this and the fact that accelerometer data were only available for about one third of the study population, the protective effect on IR found for PA is certainly noteworthy, despite the lack of a clear trend.

The associations observed in this study confirm previous findings, which however are mainly based on studies in older children/ adolescents and on cross-sectional data. In a representative subsample of diabetes-free US-adolescents aged 12–19 years who participated in the National Health and Nutrition Examination Survey (NHANES), obesity was by far the most important determinant of insulin resistance, independent of sex, age, or race/ethnicity, but data on physical activity was not included in the analysis[10]. Subcutaneous adiposity was also the most

significant covariate for HOMA-IR in a family-based US-American study, including children and adolescents from 6 years on.[11] In a cross-sectional analysis of the IDEFICS baseline survey, physical inactivity was shown to be associated with a cluster of CVD risk factors including IR[15]. In accordance with the evidence from our analysis, a few other studies published data on the association of dietary factors like intake of total energy, total fat or saturated fat with IR in children and adolescents[13,14].

There is a solid body of evidence for the association of obesity and physical inactivity with IR, especially in adults, but there is a controversy on whether the influence of sedentary lifestyle on IR is mediated by obesity, whether both are independent predictors of IR or whether the risk for IR involved with obesity is modified by PA. In our study, the association of PA with IR is attenuated only slightly when only children with normal weight at baseline are considered, i.e. PA reduces the risk of developing IR, also for children with normal weight at baseline which indicates that the effect of physical inactivity is not just mediated by obesity. This is also confirmed by the results of the multivariate model.

Other studies have also shown that increased PA and cardiorespiratory fitness can reduce the risk of cardiovascular disease, type 2 diabetes, and MS.[25,24,15,45,42] Obesity might thus be the main determinant of IR, but its effect can be attenuated by a sufficient amount of PA. A review[43] on the therapeutic power of PA in children suggests that PA may have greater influence on body composition and cardiovascular risk factors than dieting as it possibly modulates the fuel metabolism. The increased fat oxidation by PA might be the basis for prevention and restoration of insulin sensitivity and reduction of metabolic syndrome in obese children.

The main strengths of this study are the large study size, its longitudinal design, the highly standardised data collection across different European countries and the young age of the examined children, as well as the fact that physical activity was measured objectively by activity monitors. There are on the other hand also some limitations that should be mentioned: Pubertal stage was not assessed in the IDEFICS surveys and it can be suspected that a considerable proportion of the older children might have already started into puberty at follow-up. [46,38] Nevertheless, as mentioned above, the elevated IR risk seen in girls remained unchanged when older children (>7 years) where excluded. Food consumption scores used in this study were based on a food frequency questionnaire which did neither include school meals nor portion sizes and are thus based on average quantities which might reduce the truly existing differences.

In summary, to our knowledge, this study is the first longitudinal study on IR in a preadolescent children's population. It supports the available evidence, that overweight and obesity are the main determinants of IR, while physical activity seems to ameliorate the risk, independent of weight status. Thus, reduction of weight is an important measure in the fight against IR in children, but children should above all be encouraged to engage in regular physical activity, as this will keep them metabolically healthy even under the presence of overweight/obesity.

Acknowledgement

The authors also wish to thank the IDEFICS children and their parents for their participation.

