Development and validation of a low-volume LC-HRMS assay for the analysis of aldosterone, its precursor and main metabolite tailored for paediatric research in a GCLP-compliant environment

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I. Erklärung zu Dissertation

Hiermit versichere ich an Eides statt, dass die vorgelegte Dissertation mit dem Titel:

Development and validation of a low-volume LC-HRMS assay for the analysis of aldosterone, its precursor and main metabolite tailored for paediatric research in a GCLP-compliant environment

von mir selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf erstellt worden ist. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keinen erfolglosen Promotionsversuch unternommen.

Düsseldorf, den Nina Makowski

II. Danksagung

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III. Zusammenfassung

Das Renin–Angiotensin–Aldosteron Systems (RAAS) ist ein humoraler Regelkreis, der maßgeblich zur Herz-Kreislauf-Gesundheit beiträgt. Eine Fehlregulierung dieses empfindlichen Systems hat schwerwiegende Folgen und führt beispielsweise zu Bluthochdruck, Herzinsuffizienz und letztendlich zu einer erhöhten Mortalität. Aldosteron ist ein wichtiger Effektor dieses Systems und der pharmakotherapeutische Eingriff in das RAAS kann zweifelsfrei die Morbidität und Mortalität von Erwachsenen senken. Bei Kindern ist der Einfluss von Aldosteron noch nicht ausreichend erforscht, um verlässliche Aussagen treffen zu können.

Im ersten Teil dieser Arbeit werden die Ergebnisse eines Literatur-Reviews zu den derzeitig veröffentlichten Aldosteronkonzentrationen in gesunden Kindern und Kindern mit Herzinsuffizienz präsentiert. Dabei wurden in beiden Patientengruppen Altersabhängigkeit der Aldosteronkonzentrationen und eine niedrige Datendichte bei Kindern im Alter von unter einem Jahr deutlich. Durch Heterogenität der Daten wurde der dringende Bedarf qualitätsgesicherter Forschung, um eindeutige Schlussfolgerungen ziehen zu können, ersichtlich.

Um solche qualitätsgesicherte Forschung vorzubereiten, wurde ein maßgefertigtes, an die Bedürfnisse der Bioanalytik in pädiatrischen Zulassungsstudien, angepasstes Qualitätssicherungssystem entwickelt. Die erfolgreiche Umsetzung dieses Systems wurde am Beispiel der Anwendung auf die Aldosteronmessungen in einer solchen Studie gezeigt. Das entwickelte System ermöglichte die Erhebung von qualitätsgesicherten, leitlinienkonformen Aldosteronkonzentrationen. Gleichzeitig ist dabei die Überwachung der Datenerhebung möglich, um mögliche Qualitätsschwankungen zu erkennen und Gegenmaßnahmen zu ergreifen und auf diese Weise robuste Daten zu gewährleisten.

Um die Erhebung von fehlenden qualitätsgesicherten, pädiatrischen Daten zu ermöglichen, wurde eine bioanalytische Methode zur Bestimmung der Aldosteron-, 18-Hydroxycorticosteron- und Tetrahydroaldosteron-konzentrationen im Serum mittels Hochleistungsflüssigkeitschromatographie

gekoppelt mit hochauflösender Massenspektrometrie entwickelt und nach aktuellen, internationalen Leitlinien validiert. Die Anwendung, auch in schwer erkrankten Kinderkollektiven, ist durch das geringe benötigte Probenvolumen möglich. Die gleichzeitige Bestimmung von Aldosteron, seiner Vorstufe und seines Hauptmetaboliten ermöglicht neue Einblicke in den heranwachsenden pädiatrischen Organismus.

IV. Summary

The Renin–Angiotensin–Aldosterone System (RAAS) is a humoral circuit decisive for cardiovascular health. Dysregulation of this sensitive system has severe consequences and can, for example, lead to hypertension, heart failure and subsequently to higher mortality. Aldosterone is an important effector of this system, and the positive effect of RAAS-regulating pharmacotherapy on morbidity and mortality has been proven in adults. The impact of aldosterone on the paediatric organism is not yet sufficiently investigated, thus, a reliable statement on it cannot be made.

In the first part of this thesis, the results of a literature review on the currently published aldosterone concentrations in healthy children and children with heart failure are presented. An age dependency of aldosterone levels and low data density in children under the age of one year became apparent in healthy as well as diseased children. The heterogeneity of the data showed the urgent need for quality-assured research to enable unambiguous conclusions.

Further, a customised quality assurance system, tailored for the need of bioanalytics in paediatric pivotal studies, was developed to enable such quality-assured research. The successful implementation of this quality system was demonstrated using as an example its application to aldosterone measurements in such a clinical investigation. The developed system enabled the acquisition of quality-assured, guideline-conforming aldosterone concentrations. At the same time, monitoring of the data acquisition is possible, which enables detection of possible quality fluctuations and to take countermeasures to ensure reliable data. Moreover, a bioanalytical method for the reliable determination of serum concentrations of aldosterone, its precursor 18-hydroxycorticosterone, and its main metabolite tetrahydroaldosterone via high performance liquid chromatography coupled to high resolution mass spectrometry was developed and validated according to current, international guidelines to enable the collection of needed high-quality paediatric data. The application, even in severely diseased children, has been enabled by the minimal sample volume utilised. The simultaneous determination of aldosterone, its precursor and its main metabolite can provide new insights into the maturing paediatric organism.

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VI. List of abbreviations

ACE-I	Angiotensin–Converting–Enzyme Inhibitor
ACTH	Adrenocorticotropic Hormone
CHD	Congenital Heart Defects
CS	Calibration Curve Standard
CV	Coefficient of Variation
CYP	Cytochrome P450
DCM	Dilative Cardiomyopathy
ELISA	Enzyme-linked Immunosorbent Assay
EMA	European Medicines Agency
FDA	Food and Drug Administration
GCLP	Good Clinical Laboratory Practice
HPLC	High-Performance Liquid Chromatography
IS	Internal Standard
ISR	Incurred Sample Reanalysis
LC-HRMS	Liquid Chromatography–High Resolution Mass Spectrometry
LC-MS/MS	Liquid Chromatography Tandem-Mass Spectrometry
LENA	Labeling of Enalapril from Neonates up to Adolescents
LLOQ	Lower Limit of Quantification
MeSH	Medical Subject Headings
MRA	Mineralocorticoid Receptor Antagonist
PQ	Performance Qualification
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
QC	Quality Control Sample

RAAS	Renin–Angiotensin–Aldosterone System
S/N	Signal-to-Noise Ratio
SD	Standard Deviation
SE	Standard Error
TOF	Time-of-Flight
ULOQ	Upper Limit of Quantification

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1. Introduction

1.1. Aldosterone and its physiological function

Aldosterone is known to be the effector of the renin–angiotensin–aldosterone system (RAAS). This humoral circuit function regulates the cardiac function via blood pressure, fluid volume, and electrolyte balance (Muñoz-Durango et al., 2016). An overview of the RAAS and its influence on aldosterone production is presented **Figure 1-1**. Disturbance in this finely tuned system leads to a multitude of risks for the cardiovascular as well as renal system and subsequently to the development of diseases like hypertension, heart failure, or chronic kidney disease (He and Anderson, 2013; Hostetter, 2003).

Aldosterone has been intensely investigated in adults; its physiological function and diverse impacts on the organism have become evident in the adult organism. Furthermore, significant innovations in the pharmacotherapy due to the implementation of mineralocorticoid antagonists, which decrease the deleterious effect of aldosterone, led to an improvement of morbidity and mortality in cardiovascular diseases like heart failure (Pitt et al., 1999; Pitt et al., 2003). In children, however, not as much is known about the aldosterone system. Its influence on physiological and pathological developments has not been sufficiently investigated. Further, data on the development of the metabolising pathways responsible for the aldosterone production and metabolism are also lacking. Similarities and differences from the adult population can help to derive suitable therapies or to develop targeted approaches. Thus, more research is necessary to clarify the role of aldosterone in the maturing organism. It can be anticipated that the aldosterone system and metabolism changes with the maturation of the organism. Still, it needs to be investigated if aldosterone has the same decisive role in children as it does in adults.

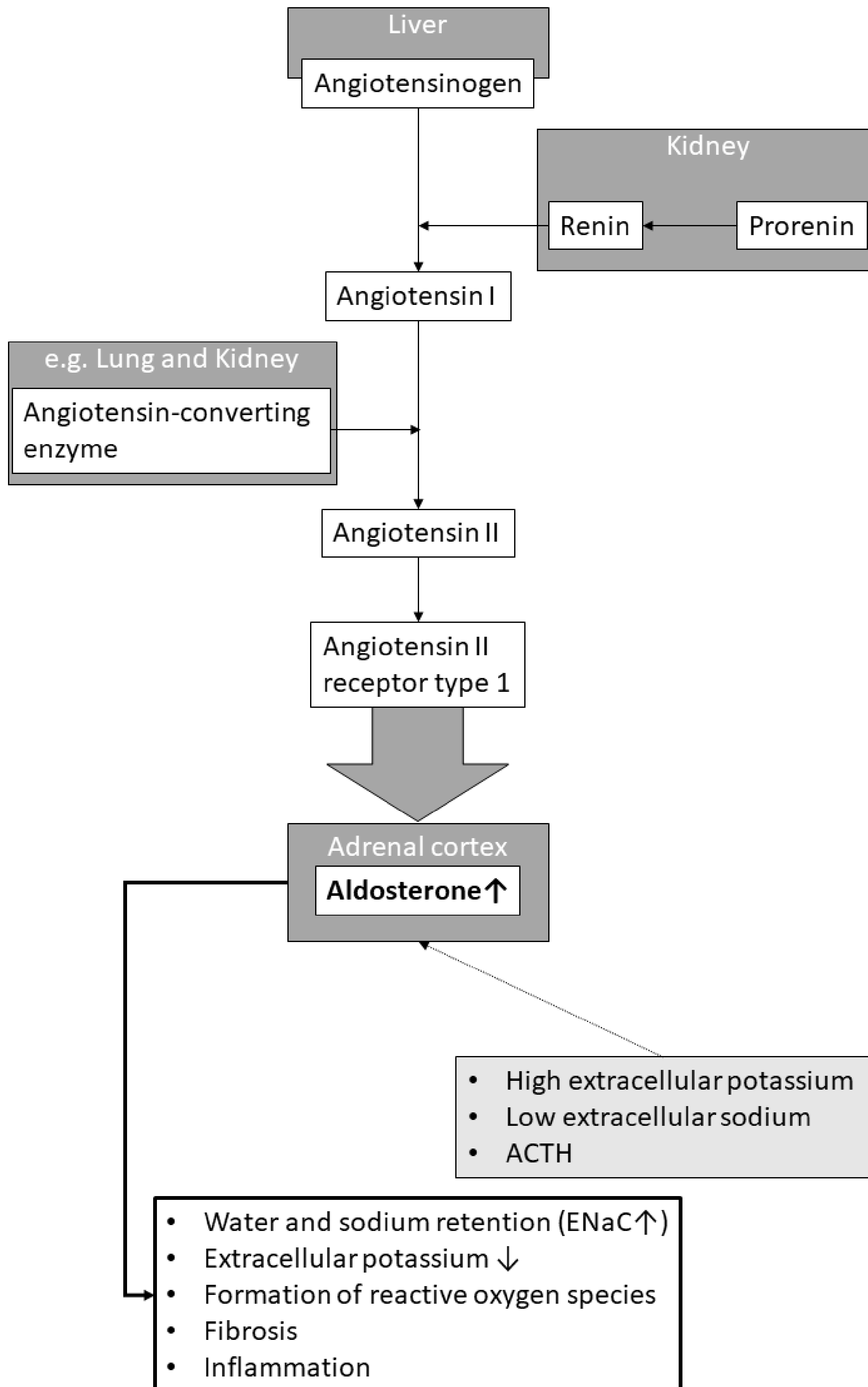

Figure 1-1. The renin–angiotensin system and its influence on aldosterone production. Further indicated are other endogenous triggers of aldosterone production and the endogenous effects of aldosterone. ENaC: Epithelial sodium channel; NO: Nitrogen monoxide; ACTH: Adrenocorticotropic hormone