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Tables and Figures

Table 1: Characteristics of study population

	Baseline survey T0			Follow-up surveyT1		
	All	**Boys**	**Girls**	**All**	**Boys**	**Girls**
N	3348	1743	1605	3348	1743	1605
Age (years) [a]	6.4 (± 1.7)	6.3 (± 1.7)	6.4 (± 1.6)	8.4 (± 1.7)	8.3 (± 1.7)	8.4 (± 1.6)
Anthropometry						
Weight (kg) [a]	23.9 (± 6.9)	24.0 (± 6.9)	23.9 (± 6.9)	30.6 (± 9.1)	30.7 (± 9.1)	30.6 (± 9.1)
Height (cm) [a]	120.0 (± 12.0)	120.1 (± 12.0)	119.9 (± 12.0)	132.3 (± 11.3)	132.4 (± 11.2)	132.3 (± 11.4)
BMI (kg/m^2) [a]	16.3 (± 2.4)	16.3 (± 2.3)	16.3 (± 2.4)	17.2 (± 3.0)	17.1 (± 3.0)	17.2 (± 3.0)
Waist circumference (cm) [a]	54.7 (± 6.7)	55.0 (± 6.8)	54.4 (± 6.6)	59.1 (± 8.4)	59.4 (± 8.5)	58.9 (± 8.3)
Weight-to-height ratio (kg/cm) [a]	0.20 (± 0.04)	0.20 (± 0.04)	0.20 (± 0.04)	0.23 (± 0.05)	0.23 (± 0.05)	0.23 (± 0.05)
Sum of skinfolds (subscap. and tric.) (mm) [b] *	15.8 (13.3, 19.9)	14.8 (12.6, 18.0)	17.2 (14.4, 21.7)	17.4 (14.0, 24.3)	15.9 (13.0, 21.7)	19.3 (15.4, 26.7)
Fat free mass (%) from BIA [a] *	70.0 (± 8.8)	72.8 (± 6.8)	66.9 (± 9.5)	70.7 (± 8.9)	73.3 (± 8.5)	68.0 (± 8.3)
Biochemical markers						
Fasting glucose (mg/dl) [a]	83.9 (± 9.4)	85.1 (± 9.5)	82.6 (± 9.0)	86.7 (± 9.2)	87.9 (± 9.1)	85.4 (± 9.2)
Fasting insulin (µIU/ml) [b]	3.5 (2.2, 5.3)	3.3 (2.0, 5.2)	3.7 (2.4, 5.5)	5.3 (3.6, 7.7)	4.9 (3.3, 7.0)	5.9 (4.0, 8.6)
HOMA-IR [b]	0.7 (0.4, 1.2)	0.7 (0.4, 1.1)	0.8 (0.5, 1.2)	1.1 (0.8, 1.7)	1.1 (0.7, 1.6)	1.3 (0.8, 1.8)
Other variables						
Time spent with audio-visual media (h/day) [b] *	1.5 (1.0, 2.2)	1.6 (1.0, 2.2)	1.4 (1.0, 2.0)	1.9 (1.2, 2.5)	1.9 (1.2, 2.8)	1.8 (1.0, 2.4)
Number of media in the child's bedroom [a] *	0.8 (± 1.2)	0.9 (± 1.3)	0.7 (± 1.2)	1.0 (± 1.4)	1.1 (± 1.4)	1.0 (± 1.4)
Fat consumption propensity score [a] * #	25.5 (± 9.3)	25.4 (± 9.4)	25.5 (± 9.2)	not available	not available	not available
Sugar consumption propensity score [a] * #	25.0 (± 11.3)	25.3 (± 11.5)	24.7 (± 11.2)	not available	not available	not available
Highest parental education (ISCED-level) [a] *	4.0 (± 1.2)	4.0 (± 1.2)	3.9 (± 1.2)	4.0 (± 1.2)	4.0 (± 1.2)	4.0 (± 1.2)
N with SACINA data	1836	961	875	1450	763	687
Sleep duration on weekdays	10.2 (± 0.9)	10.2 (± 1.0)	10.1 (± 0.9)	9.8 (± 1.0)	9.9 (± 1.0)	9.8 (± 1.0)
N with accelerometer data	1967	1029	938	1793	920	873
Time spent in MVPA (average minutes/day) [a]	41.2 (± 21.2)	46.0 (± 22.8)	36.0 (± 18.0)	43.4 (± 22.6)	48.5 (± 24.6)	38.0 (± 19.0)

[a] Data are presented as mean (± standard deviation), [b] Data are presented as median (25th, 75th percentile),

* Variables with missing data, descriptive statistics based on slightly smaller numbers of children (N≥3055)

**MVPA: moderate to vigorous physical activity

Propensity to consume items high in fat or sugar resp., relative to frequency of all items on food frequency questionnaire

Table 2: Prevalence of insulin resistance at follow-up by BMI categories according to Cole & Lobstein (2012)

Country	Insulin resistance	Thin	Normal weight	Overweight	Obese	All
Italy	+	1	27	44	64	136
	(HOMA-IR ≥ p95*)	*8.3%*	*13.9%*	*35.5%*	*68.0%*	*32.1%*
	-	11	167	80	30	288
	(HOMA-IR < p95*)	*91.7%*	*86.1%*	*64.5%*	*31.9%*	*67.9%*
	All	12 (*2.8%)*	194 (*45.8%)*	124 (*29.3%)*	94 (*22.2%)*	424 (*100.0%)*
Estonia	+	3	35	18	11	67
	(HOMA-IR ≥ p95*)	*7.1%*	*11.9%*	*40.9%*	*84.6%*	*17.0%*
	-	39	260	26	2	327
	(HOMA-IR < p95*)	*92.9%*	*88.1%*	*59.1%*	*15.4%*	*83.0%*
	All	42 (10.7%)	295 (*74.9%*)	44 (*11.2%)*	13 (*3.3%)*	394 (*100.0%)*
Belgium	+	2	11	11	5	29
	(HOMA-IR ≥ p95*)	*4.2%*	*3.7%*	*42.3%*	*55.6%*	*7.6%*
	-	46	289	15	4	354
	(HOMA-IR < p95*)	*95.8%*	*96.3%*	*57.7%*	*44.4%*	*92.4%*
	All	48 (*12.5%)*	300 (*78.3%)*	26 (*6.8%)*	9 (*2.4%)*	383 (*100.0%)*
Sweden	+	2	30	19	5	56
	(HOMA-IR ≥ p95*)	*3.8%*	*7.7%*	*36.5%*	*62.5%*	*11.1%*
	-	50	361	33	3	447
	(HOMA-IR < p95*)	*96.2%*	*92.3%*	*63.5%*	*37.5%*	*88.8%*
	All	52 (*10.3%)*	391 (*77.7%)*	52 (*10.3%)*	8 (*1.6%)*	503 (*100.0%)*
Germany	+	2	27	16	8	53
	(HOMA-IR ≥ p95*)	*5.4%*	*13.1%*	*39.0%*	*88.9%*	*18.1%*
	-	35	179	25	1	240
	(HOMA-IR < p95*)	*94.6%*	*86.9%*	*61.0%*	*11.1%*	*81.9%*
	All	37 (*12.6%)*	206 (*70.3%)*	41 (14.*0%)*	9 (*3.1%)*	293 (*100.0%)*
Hungary	+	8	89	46	30	173
	(HOMA-IR ≥ p95*)	*6.7%*	*18.2%*	*44.7%*	*69.8%*	*22.9%*
	-	112	399	57	13	581
	(HOMA-IR < p95*)	*93.3%*	*81.8%*	*55.3%*	*30.2%*	*77.1%*
	All	120 (*15.9%)*	488 (*64.7%)*	103 (*13.7%)*	43 (*5.7%)*	754 (*100.0%)*
Spain	+	0	31	33	17	81
	(HOMA-IR ≥ p95*)	*0.0%*	*7.5%*	*27.1%*	*50.0%*	*13.6%*
	-	28	382	89	17	516
	(HOMA-IR < p95*)	*100.0%*	*92.5%*	*73.0%*	*50.0%*	*86.4%*
	All	28 (4.*7%*)	413 (*69.2%)*	122 (*20.4%)*	34 (*5.7%)*	597 (*100.0%)*
All countries	+	8	250	187	140	595
	(HOMA-IR ≥ p95*)	*2.2%*	*10.9%*	*36.5%*	*66.7%*	*17.8%*
	-	349	2037	325	70	2753
	(HOMA-IR < p95*)	*97.8%*	*89.1%*	*63.5%*	*33.3%*	*82.2%*
	All	339 (*10.1%)*	2287 (*68.3%)*	512 (*15.3%)*	210 (*6.3%)*	3348 (*100.0%)*

* Age- and sex-specific 95th percentiles (p95) from Peplies et al. 2014 [37]

Table 3: Two-year change of HOMA-IR by changes of weight status

BMI* at baseline	BMI* at follow-up	N	Two-year change of HOMA-IR delta z-IR** (Std$^{\#}$)
Overweight/obese	Thin/normal weight	75	-0.19 (1.1)
Thin/normal weight	Thin/normal weight	2539	0.20 (1.2)
Overweight/obese	Overweight/obese	513	0.46 (1.1)
Thin/normal weight	Overweight/obese	221	0.64 (1.2)
All		3348	0.26 (1.2)

* weight status according to extended IOTF criteria (Cole, 2012)

** delta z-IR = z-score IR (T1) - z-score IR(T0)

$^{\#}$ standard deviation

Table 4: Determinants of insulin resistance (HOMA-IR≥ p95*) - crude odds ratios from univariate logistic regression analyses (children without IR at T0)