Aldosterone has been an object of research since Simpson et al. first extracted it in 1953 (SIMPSON et al., 1953). Aldosterone is the most potent endogenous mineralocorticoid, and for a long time, aldosterone was thought to be synthesized exclusively in the adrenal cortex zona glomerulosa. However, new evidence suggests that aldosterone is produced in many other cells (e.g. endothelium, brain cells, adipocytes) (Nguyen Dinh Cat et al., 2011; Briones et al., 2012; Lyngsø et al., 2016). These innovations illustrate that still much is unknown about this long known and well-investigated hormone. Like all other endogenous glucocorticoids and mineralocorticoid steroids, it is synthesized in multiple steps from cholesterol. Details of the aldosterone synthesis are outlined in **Figure 1-2**. The final steps of the aldosterone production are catalysed by the cytochrome P450 (CYP) 11B2 (aldosterone synthase). This enzyme is located in the mitochondria of cells and has an 18-hydroxylase and a methyloxidase function (Payne and Hales, 2004). In the last step of the aldosterone synthesis, aldosterone's direct precursor, 18-hydroxycorticosterone is converted into aldosterone exclusively through the 18-oxidase activity of the aldosterone synthase (Roumen et al., 2011; Hattangady et al., 2012). The main elimination organ of aldosterone is the kidney, after glucuronidation (Phase II) in the liver, aldosterone and its metabolites are excreted renally.

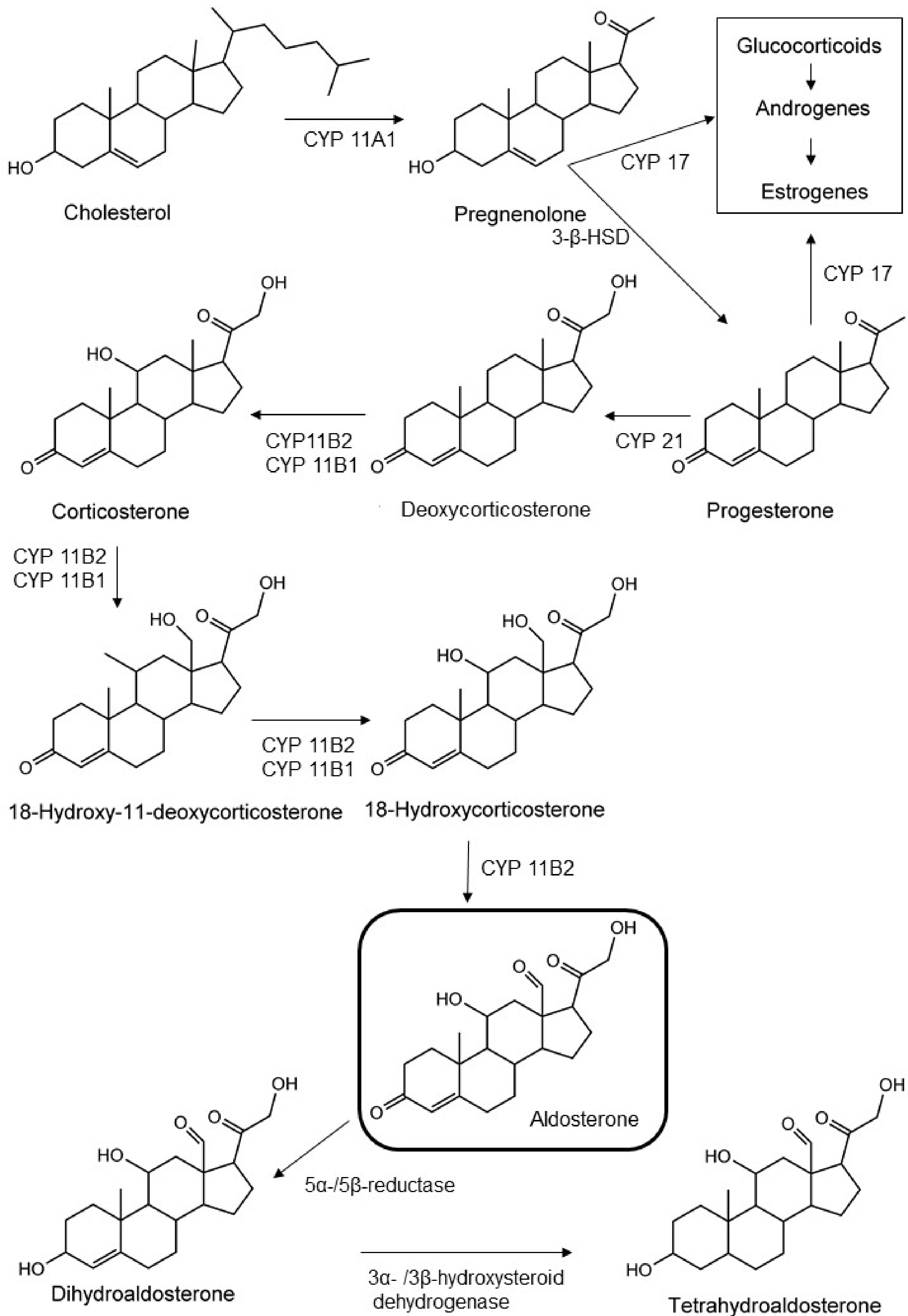

Figure 1-2. Endogenous synthesis of mineralocorticoids and aldosterone metabolism. HSD: Hydroxysteroid dehydrogenase (Hattangady et al., 2012; Roumen et al., 2011).

As shown in **Figure 1-1**, aldosterone production is regulated by different endogenous parameters, the most important ones being angiotensin II, extracellular potassium levels and adrenocorticotropic hormone (ACTH). Aldosterone is predominantly released from the zona glomerulosa and can act via mineralocorticoid receptor-dependent or -independent pathways (Brown, 2005).

Aldosterone, as an endogenous hormone, underlies the circadian rhythm (Doi et al., 2010). In a healthy adult, the highest aldosterone values are observed in the early morning. The aldosterone levels decrease until the evening and then start increasing again during the night (Cugini et al., 1981; Schaefer et al., 2017a). This has also been shown in healthy school children and adolescents (Rittig et al., 2006). This circadian rhythm, however, is influenced by different factors like age, sodium intake and pharmacotherapy (Lamarre-Cliche et al., 2005), and thus these factors need to be taken into consideration during sampling and when evaluating aldosterone values.

Despite the susceptibility of the serum aldosterone concentration to age, time of sampling, and posture, it is used as a valuable marker for different endocrine, renal as well as cardiac diseases (Briet and Schiffrin, 2013; Brown, 2013; Pitt et al., 1999). Its influence on cardiovascular risks has been investigated in large randomised clinical studies, and the effect of high aldosterone values on morbidity and mortality has been proven (Pitt et al., 2003; Pitt et al., 1999). Moreover, in patients with hyperaldosteronism due to adrenal hyperplasia or aldosterone-producing adenoma, chronically elevated aldosterone levels have been identified as the cause of hypertension or congestive heart failure with left ventricular hypertrophy (Freel and Connell, 2004; He and Anderson, 2013) . Aldosterone's importance and influence on the organism is undeniable, which make it a versatile object of research.

1.2. Paediatric heart failure

The European Society of Cardiology defines heart failure as '[...] a clinical syndrome characterized by typical symptoms (e.g. breathlessness, ankle swelling and fatigue) that may be accompanied by signs (e.g. elevated jugular venous pressure, pulmonary crackles and peripheral oedema) caused by a structural and/or functional cardiac abnormality, resulting in a reduced cardiac output and/or elevated intracardiac pressures at rest or during stress' (Ponikowski et al., 2016). The main reasons for heart failure in adults are chronic hypertension or ischaemic heart diseases (Nandi and Rossano, 2015). Therefore, heart failure is newly diagnosed in adults mostly in the second half of life.

The pathophysiology and presentation of symptoms in paediatric heart failure, however, differ distinctively from the adult population. The cause of paediatric heart failure is most often congenital heart defects (CHD) (e.g. left-to-right shunts, single ventricle defects or aortic regurgitation) (Nandi and Rossano, 2015; Hsu and Pearson, 2009a). Heart failure, in children with structurally normal hearts, can be induced by cardiomyopathies (dilated, hypertrophic, or restricted) or it can develop due to secondary causes (e.g. viral infections or exposure to toxins) (Hsu and Pearson, 2009a). Paediatric patients with heart failure show impaired exercise tolerance (e.g. exhaustion while feeding) and thus present with a failure to thrive as well as respiratory distress. Many cases of paediatric heart failure are diagnosed in the first year of life, instead of damage building up over decades in the adult heart failure. Paediatric heart failure can have far-reaching consequences for patients and their caregivers due to its strong impact on the paediatric organism. For example, children with dilated cardiomyopathy (DCM) have a 40% risk of mortality or the need for heart transplantation two years after presentation, twice as much as the rate of normalisation (Lee et al., 2017). The incidence of paediatric heart failure may be low, but the impact on the patients' morbidity and mortality, with over 250,000 deaths per year in the US, is extensive (Nandi and Rossano, 2015; Rosenthal et al., 2004). However, higher than the overall mortality of 6–7% of paediatric patients suffering from heart failure of all causes is the associated morbidity (Nandi and Rossano, 2015).