	Children of all weight groups				Normal weight children only			
	N	%	OR	95% CI	N	%	OR	95% CI
Sex	**3152 (all)**				**2297 (all)**			
Female	1508	47.8	**1.5**	**1.3-1.9**	1078	46.9	**1.4**	**1.1-1.9**
Male	1644	52.2	Ref.		1219	53.1	Ref.	
Age group	**3152 (all)**				**2297 (all)**			
3-<4 years	353	12.5	**0.4**	**0.3-0.7**	271	11.8	0.7	0.4-1.1
4-<5 years	486	15.4	**0.5**	**0.4-0.8**	348	15.2	0.7	0.5-1.1
5-<6 years	344	10.9	**0.5**	**0.3-0.7**	259	11.3	**0.5**	**0.3-0.9**
6-<7 years	525	16.7	**0.7**	**0.5-0.9**	388	16.9	0.7	0.5-1.1
7-<8 years	841	26.7	0.8	0.7-1.1	608	26.5	0.9	0.6-1.2
8-<10 years	603	19.1	Ref.		423	18.4	Ref.	
Age (continuous, unit change from mean)	**3152 (all)**		**1.2**	**1.1-1.3**	**2297 (all)**		**1.1**	**1.0-1.2**
Country	**3152 (all)**				**2297 (all)**			
Belgium	389	12.3	Ref.		202	8.8	Ref.	
Sweden	358	11.4	1.5	0.8-3.1	276	12.0	1.6	0.7-3.7
Spain	370	11.7	**2.0**	**1.1-3.8**	297	12.9	1.6	0.7-3.6
Estonia	495	15.7	**2.3**	**1.2-4.5**	390	17.0	**2.6**	**1.2-5.6**
Germany	269	8.5	**2.6**	**1.4-5.1**	206	9.0	**2.6**	**1.2-5.9**
Hungary	704	22.3	**3.5**	**1.9-6.4**	502	21.9	**3.7**	**1.8-7.8**
Italy	567	18.0	**5.7**	**3.1-10.5**	424	18.5	**3.7**	**1.7-8.0**
ISCED[##]	**3114 (all)**				**2269 (all)**			
Low (1-2)	215	6.9	**2.6**	**1.9-3.6**	135	6.0	**2.2**	**1.4-3.5**
Medium (3-4)	1563	50.2	**1.8**	**1.4-2.3**	1121	49.4	**1.6**	**1.2-2.2**
High (5-6)	1336	52.9	Ref.		1013	44.7	Ref.	
BMI (Cole)	**3152 (all)**							
Thin	362	11.5	**0.5**	**0.3-0.9**				
Normal weight	2297	72.9	Ref.					
Overweight	345	11.0	**4.1**	**3.3-5.0**				
Obese	148	4.7	**10.8**	**8.2-14.0**				
BMI z-score (Cole)	**3152 (all)**							
1st Quartile (≤ -0.49)	813	26.2	**0.7**	0.5-1.1				
2nd Quartile (-0.49 - ≤ 0.19)	802	25.6	Ref.					
3rd Quartile (0.19 - ≤ 0.97)	811	23.1	**1.2**	0.9-1.8				
4th Quartile (≥ 0.97)	726	25.1	**4.9**	**3.7-6.6**				
BMI z-score (Cole) (unit change from mean)	**3152 (all)**		**2.5**	**2.2-2.8**				
Waist z-score (Cole)	**3152 (all)**							
1st Quartile (≤ -0.66)	826	26.2	0.8	0.5-1.2				
2nd Quartile (-0.66 - ≤ 0.09)	791	25.1	Ref.					
3rd Quartile (0.09 - ≤ 0.95)	808	25.6	**1.5**	**1.0-2.1**				
4th Quartile (≥ 0.95)	727	23.1	**5.7**	**4.1-7.7**				
Waist z-score (Cole) (unit change from mean)	**3152 (all)**		**2.2**	**2.0-2.4**				
Audio-visual media time	**3152 (all)**				**2297 (all)**			
≤ 1 hours/day	1062	33.7	Ref.		774	33.7	Ref.	
1 - ≤2 hours/day	1221	38.7	**1.4**	**1.1-1.9**	899	39.1	**1.4**	**1.0-2.1**
2 - ≤3 hours/day	603	19.1	**1.7**	**1.3-2.3**	439	19.1	**1.8**	**1.2-2.6**
> 3 hours/day	266	8.4	**2.1**	**1.5-2.9**	185	8.1	**2.3**	**1.5-3.6**
Media in bedroom	**3090 (all)**				**2250 (all)**			
0 media	1902	61.5	Ref.		1429	63.5	Ref.	
1-2 media	868	28.1	**1.8**	**1.4-2.2**	609	27.1	**1.8**	**1.3-2.3**
3 media	320	10.4	**2.6**	**2.0-3.4**	212	9.4	**2.4**	**1.7-3.4**
Sugar consumption propensity score (N=3125)[#]	**3152 (all)**				**2297 (all)**			
1st Quartile (≤ 16.9)	842	26.7	Ref.		625	27.2	Ref.	
2nd Quartile (16.9 - ≤ 24.1)	832	26.4	1.0	0.8-1.4	557	24.3	1.0	0.7-1.5
3rd Quartile (24.1 - ≤ 32.4)	766	24.3	1.1	0.8-1.4	562	24.5	1.0	0.7-1.5
4th Quartile (≥ 32.4)	713	22.6	1.2	0.9-1.6	553	24.1	1.4	1.0-1.9
Fat consumption propensity score (N=3125) [#]	**3152 (all)**				**2297 (all)**			
1st Quartile (≤ 19.0)	901	28.6	Ref.		595	25.9	Ref.	
2nd Quartile (19.0 - ≤ 24.8)	790	25.1	0.8	0.6-1.0	543	23.6	1.1	0.8-1.6
3rd Quartile (24.8 - ≤ 31.3)	748	23.7	0.8	0.6-1.0	574	25.0	1.1	0.8-1.6
4th Quartile (≥ 31.3)	713	22.6	0.9	0.7-1.2	585	25.5	1.1	0.8-1.6
Sleep duration on weekdays (N=1730)	**1730 (all)**				**1253 (all)**			
Average sleep time < 9h	104	6.0	**1.8**	**1.2-2.7**	58	4.6	1.5	0.8-2.8
Average sleep time ≥ 9h	1626	94.0	Ref.		1195	95.4	Ref.	
Time spent in MVPA**	**1042 (all)**				**771 (all)**			
1st Quartile (≤ 27 min./day)	259	24.9	Ref.		203	26.3	Ref.	
2nd Quartile (27 - ≤ 38.7 min./day)	256	24.6	0.8	0.5-1.2	193	25.0	0.9	0.5-1.4
3rd Quartile (38.7 - ≤ 54.6 min./day)	264	25.3	**0.4**	**0.2-0.7**	193	25.0	**0.4**	**0.2-0.9**
4th Quartile (≥ 54.6 min./day)	263	25.2	**0.6**	**0.4-1.0**	182	23.6	0.7	0.3-1.0

* Age- and sex-specific 95th percentiles (p95) from Peplies et al. 2014 [37]

** MVPA: moderate to vigorous physical activity

Propensity to consume items high in sugar or fat resp., relative to frequency of all items on food frequency questionnaire

Maximum ISCED level of both parents was considered.

Table 5: Determinants of insulin resistance (HOMA-IR≥ p95*) - results from multivariate mixed logistic regression models, including country as a random effect to account for the clustered study design.

N=1083	Model 1** (with BMI z-score)		Model 2** (with waist z-score)	
	OR	**95% CI**	**OR**	**95% CI**
BMI z-score (Cole) (unit change from mean)	**2.6**	**2.1-3.1**		
Waist z-score (Cole) (unit change from mean)			**2.2**	**1.9-2.6**
Time spent in MVPA* **				
1st Quartile (≤ 27 min./day)	Ref.		Ref.	
2nd Quartile (27 - ≤ 38.7 min./day)	0.9	0.6-1.5	1.1	0.7-1.7
3rd Quartile (38.7 - ≤ 54.6 min./day)	**0.5**	**0.3-0.9**	**0.5**	**0.3-0.9**
4th Quartile (≥ 54.6 min./day)	0.7	0.5-1.1	0.7	0.5-1.1
Sex (female versus male)	**2.2**	**1.5-3.1**	**2.5**	**1.8-3.6**
Age (unit change from mean)	1.0	0.9-1.2	1.0	0.9-1.1
ISCED#				
Low (1-2)	1.2	0.7-2.2	1.3	0.7-2.3
Medium (3-4)	1.2	0.9-1.8	1.2	0.8-1.8
High (5-6)	Ref.	Ref.	Ref.	Ref.
Audio-visual media time (h/d) (unit change from mean)##	**1.2**	**1.0-1.4**	**1.2**	**1.0-1.4**
Fat consumption propensity score### (unit change from mean)	**1.2**	**1.0-1.4**	**1.2**	**1.0-1.4**

* Age- and sex-specific 95th percentiles (p95) from Peplies et al. 2014 [37]
** Analyses were adjusted for all parameters in the respective column
*** MVPA: moderate to vigorous physical activity
# Maximum ISCED level of both parents was considered.
Number of media in bedroom' was dropped in favour of audio-visual media time as both measure a similar construct, thus to avoid collinearity.
Propensity to consume items high in or fat, relative to frequency of all items on food frequency questionnaire. The score was included into the model in a modified form: it was divided by 10 to obtain meaningful effect estimates – one unit in the multivariate model thus represents 10 units of the original score used in the univariate model.