Corrective surgery and pharmacotherapy are current tools being utilised to reduce this burden. Cardiac surgery has shown to be possible and is as beneficial in very young children under the age of 1 year as in older children (Hsu and Pearson, 2009b). However, despite surgery for the improvement of the patient's health status and the prevention of death, additional pharmacotherapy is often indispensable. The pharmacotherapy, however, is not well investigated, despite the broad and evidence-based range of pharmacotherapy available for adults. The International Society for Heart and Lung Transplantation released a guideline for the management of paediatric heart failure, but as far as the pharmacotherapy is concerned all recommendations are based on only one randomised trial, multiple non-randomised trials (both evidence level B) or expert consensus (level C) (Rosenthal et al., 2004; Kirk et al., 2014). This lack of knowledge about safe and effective pharmacotherapy is caused by the lack of paediatric research. Thus, paediatric pharmacotherapy for heart failure is still in large part derived from adults although it is known that the paediatric organism and the pathophysiology of the disease differ. Thus, research that focuses on the particular needs and characteristics of the paediatric population is urgently needed to enable children access to a safe and evidence-based pharmacotherapy (Kearns et al., 2003).

1.3. Research in children

Because the paediatric organism differs strongly from the adult one, obtained data in adults and its knowledge on physiological and pharmacological responses cannot be linked to paediatrics aiming a safe and rational drug therapy (Ginsberg, 2002; Barker et al., 2018). Differences in the body water and fat portions, as well as differences in the metabolic system due to the maturing of the paediatric organism, are reasons for the limited comparability to adults (Cock et al., 2011). These parameters have a pivotal influence on the blood concentrations of drugs and, thus, the pharmacotherapy. Hence, pharmacokinetic data are needed to understand the behaviour of a drug in a child. In addition, pharmacodynamic data are necessary for a better understanding of the effect that the drug has on the paediatric organism and to reveal new insights into the development of the paediatric organism. For example, metabolic pathways have been found to differ decisively from the adult ones and develop over time. In young children, sulfation is the primary metabolic pathway when glucuronidation and CYP metabolism still need to mature (Cock et al., 2011). In the case of aldosterone, renal elimination is the main metabolic pathway, after metabolism in the liver to tetrahydroaldosterone and glucuronidation in the kidneys. (Abdelhamid, 2003). In young children, these pathways are maturing, and consequences of this alteration on frame conditions for pharmacotherapy and the impact on the aldosterone system are still insufficiently investigated.

The lack of paediatric data is, among other things, a consequence of the ethical restrictions in paediatric research. These restrictions are necessary for the safeguarding of the vulnerable paediatric population but present as obstacles for investigators. A physiologically based example is the restricted volume of blood available for the determination of bioanalytical data. Because most of the bioanalytical assays have been established for the determination of aldosterone in an adult population and thus utilize sample volumes well tolerated by adults, they are inappropriate for the paediatric population, especially when taking into consideration preterms and newborns. When sampling blood from children, it is not advised to sample more than 1% of the blood volume at a single sampling time point and not more than 3% in four weeks (Committee for Medicinal Products for Human Use, Ad-hoc group for the development of implementing guidelines

for Directive 2001/20/EC, 2008). This equates to around 2400 µL of whole blood available for sampling from a child with a weight of 3 kg at a single time point. Methods available in the clinical routine require about 33–100% of the blood volume available from a child of 3 kg for a single determination of aldosterone (MVZ Labor Dr. Limbach Heidelberg; Universitätsklinikum Leipzig Institut für Laboratoriumsmedizin, Klinische Chemie und Molekulare Diagnostik; Mayo Clinic Laboratories; ROE Laboratorio Clinico). This procedure is neither acceptable for multiple sampling time points nor for very small children, taking into consideration that children requiring aldosterone determination are not only healthy children undergoing a routine check-up but rather diseased children who need the regular monitoring of different blood parameters simultaneously. Further obstacles for research in children are the smaller patient pool available, from which the recruitment is additionally more challenging in comparison to the recruitment in adult studies. A further hurdle is a corresponding smaller market size, which mostly does not compensate for the higher costs for the funding of paediatric trials (Caldwell et al., 2004).

Despite all these pitfalls and obstacles, paediatric research is crucial for the safeguarding of the paediatric population and needs to be conducted with the highest quality possible because sample sizes are small, and the data acquired in the first study population may not be investigated a second time. Thus, the acquired data need to be reliable because data obtained with such investigations might act as a basis for far-reaching therapeutic decisions.

1.4. The LENA project

The LENA (Labeling of Enalapril from Neonates up to Adolescents) project was an investigator-driven paediatric investigation funded by the European Commission (Seventh Framework Programme [FP7/2007-2013] under grant agreement n°602295 [LENA]). LENA aimed to generate data of an orally disintegrating mini-tablet containing the angiotensin–converting–enzyme inhibitor (ACE-I) enalapril, which was administered to children suffering from heart failure. These data were needed for the licensing of those mini-tables, specifically developed for the paediatric population, to improve the pharmacotherapy available for children with heart failure substantially.

The LENA project did not only aim to gather pharmacokinetic data on enalapril and its active metabolite enalaprilat in children suffering from heart failure but further investigated the impact of the pharmacotherapy on pharmacodynamic parameters. In the context of this explanatory pharmacodynamic investigations, RAAS parameters (e.g. aldosterone, angiotensin, renin) were investigated to build the basis for a safe pharmacotherapy and gain new insight into the pharmacodynamics of the paediatric population suffering from heart failure (Bajcetic et al., 2019). One hundred and two diseased children suffering from CHD or DCM participated in the studies. Over 70% of the LENA study population was under the age of 1 year, demanding suitable bioanalytical methods and newly developed quality assurance strategies. Hence, bioanalytical methods tailored for this paediatric population had to be developed using only a small amount of blood to be able simultaneously to investigate pharmacokinetics and pharmacodynamics alongside required safety parameters in this severely diseased population.

1.5. Bioanalytical determinations in the paediatric population

Currently, aldosterone determinations are conducted with several bioanalytical methods. Radioimmunoassays, chemiluminescence immunoassays, as well as enzyme-linked immunosorbent assays (ELISAs) are widely utilised (Carvajal et al., 2018; Burrello et al., 2016; Schaefer et al., 2017a). These immunoassay approaches are based on the specific antigen-antibody reaction, which enables a sensitive determination of the analyte of interest in complex samples. Reducing the required blood volume is a key element in paediatric research and needs to come along with reliable data generation (Schaefer et al., 2017a; Schaefer et al., 2017b). Nevertheless, especially for endogenous compounds, cross-reactions are possible due to structurally related molecules to the antigen, which can lead to an overestimation of the actual concentration. Further, classical 96-well immunoassays can only provide information on one specific analyte and cannot simultaneously detect several analytes. Therefore, the explanatory power of these assays is limited.

A more advanced bioanalytical approach is the determination via liquid chromatography tandem-mass spectrometry (LC-MS/MS). Several assays for the determination of aldosterone were developed (Häkkinen et al., 2018; Turpeinen et al., 2008; Hinchliffe et al., 2013), but the required blood volume varied between 0.15 to 1.2 mL of whole blood (Häkkinen et al., 2018; Mayo Clinic Laboratories). The latter is too high for sophisticated pharmacokinetic investigations with comprehensive sampling points. Applications of LC-MS/MS approaches in paediatric research for the determination of aldosterone have been reported (Soldin et al., 2009; Travers et al., 2017). However, the use is limited, although the technique has the potential to offer a deeper insight into the sample matrix and its composition by simultaneous analyte determination.

The application of Liquid Chromatography–High Resolution Mass Spectrometry (LC-HRMS) appears a promising approach to obtain data with the advantages of the LC-MS/MS methods but offers in parallel the comprehensive screening even of yet unknown compounds via the highly selective time–of–flight (TOF) detector. This allows for a deeper insight into the sample and acquisition of data that might at the moment not be in a correlation of the analyte of interest (AB Sciex, 2018; Gross, 2013). However, if future research generates such a hypothesis, a

retrospective analysis could be initiated, which might be of high advantage in paediatric research where samples are limited, and investigation of paediatric populations mostly be conducted once only. The determination of the exact mass is another advantage of this approach. Unlike the conventional LC-MS/MS methods, the LC-HRMS approach can reliably determine the mass of a molecule further than just the first decimal due to a high resolution of about 5 ppm (Pleil and Isaacs, 2016). Thus, the distinction of analytes with similar masses are possible, which further expands the explanatory power of HRMS approaches.

Especially the fact that investigation of paediatric populations mostly is conducted once only should be an incentive to conduct the most comprehensive investigation of the available the samples to build a reliable base for knowledge generation on this vulnerable population. Hence, the most sophisticated and most meaningful bioanalytical approach available should be utilised in paediatric research to generate high-quality data and facilitate new insights into the paediatric samples. Therefore, low-volume LC-HRMS methods for the use in paediatric populations are needed. Nevertheless, the use of this advanced approach is only a first step to the generation of high-quality data. Validation and further quality assurance means, meeting the demands of regulatory authorities, are needed to generate urgently needed reliable data.

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1.6. The aim of this thesis

The aim of this three-part thesis is to gain new insights into the—in adults well-known—aldosterone system and to provide new tools for quality assurance, expand knowledge about the development of the paediatric aldosterone system, as well as provide a reliable, high-quality bioanalytical assay, thus, enabling the investigation of the development of the RAAS in children.

First, a literature search of the currently available data on aldosterone values in healthy children and children suffering from heart failure should be conducted, aiming to compile the available data and identify controversial areas as well as areas of low data density.

Second, a quality assurance approach customised for the bioanalytics in paediatric drug development for drug approval should be developed with the aim of enabling high-quality research in children and to bridge the requirements of the authorities and the exceptional circumstances and obstacles involved in paediatric research.

Lastly, to enable new insights into the paediatric collective, a bioanalytical method customised for paediatric research, an LC–HRMS assay for the determination of aldosterone, its precursor (18-hydroxycorticosterone) and main metabolite (tetrahydroaldosterone) should be developed and validated according to international guidelines.

Each chapter will give a short specific introduction to the topic at hand, the methodical tools utilised as well as the results, and is closed with a discussion. A final overall conclusion and perspective will be given at the end of this thesis.

2. Current literature on aldosterone values in healthy children and children suffering from heart failure

2.1. Introduction

Aldosterone plays an essential role in the healthy and diseased cardiac system as part of the RAAS. Aldosterone has been an object of research since its isolation in the early 1950s (SIMPSON et al., 1953; SIMPSON et al., 1954) but is still a topic of current investigations (Travers et al., 2018a). Physiologically, aldosterone regulates the electrolyte homeostasis and blood pressure but triggers also fibrosis, inflammation and endothelial dysfunction (Briet and Schiffrin, 2013; Brown, 2005; Vogt and Burnier, 2009).

In research on cardiovascular, renal and endocrinologic disorders, aldosterone levels are commonly determined to gain further insights on its role in the investigated disease (Funder et al., 2016; Capelli et al., 2019; Kolodziejczyk et al., 2018). Aldosterone reference values for adults have been repeatedly reported and seem well-founded as well as comparable across different sources (O'Shea et al., 2016; Kerstens et al., 2011; Hannemann et al., 2010). However, the information on aldosterone concentrations in children is not as extensive as in adults. Nevertheless, well-founded knowledge about the maturing cardiovascular system and their important effectors like aldosterone is needed to enable a safe and rational pharmacotherapy for the vulnerable paediatric population. The role of aldosterone in paediatric heart failure is, for example, not yet clarified. Paediatric heart failure is characterised by diverse aetiologies (e.g. DCM, CHD) and, thus, the effect of aldosterone may also differ within this broad clinical picture. Thus, data, as well as treatment strategies, cannot be derived from adults in whom heart failure is caused, for example, by long-term hypertension or coronary heart disease.

The lack of data is, among other things, caused by the obstacles involved in paediatric research. Because the paediatric population is in particular need of protection, the risks and benefits of a study as well as of any associated intervention need to be considered. Healthy control groups are lacking in paediatrics studies due to the ethical considerations (Smyth and Weindling,

1999). In addition, small-sized studies are common for paediatric research. Thus, the compilation of available data sets facilitates an overall impression of the aldosterone values reported and supports a more meaningful classification of new data sets.

An overview of currently available aldosterone data found in healthy children as well as children suffering from heart failure can strengthen the knowledge of aldosterone in the maturing and diseased paediatric organism. The presented review enables a comprehensive view on the alteration of the aldosterone values in children and can be used to identify less investigated age groups, which might encourage further investigations for a better understanding of the paediatric organism. Moreover, these data can support the integration of data acquired in current paediatric research into the already available data.

2.2. Methods

A literature review was conducted to generate a compilation of aldosterone values for the healthy paediatric population and children suffering from heart failure. The search was carried out in Medline via PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) in January 2019 according to the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement (Moher et al., 2009). Two separate searches were conducted utilizing the following MeSH (Medical Subject Headings) terms:

- "Aldosterone AND (Congenital heart disease OR Dilated cardiomyopathy OR heart failure) AND (Neonate OR Toddler OR Child OR Paediatric OR Infant)"
- "Aldosterone AND Healthy AND (Neonate OR Toddler OR Child OR Paediatric OR Infant)"

Children, born after ≥37 gestational weeks with a birthweight ≥2.5 kg, suffering from heart failure due to CHD or DCM, not in need of intensive care support due to end-stage heart failure and not suffering from severe renal impairment (glomerular filtration rate <30 mL/1.73 m^2), were included. Children with uncorrected primary obstructive valvular disease, significant systemic ventricular outflow obstruction, restricted or hypertrophic cardiomyopathy, and any kind of additional disease in combination with heart failure were excluded. Further, children classified as healthy in the corresponding publication who were admitted to the hospital for minor injuries or non-cardiovascular related health issues were excluded because the influence of their condition on the aldosterone values was not assessable.

Records that were not in English or German were excluded as well as records not containing aldosterone values or records containing imprecise data (e.g. the age not stated, logarithmic values given, statistical operator not given, only graphical data given which were not fit for extraction due to overlapping), adult, in vivo, animal data, data from a matrix different from serum, plasma, or whole blood. If no numerical data were available, graphical data were extracted if possible and the mean of three extractions was included in the data compilation.

The mean and standard deviation (SD) were manually calculated for individual data points stated in the publication.

Relevant data were included independently from sample size or size of the age group. Each applicable age group state in one record was included separately. Aldosterone values not expressed as pg/mL were converted (e.g. from pmol/L (1 pg/mL = 2.775 pmol/L)).

Software

Data only available in graphs were extracted via GetData Graph Digitizer (Version 2.26.0.20) if possible. The graphical representation and the corresponding calculations were performed utilizing OriginPro® 2018b (OriginPro 2018b (64-bit) b9.5.5.409 (Lehre)).

2.3. Results

In the systematic literature search, a total of 307 records were identified. After the removal of eleven duplicates, 296 records remained for screening. After the title/abstract screening, 141 records were excluded. The full text of the resulting 155 records was assessed for eligibility. Forty-four records, published between 1975 and 2018, were identified (see **Figure 2-1**). Thus, data from 3453 children, between the age of 0 and 18 years, were compiled. Tables summarising the identified data for healthy children and children suffering from heart failure are given in **Appendix 1** and **Appendix 2**.

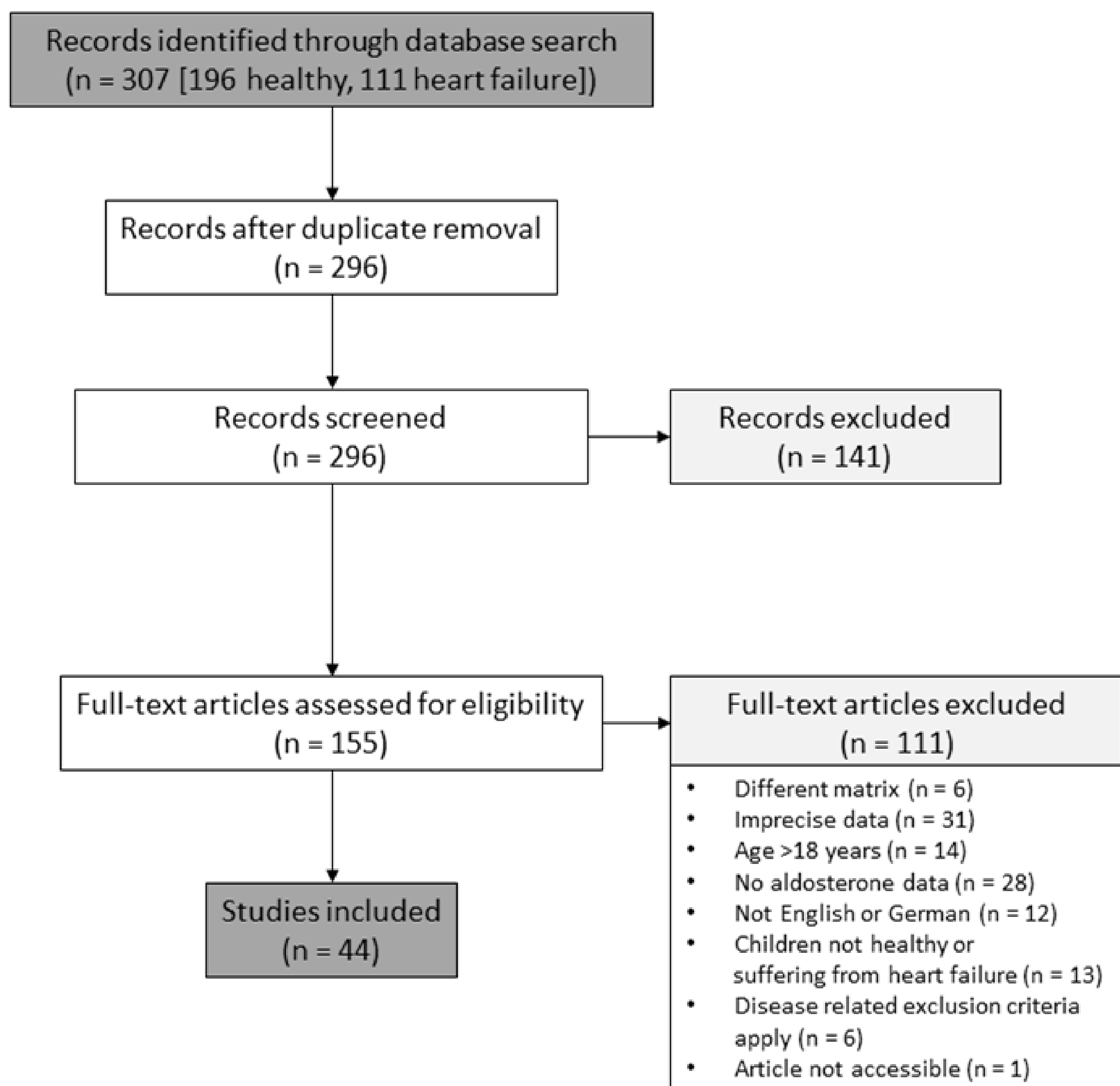


Figure 2-1. PRISMA flow diagram for the systematic literature research for aldosterone in healthy children and children with heart failure (Moher et al., 2009).

Healthy paediatric population

Aldosterone data extracted from 33 identified full text articles representing 2985 healthy children were compiled. The age groups as presented by the authors and the corresponding aldosterone values as presented in the publications were included in the data compilation as data points. Thus, multiple data points per publication were possible, if data for multiple age groups were given in the corresponding publication (see **Appendix 1** and **Appendix 2**). Sixty-eight data points were compiled for the healthy paediatric population. The details of the extracted data are given in **Appendix 1** and the graphical representation of the data is shown in **Figure 2-2**. The following evaluation of the data was conducted in accordance with the age groups recommended by the European Medicine Agency (EMA) (Committee for Medicinal Products for Human Use, Ad-hoc group for the development of implementing guidelines for Directive 2001/20/EC, 2008) to facilitate a comprehensive overview and a structured comparison of the data.

Newborns (1 – 27 days)

Fourteen data points, representing 195 healthy children, were extracted from seven publications stated age groups in accordance to the EMA definition of newborns (Dotsch et al., 2005; Garcia del Rio et al., 1982; Gemelli et al., 1991; Hubl et al., 1978; Sulyok et al., 1979; Sulyok et al., 1980; Zeevi et al., 1998). A high variation in the mean values, ranging between 340 and 2982 pg/mL, was observed (Gemelli et al., 1991; Dotsch et al., 2005). The mean and median of the identified mean values of these publications were 691 pg/mL and 1069 pg/mL indicating the broad range and high variation in this very young age group. Nevertheless, Hubl et al. (1978) showed a constant decrease in aldosterone values from the first 12 h after birth up to the fifth day after birth. The aldosterone value decreased from 795.3±226.8 to 441.8±182.9 pg/mL (Mean±SD). At the fourth days of live Hubl et al. (1978) reported aldosterone values of 501.9±261.7 (Mean±SD), in contrast to the higher values found by Gemelli et al. (1991) of 2982±1909 pg/mL. Mean aldosterone concentrations on the sixth day of life of 2110 pg/mL, presented by Sulyok et al. (1979), support the results presented by Gemelli et al. (1991).

Infants and toddlers (28 days – 23 months)

Data for 128 children between the age of 28 days and 23 month defined as infants and toddlers by the EMA were stated in six publication resulting in seven data points (Dillon and Ryness, 1975; Fiselier et al., 1983; Fiselier et al., 1984; Gjuric et al., 1982; Hubl et al., 1978; Lashansky et al., 1992). The mean values found for this population seem to reveal a more uniform trend over different data points than observed in neonates. Mean aldosterone values between 279 and 328 pg/mL were reported in five data points (Dillon and Ryness, 1975; Hubl et al., 1978; Lashansky et al., 1992). Nevertheless, the variation in data was still high. For example, ranges from 59 to 1055 pg/mL (Dillon and Ryness, 1975) and 207 – 1927 pg/mL (Gjuric et al., 1982) have been described.

Children (2–10 years)

Thirteen publications collected aldosterone data in children between the age of 2 to 10 years. Fifteen data points, representing a total of 244 children, were extracted (Abd-Allah et al., 2004; Dillon and Ryness, 1975; El-Raziky et al., 2005; Hubl et al., 1978; Nalcacioglu et al., 2018; Nicolaidou et al., 2003; Parth et al., 1976; Sigirci et al., 2006; Tiosano et al., 2011; Lashansky et al., 1992; Zeevi et al., 1998) . The reported mean values of the aldosterone concentration reported by the different authors ranged between 53 and 191 pg/mL. The overall mean and median calculated for those aldosterone mean values are 119 and 111 pg/mL, which indicates a more unified concentration range in comparison to the children under the age of 2 years (Abd-Allah et al., 2004; Dillon and Ryness, 1975; El-Raziky et al., 2005; Hubl et al., 1978; Nicolaidou et al., 2003; Parth et al., 1976; Sigirci et al., 2006; Tiosano et al., 2011; Lashansky et al., 1992; Zeevi et al., 1998). The stated ranges of aldosterone concentrations which varied from 0.77 up to 214 pg/mL (Nalcacioglu et al., 2018) and 50 up to 440 pg/mL (Fiselier et al., 1984) seem to confirm this picture.

Adolescents (11–18 years)

Data for adolescents were given in five publications. Five data points representing 165 children between the age of eleven to eighteen years were compiled (Lee et al., 2009; Rittig, 2010; Sigirci et al., 2006; Tiosano et al., 2011; Wyller et al., 2010). The published mean values for this population seem to be similar to the values

found for children at the age of 2-10 years and range between 13.9 to 146 pg/mL aldosterone (Rittig, 2010; Sigirci et al., 2006). Further, the observed variations seem small and underline a consistency in the compiled data.

The most age groups consisted of children ≥3 years. Aldosterone data of 2587 children over the age of 3 years were summarised in 43 data points were reported in the extracted publications. On the other hand, only 24 of the extracted age groups included children ≤ 1 year of age. Therefore, a total of only 389 children ≤1 year are represented in the data compilation. The studies with the largest number of patients (n ≥ 195) were carried out in older children and adolescents. In five large studies, aldosterone values in a total of 1248 school children and adolescents were investigated (Tu et al., 2017; Tu et al., 2014; Martinez-Aguayo et al., 2010; Pratt et al., 2000; Harshfield et al., 1993). These findings reflect an imbalance in data density.

The overall number of data points seems considerably for children, but as the explanatory power is closely linked to the study size and the age range, these factors need to be kept in mind for a comprehensive assessment of the data. The overall sample size, however, is small in comparison to adults (Hannemann et al., 2010). Almost 70% of age groups represent a sample size of fewer than 25 children. Details of the sampling procedure and the matrix collected are also listed in **Appendix 1**. The use of plasma seems to be more common than the use of serum. Further, the sampling was conducted as reported, predominantly in the morning. Adult data indicates the influence of the circadian rhythm on aldosterone values, which has not been intensively investigated in children but has to be recognised as a potential source of variation.

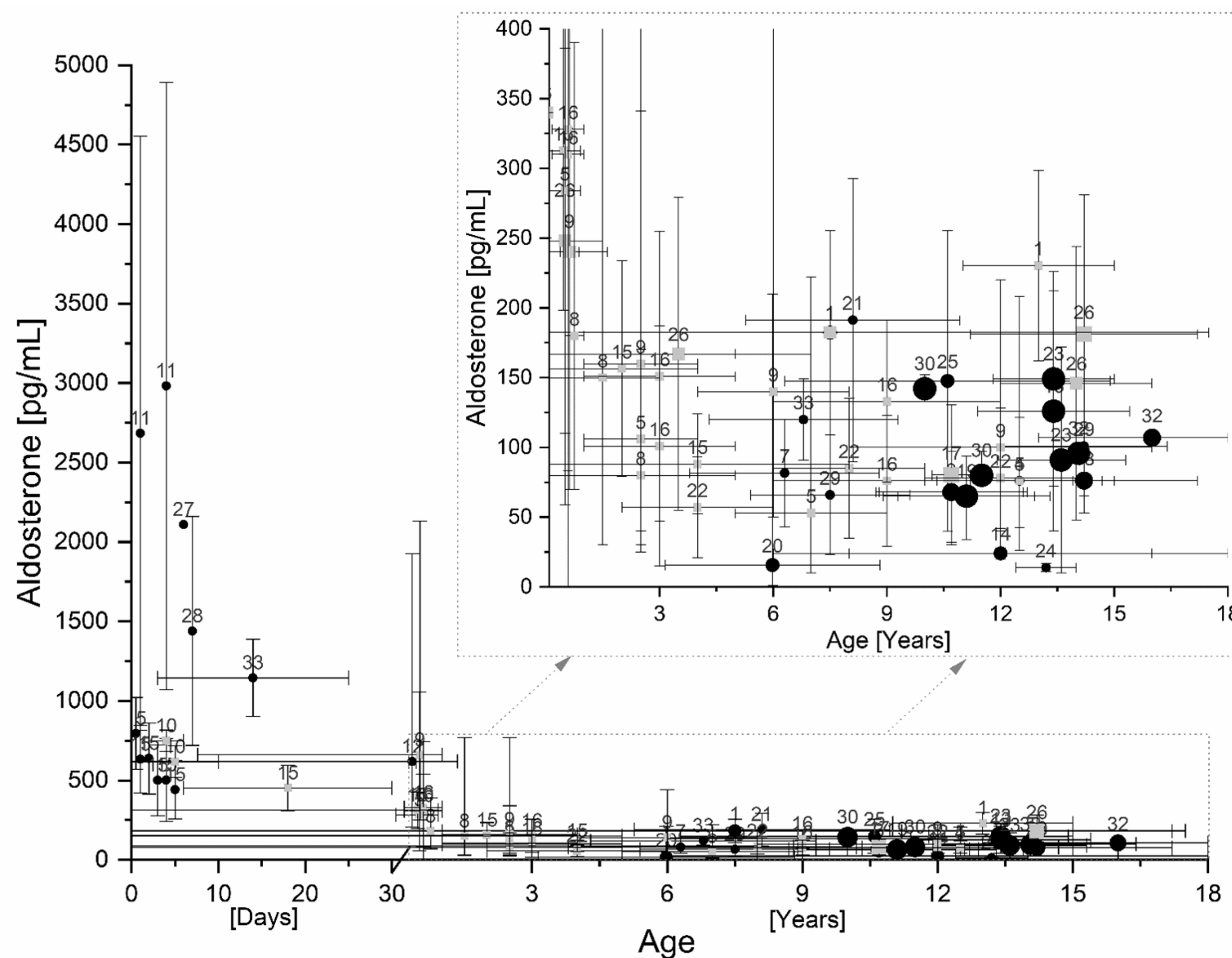


Figure 2-2. Compiled data points identified for the healthy population (N = 33; n = 2985) in 33 full text articles. The black dots represent the point estimator for each given age group, its size indicates the sample size (●: n = 195 – 314, ●: n = 50 – 99, ●: n = 25 – 49, ●: n = 1 – 24). The grey squares indicate the fictional calculated estimator needed for graphical representation of for data sets stating only an age range (■: n = 50 – 99, ■: n = 25 – 49, ■: n =1 – 24). The x- and y-error bars represent the range/ standard deviation/ standard error/ interquartile range/ 95% confidence interval of the age and aldosterone concentration given. The graph in the dotted grey square present a close-up view of the data for children from 1 month to 18 years. The graph is based on data from the following publications: 1: Abd-Allah et al., 2004; 2: Alpert et al., 1979 (‡); 3: Campino et al., 2013; 4: Carvajal et al., 2018; 5: Dillon and Ryness, 1975; 6: Dotsch et al., 2005; 7: El-Raziky et al., 2005; 8: Fiselier et al., 1983; 9: Fiselier et al., 1984; 10: Garcia del Rio et al., 1982; 11: Gemelli et al., 1991; 12: Gjuric et al., 1982; 13: Harshfield et al., 1993; 14: Hjortdal et al., 2000; 15: Hubl et al., 1978; 16: Lashansky et al., 1992; 17: Lee et al., 2009; 18: Mangge et al., 2013; 19: Martinez-Aguayo et al., 2010; 20: Nalcacioglu et al., 2018; 21: Nicolaidou et al., 2003; 22: Parth et al., 1976; 23: Pratt et al., 2000; 24: Rittig, 2010; 25: Siahanidou et al., 2000; 26: Sigirci et al., 2006; 27: Sulyok et al., 1979 (†);28: Sulyok et al., 1980; 29: Tiosano et al., 2011 (†); 30: Tu et al., 2014 (†); 31: Tu et al., 2017; 32: Wyller et al., 2010; 33: Zeevi et al., 1998. Details are given in **Appendix 1**. †: Data extracted from graph (mean of n=3); ‡: Individual values stated in the publication

Paediatric population suffering heart failure

Aldosterone values in children suffering from heart failure were identified and extracted from twelve full text articles published between 1979 – 2016. Details are listed in **Appendix 2**. Twenty-five data points were included, representing 468 children suffering from heart failure. The majority of patients cover the age range from one to six years. Children suffering from heart failure due to CHD and DCM have been combined, because only two of the identified 12 studies investigated children suffering from DCM, and further, only one of those two studies included DCM patients exclusively. As the objective of the majority of the extracted studies was the investigation of an intervention (e.g. surgery, change in the drug regime), most papers give aldosterone values for the investigated paediatric population before and after an intervention. Presented in **Figure 2-3** is the overall picture of aldosterone values compiled for children suffering from heart failure (if applicable data before study intervention were included).

Newborns (1 – 27 days)

Two of the identified studies investigated the impact of CHD correction surgeries on the aldosterone levels in newborns with heart failure (Anand et al., 1990; Zeevi et al., 1998). Anand et al. (1990) reported a decrease in aldosterone values from 1009±324 pg/mL before surgery to 324±144 pg/mL (mean±standard error [SE]) 24 hours after surgery. Similar results have been reported some years later by Zeevi et al. (1998). Before the correction of CHDs, aldosterone values of 1676±169 pg/mL were observed, and 7 to 30 days after surgery these values dropped to 506±181 pg/mL (mean±SE) (Zeevi et al., 1998).

Infants and toddlers (28 days – 23 months)

Two of the 12 studies investigated aldosterone levels in infants and toddlers. In one of those studies, the impact of treatment with digoxin and diuretics on the aldosterone levels of infants and toddlers, suffering from CHD, was assessed (Baylen et al., 1980). Before the introduction of the therapy, a mean aldosterone value of 1506 pg/mL was reported. Six days after introducing medication, the aldosterone value raised to a mean of 2605 pg/mL. Distinctively different aldosterone values have been obtained by a study investigating the effect of a bidirectional Glenn procedure on the aldosterone levels in this age group. The

aldosterone value did not differ strongly before, and 5 days after the intervention, values of 280±40 pg/mL and 270±40 pg/mL (mean±SE) were obtained (Mainwaring et al., 1994).

Children (2–10 years)

The most data was found for this age group of children from 2 to 10 years. Five studies, which enrolled a total of 106 children, investigated the effect of surgical and pharmacological interventions on the aldosterone values of children suffering from heart failure (Ationu et al., 1993; Mainwaring et al., 1994; Francois et al., 2009; Saiki et al., 2016; Senzaki et al., 2008). The studies assessing the effect of surgical impact report different, but stable values. Ationu et al. report the values of 434±26 and 495±47 pg/mL (mean ±SE) aldosterone before and 24 hours after surgery (Ationu et al., 1993). Lower and equally stable aldosterone levels of 220±40 and 290±40 pg/mL (mean ±SE) were described by Mainwaring et al. (1994). Distinctively higher aldosterone values before and after surgery were also detected. These high aldosterone concentrations of 1090±760 pg/mL decreased to 270±280 pg/mL (mean ±SD) after surgery and a six-month treatment with the ACE-I lisinopril.

Adolescents (11–18 years)

No applicable data were identified within the extracted records for this age group.

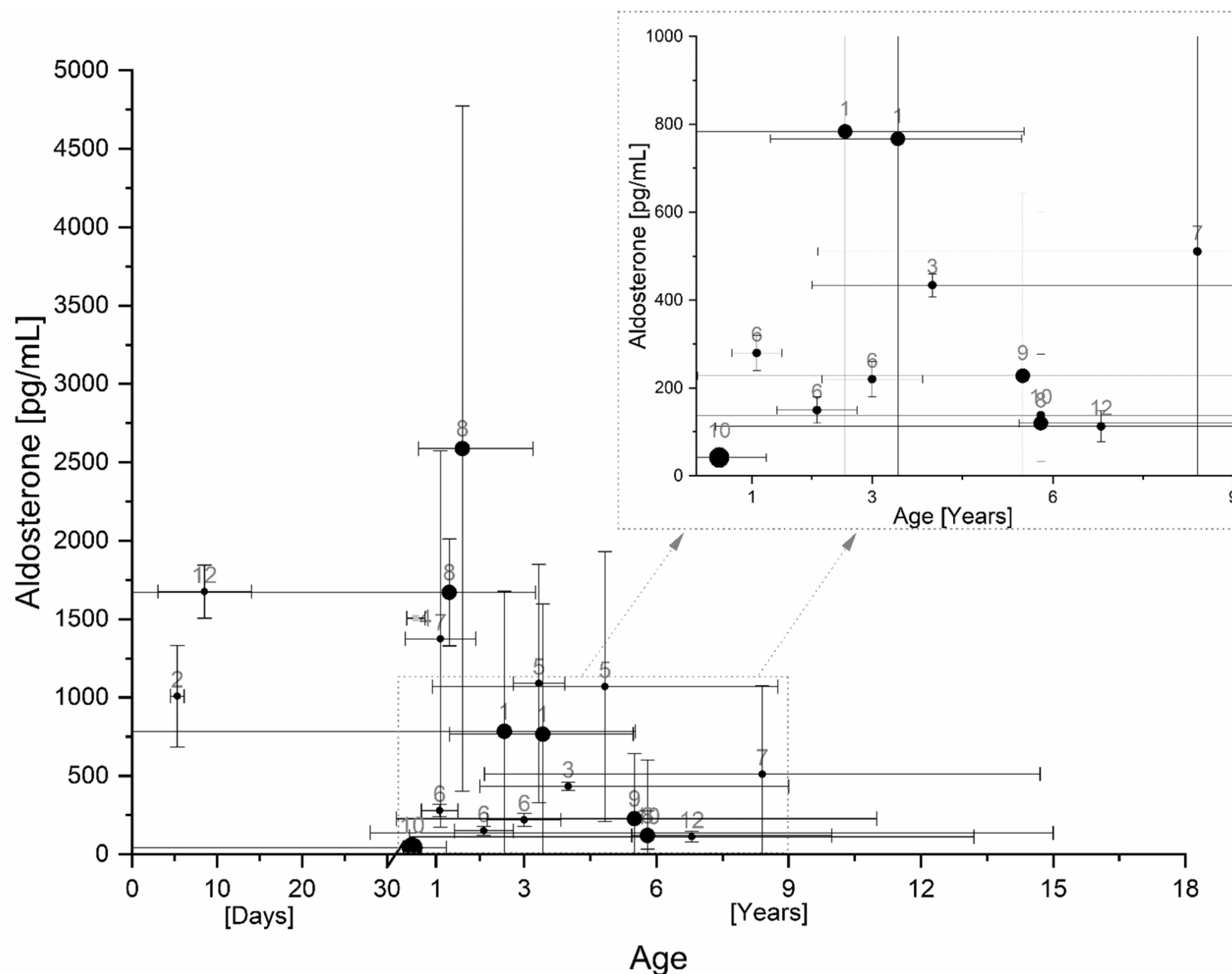


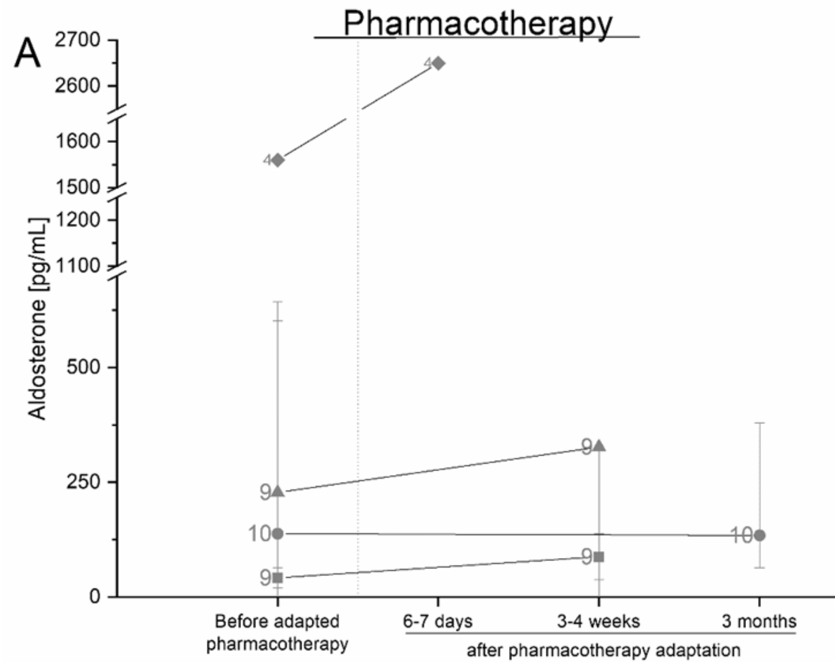
Figure 2-3. Compiled data points identified for the paediatric population suffering from heart failure (Congenital heart defects and dilative cardiomyopathy) (N = 12; n = 468). The black dots represent the point estimator for each given age group, its size indicates the sample size (⬤: n = 50 – 99, ●: n = 25 – 49, •: n = 1 – 24). The x- and y-error bars represent the range/ standard deviation/ standard error/ interquartile range of the age and aldosterone concentration given. The grey squares indicate the fictional calculated estimator needed for graphical representation of for data sets stating only an age range (■: n= 1 – 24) The graph in the dotted grey square presents a close-up view for a better understanding of the data for children from 1 month to 9 years. The graph is based on data from the following publications: 1: Alvarez Kindelan et al. 1994; 2: Anand et al. 1990; 3: Ationu et al. 1993; 4: Baylen et al. 1980; 5: Francois et al. 2009; 6: Mainwaring et al. 1994; 7: Masutani et al. 2013; 8: Saiki et al. 2016; 9: Senzaki et al. 2008; 10: Stern et al. 1990; 11: Wimmer et al. 1979(†); 12: Zeevi et al. 1998. Details are given in **Appendix 2**. †: Data extracted from graph (mean of n=3)

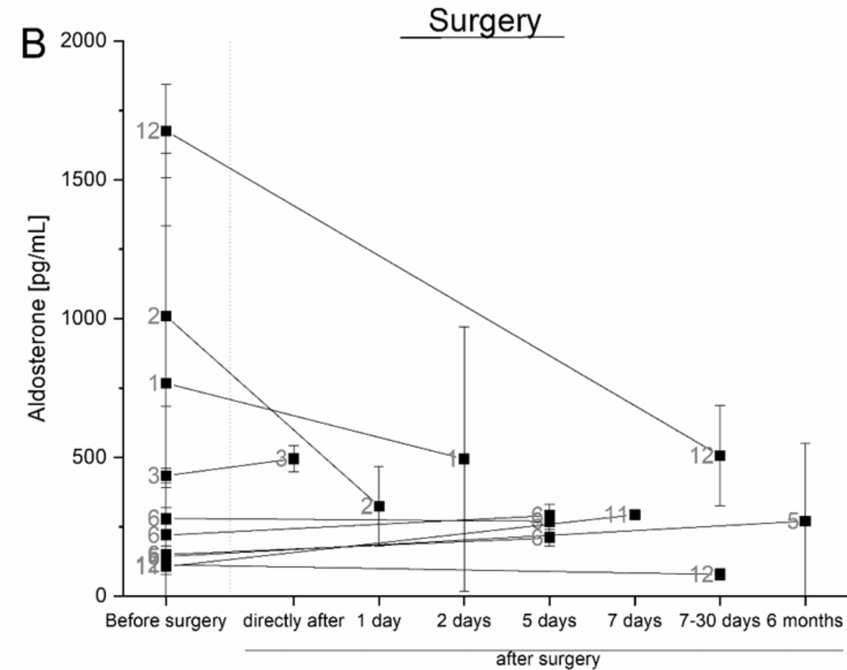
Influence of pharmacotherapy and surgery on aldosterone values on paediatric heart failure

Sub-analysis of the different interventions and their impact on the aldosterone levels are presented in **Figure 2-4**. RAAS modulation is a fundamental part of the drug therapy in adults suffering from heart failure. The pathophysiology of the disease may differ strongly, but the beneficial effects of drug therapy with ACE-I, diuretics, and mineralocorticoid receptor antagonist (MRA) have also been anticipated in the paediatric population. However, five of the included articles have investigated the effect of other drugs modulating the RAAS and their influence on the paediatric aldosterone levels (Baylen et al., 1980; Senzaki et al., 2008; Stern et al., 1990; Francois et al., 2009; Wimmer et al., 1979). Two studies indicated that the introduction of diuretics (torasemide) or digoxin/diuretics increased the aldosterone values of the investigated population. The introduction of torasemide raised the aldosterone values of children naive to diuretics from 44±22 to 88±50pg/mL and from 228±415 to 327±528 pg/mL in children already medicated with a different diuretic than torasemide (Senzaki et al., 2008). Similar effects were observed after the treatment with digoxin and diuretics. Mean aldosterone values of children between the age of 4 – 9 months increased from 1506 to 2605 pg/mL after the introduction of diuretics or digoxin (Baylen et al., 1980).On the other hand, the use of captopril decreased aldosterone values after three months (Stern et al., 1990). These results are consistent with the mechanism of action of ACE-I derived from adults (Brown and Vaughan, 1998). This observation is further supported by another independent study investigating the effect of the treatment with lisinopril, an ACE-I, after cardiac surgery in children suffering from heart failure (Francois et al., 2009). The authors reported a decrease from 1070±860 to 380±760 pg/mL (mean±SD) aldosterone after six months of lisinopril therapy after surgery. The aldosterone values of the control group decreased from 1090±760 pg/mL to 270±280 pg/mL (mean±SD) six months after surgery without the introduction of lisinopril. No studies investigating the influence of enalapril on children with heart failure were identified. In another study, the influence of spironolactone, an MRA proved to extend survival in adults suffering from heart failure (Pitt et al., 1999), was investigated in children after CHD correction surgery. The mean aldosterone values of the children not receiving spironolactone raised from 160.7 to 160 pg/mL seven days after

surgery. Less intensely, than the mean aldosterone values of children receiving spironolactone, which raised from 106.7 to 293 pg/mL (Wimmer et al., 1979).

The trend for the influence of the different surgery techniques (e.g. Fontan procedure, bidirectional Glenn procedure, or repair of ventricle septum defect) on the aldosterone values is not as clear as one of the drug interventions. Trends of decreasing and increasing aldosterone levels were observed regardless of the investigated age groups. Zeevi et al. reported a decrease of aldosterone values from 113±7 pg/mL to 78±20 pg/mL (mean±SD) in children at the age of 6.8±6.4 years (mean±SD) and from 1676±169 pg/mL to 506±181 pg/mL (mean±SD) for younger children at the age of 8.5±5.5 days after CHD correction surgery (Zeevi et al., 1998). On the other hand, increased aldosterone values after surgery were reported. A slight increase from 220±40 to 290±40 pg/mL (mean±SE) five days after Fontan procedure and from 150±30 to 210±30 pg/mL (mean±SE) aldosterone five days after repair of ventricular septum defect was described (Mainwaring et al., 1994). Likewise, an increase of aldosterone values from 434±26 to 495±47 pg/mL (mean±SE) after CHD correction has been observed. These different observations may be due to the different underlying aetiologies of paediatric heart failure.





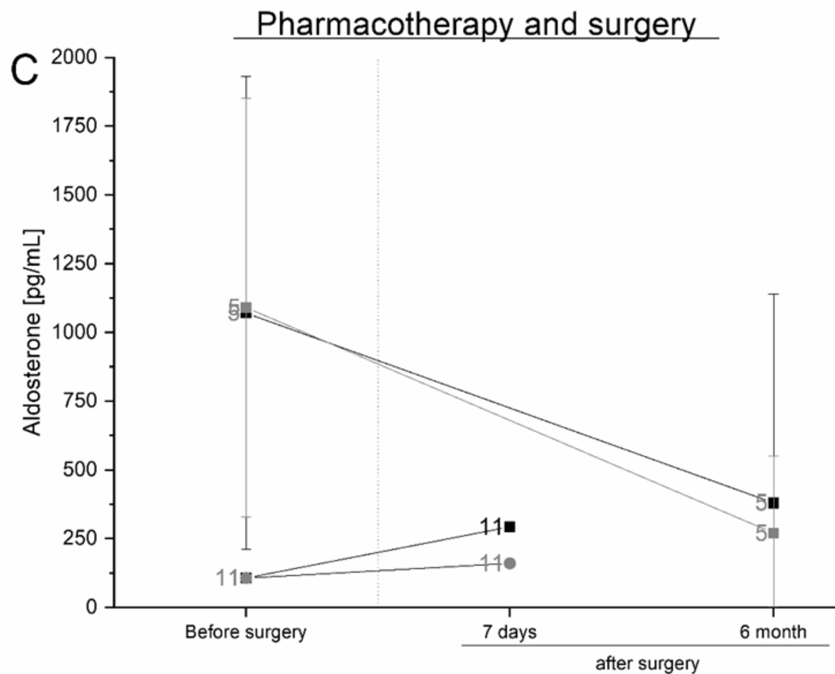


Figure 2-4. Influences of interventions on aldosterone values in children suffering from heart failure.

A: ♦: Introduction of digoxin and diuretics to patients with moderate to severe heart failure (4:Baylen et al., 1980); ■: Introduction of torasemide to patients naive to diuretics or ▲non-naive to diuretics (9: Senzaki et al., 2008); ●: Introduction of captopril to patients with heart failure due to dilated cardiomyopathy (10: Stern et al., 1990).

B: Aldosterone values before and after surgery (1: Alvarez Kindelan et al., 1994; 2: Anand et al., 1990; 3: Ationu et al., 1993; 5: Francois et al., 2009; 6: Mainwaring et al., 1994; 11: Wimmer et al., 1979; 12: Zeevi et al., 1998).

C: ■/■: Comparison of the influence of the introduction of Lisinopril 36h after surgery (■) and no lisinopril introduction after surgery (■) (5: Francois et al., 2009). ●/●: Comparison of aldosterone values after surgery under the influence of spironolactone introduced after surgery (●) or without the introduction of spironolactone (●) (11: Wimmer et al., 1979).

2.4. Discussion

A total sample size of 2985 healthy and 468 children suffering from heart failure was obtained. A trend to higher aldosterone values in children under the age of one year could be derived for healthy children. A decrease in aldosterone levels up to adult values was observed between the ages of one and three years. In comparison to the values found in healthy children, the aldosterone levels in children with heart failure appear to be generally higher than their corresponding healthy counterparts.

The number of participants per study ranged between 6 and 540 healthy children and 9 and 102 children suffering from heart failure. These studies reported data for age groups consisting of 2–314 healthy and 5–62 children suffering from heart failure. Small samples sizes under 25 children per data point have been identified as the most common. These sample sizes are conditioned by ethically funded restrictions in paediatric research, especially in young, healthy children (Caldwell et al., 2004). Thus, to allow meaningful evaluation, the researcher formed broader age groups to gain larger sample sizes. However, this approach can undermine the explanatory power of the resulting data. Furthermore, over 75% of the data found for healthy children under the age of 1 year were extracted from articles published before 1985. This further underlines the influence of the ethical considerations in paediatric research because investigations conducted in a paediatric population are not repeated. However, the research in school children and adolescents is characterised by larger sample sizes. For children with heart failure, most data points were found for children between the ages of one and six years. This may be conditioned by the time at which these children undergo surgery, which is in this age range for most paediatric patients (Chang et al., 2000).

Substantial variations of the reported aldosterone can be observed, especially for children ≤1 year-old. These variations in the reported aldosterone values can be observed within the same data set (e.g. as a high SD or a broad concentration range) and among different data sets of the same age group (e.g. sharply different mean values). The position and activity of the children during blood sampling (e.g. recumbent, screaming or crying) or the time point of the blood sampling, because aldosterone underlies a circadian rhythm (Doi et al., 2010),

can contribute to these variations and thus need to be closely monitored to establish comparable results. These variations lower the expressiveness of the data found for younger children and underline the current challenging situation of health care providers.

The trend to an age dependence can also be observed for children suffering from heart failure. Because not as many data are available for very young diseased children, the trend must be interpreted with caution. When further comparing the data sets of healthy and diseased children, the elevation of aldosterone values in the diseased population becomes apparent over the whole paediatric age range. Moreover, high variations were found in all diseased age groups, which may be due to different aetiology or severity of the heart failure or differences in the concomitant medications of the patients included in the different studies. However, the activation of the RAAS and, consequently, the release of aldosterone, is a compensation mechanism to the reduced ejection fraction of the failing heart and the corresponding lack of oxygen. Thus, it seems reasonable that heart failure leads to higher aldosterone values in children as a means to counteract the impaired cardiac function. The activation of the RAAS as a reason for higher aldosterone values in children suffering from heart failure is supported by the results found for drug introduction in this paediatric population. It has been shown in different, independent studies that the introduction of an ACE-I reduces aldosterone values (Francois et al., 2009; Stern et al., 1990). Because ACE-Is inhibit the production of angiotensin II, which leads to the release of aldosterone over the activation of the angiotensin II receptor type I, less aldosterone is released due to the down-regulation of the RAAS. A reduction of aldosterone values in children was also shown to be induced by the MRA spironolactone (Wimmer et al., 1979). The introduction of diuretics, however, seems to result in higher aldosterone levels in the paediatric population. This may be explained by an increase in extracellular potassium, which is a trigger for aldosterone production. These reactions to different drugs are in accordance with the reaction seen in adult patients.

Some limitations have to be pointed out because this review compiles many different data sets acquired over a period of 40 years. All of the data stated the assay procedure used RIA as a bioanalytical method. Nevertheless, variations

due to different vendors, sample volume used or method adaptions over time are possible. The use of plasma or serum might also lead to different resulting aldosterone values (Glinicki et al., 2015). Further, biases due to the different statistical operators given for the age and concentrations (e.g. median, mean, range) cannot be ruled out. Moreover, keeping the age dependency of aldosterone values in mind, the explanatory power of the data is possibly reduced due to the use of broad age groups. The resulting mean or median aldosterone concentration reported for a broad age range might not be able to reflect the distribution of the data and, further, lead to higher variations. This limitation mainly applies to the younger age groups in which a stronger trend to variations was observed. Most of the aldosterone values were determined from sample time points in the morning, but the influence of the time of blood withdrawal could not be studied in this data review. However, it can also be a reason for variations in the observed results. The degree to which the aldosterone values of very young children underlie this circadian rhythm is not yet known and thus cannot be estimated.

Taken together, the presented review of aldosterone values compiled for healthy children and children suffering from heart failure offers an overview on the current knowledge of the aldosterone values in healthy children as well as children suffering from heart failure and, thus, the development of the RAAS in children. Differences between age groups as well as healthy and diseased children have been identified. The influence of drug therapy on the aldosterone values of children suffering from heart failure was considered physiologically reasonable and comparable to the reaction of the adult population.

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3. GCLP-compliant quality assurance system for an aldosterone assay facilitated in a European paediatric study according to current international guidelines

3.1. Introduction

Reliability of the data collected in clinical studies is fundamental and, consequently, strictly regulated. The EMA and the US Food and Drug Agency (FDA) provide guidelines to ensure the acquisition of high-quality bioanalytical data (European Medicines Agency Committee for Medicinal Products for Human Use, 2012; U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) Center for Veterinary Medicine, 2018). These guidelines aim to assure data reliability and subsequently form, in an overarching objective, the basis for decision-making concerning safe, rational, and effective drug therapy.

In paediatrics, licensed drug therapies for very young children currently remain lacking because meaningful clinical studies are missing. The EU-funded LENA project (grant agreement n°602295) represents a rare comprehensive clinical investigation of children suffering from heart failure. It involves three linked paediatric studies investigating a novel child-appropriate drug formulation in all paediatric age groups (ClinicalTrials.gov Identifier: NCT02652728/NCT02652741/NCT02654678). Based on the small amount of applicable data regarding this vulnerable population, the primary endpoint is the evaluation of pharmacokinetics, followed by exploratory pharmacodynamic investigations of humoral parameters acting in the RAAS, which is strongly involved in adult heart failure. Paediatric clinical studies on a specific research topic are often only performed once, however, due to ethical constraints. All effort should therefore be undertaken to avoid common discontinuation in order to generate the lacking data to allow for better drug therapy (Pica and Bourgeois, 2016). Thus, new reliable data collected in accordance with state-of-the-art recommendations is key to gaining urgently needed insights into this vulnerable population.

In relation to current EMA and FDA guidance, bioanalytical validation is only a first step in the generation of reliable, high-quality data (European Medicines Agency Committee for Medicinal Products for Human Use, 2012; U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) Center for Veterinary Medicine, 2018) . Both agencies recommend monitoring each bioanalytical run using a set of calibration curve standards (CSs) and quality control samples (QCs) to assess the validity of the runs. Although recommended specifications for within-run performance can be found in the guidelines, specific limits for between-run performance and subsequent overall reliability of the bioanalytical data of clinical studies are not detailed. Additionally, the use of incurred sample reanalysis (ISR) as a means of quality assurance is strongly recommended. The implementation of ISR is a powerful tool for comparing study results measured over an extended period of time. Nevertheless, ISR remains a topic of current discussion (European Medicines Agency Committee for Medicinal Products for Human Use, 2012; U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) Center for Veterinary Medicine, 2018; Fluhler, 2018; Vazvaei, 2018) .

Recommendations for bioanalytical data acquisition outlined by the EMA focus on research in adults, however, and are not directly transferable to paediatric research. As physiological and ethical differences must be taken into account, a quality assurance (QA) approach, tailored to the particular demands of such a paediatric study population, must be implemented to facilitate high quality of the acquired bioanalytical data.

Based on the recommendations given in the guidelines of the EMA, a multi-step quality assurance approach was developed and implemented in the bioanalysis of the humoral parameter aldosterone, with the aim of guaranteeing reliable results over the whole study period of the LENA project. The quality system was based on the three mainstays of CS, QC, and ISR, but was modified to a custom-made set for the paediatric clinical studies.

3.2. Methods

Within the investigator-driven LENA project, great effort went into ensuring reliable bioanalytical data generation within the three conducted paediatric trials, with the aim of generating pharmacokinetic (primary study endpoint), pharmacodynamic, and safety data (secondary study endpoint).

Especially with the pharmacodynamic samples, on-site preparation was inevitable because the humoral parameters were characterised by rapid degradation. The clinical staff was therefore trained in a two-day, hands-on simulation training program as a customised educational approach to communication (e.g. informed consent discussion with parents), blood sampling, and sample preparation (Ciplea et al., 2018). The latter two topics were particularly crucial for assuring reliability of the acquired bioanalytical data, as the sampling regime within the LENA paediatric studies was complex due to the extensive sampling of pharmacokinetic and sensitive pharmacodynamic samples. Prior to the study start and to paediatric investigations, a feasibility study was conducted to stress the developed study concept, and confirm sample collection and on-site preparation in a reliable manner (Ciplea et al., 2018). Successfully passing the feasibility study was mandatory for each clinical site to start recruiting paediatric patients.

Similar to the steps taken to secure the training of the clinical teams, the quality of the subsequent sample analysis in the central laboratory was also secured. The collected study samples were shipped in compliance with Good Distribution Practice to the academic bioanalytical laboratory at the Institute of Clinical Pharmacy and Pharmacotherapy at the University of Duesseldorf (Germany). As a ready-to-use quality assurance system customised to paediatric research was lacking, an individual fit-for-purpose quality assurance system was developed at the bioanalytical laboratory at the Institute of Clinical Pharmacy and Pharmacotherapy, encompassing the specific demands within paediatric bioanalytics. This system was established to enable a Good Clinical Laboratory Practice-compliant determination of study samples and was affirmed through successful participation in three external ring tests and an audit.

Setting up the fit-for-purpose quality assurance system

The applied low-volume immunoassay (40-µL sample volume) for determination of aldosterone was successfully validated in accordance with EMA bioanalytical method validation guidelines (Schaefer et al., 2017a). Changes in assay kit components (e.g. new batch of antibodies) and method optimisation demanded two partial revalidations of the assay, which were successfully performed in June 2017, and between December 2017 and January 2018.

The developed fit-for-purpose quality assurance system consisted of four quality steps. First, the instrument performance of the absorbance reader (Tecan ® Infinity F50) was verified through a performance qualification (PQ) at every measurement day, as advised by the FDA guidelines (U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) Center for Veterinary Medicine, 2018). Second, freshly-prepared calibration standards were used for quantification, which were required to meet specifications given in the EMA bioanalytical guidelines. Third, all bioanalytical runs enclosed QCs of known concentration to verify reliable data generation within and between runs. If one of those aforementioned parameters did not fulfil the international bioanalytical guidelines' requirements, the analytical run was rejected and repeated. Finally, robustness regarding accuracy and precision of the aldosterone assay was monitored by ISR.

Performance qualification (PQ)

Before conducting an analytical run, the instrumental performance of the absorption multiplate reader (Tecan Infinity F50) was ensured via a customised alizarin PQ approach on each measurement day. Alizarin yellow GG solution was prepared in water and serially diluted to cover the applied range of optical density (OD). Six dilution steps reflected the absorbance range from 3.0 OD (maximum value of the device) to 0.1 OD. Five replicates of each concentration level were pipetted into a 96-well plate and absorption was determined at 450 nm. As an acceptance criterion, the variability in the OD was set to less than 10% (see Equation 3-1). Analytical or validation runs were only allowed to be conducted after a successful PQ.

$$Deviation\ PQ\ [\%] = \left(\frac{100}{determined\ OD\ of\ freshly\ prepared\ solution}\right) * determined\ OD$$

Equation 3-1. Performance qualification of precision; PQ: performance qualification; OD: optical density

Calibration curve standards (CSs)

The sole function of the calibration curve is the quantification of unknown study samples and, hence, it must be accurate and precise. Each analytical run included a fresh calibration curve encompassing six non-zero levels. Each level was measured in duplicate (12 CSs in total). The calibration curves ranged from 31.3 pg/mL to 1000 pg/mL of aldosterone. Additionally, a triplicate blank sample was included in each run.

The acceptance criteria for valid calibration curves were determined by monitoring the relative error between determined concentration and nominal concentration (see Equation 3-2). At maximum, this error could extend ±20% (±25% at the lower limit of quantification [LLOQ]). Moreover, at least one CS per concentration level (50%) and a total of at least nine CSs (75%) had to fulfil this specification to accept the calibration curve as valid.

The measured OD of calibration standards was plotted against the corresponding concentration to establish the calibration curve. For quantification, a regression model using the 4-parameter Marquardt approach without weighting was applied. A coefficient of determination (r^2) of ≥0.99 was classified appropriate for determination of unknown samples.

$$Accuracy\ [\%] = \left(\frac{measured\ concentration\ \left[\frac{pg}{mL}\right]}{nominal\ concentration\ \left[\frac{pg}{mL}\right]}\right) * 100$$

Equation 3-2. Calculation of the relative error for determination of accuracy within calibration levels.

Quality control samples (QCs)

Unlike in study samples, the concentrations of QCs are known and thus enable verification of the suitability of the calibration curve if placed into a bioanalytical run. The reliability of the data obtained per analytical run was monitored at four levels of QCs measured in duplicate. The QC levels covered the whole calibration range, with one high, two medium (high-medium and low-medium), and one low QC level, with aldosterone concentrations of 500 pg/mL, 250 pg/mL, 125 pg/mL, and 83.3 pg/mL, respectively. To evaluate the validity of the QCs, within-run accuracy and precision were assessed based on relative error and the coefficient of variation, respectively. At least one QC per concentration level (50%) was expected to lay within a ±20% range, as were the resulting mean of each concentration level and a total of at least six QCs (>67%). Calculations were conducted using Equations 3-2 and 3-3.

$$Precision\ [\%] = \left(\frac{Standard\ deviation\ of\ the\ mean\ \left[\frac{pg}{mL}\right]}{mean\ concentration\ \left[\frac{pg}{mL}\right]} \right) * 100$$

Equation 3-3. Calculation of the coefficient of variation for quality controls

The performance of QCs was also monitored based on between-run accuracy, which allows for checking assay robustness over the entire study period. As the guidelines did not provide any recommendation for the investigation of between-run accuracy in the study setting, modified Westgard rules as proposed by van Bruijnsvoort et al. were implemented. The following three rules were applied (van Bruijnsvoort et al., 2017):

1) No two consecutive results exceed the alert level (mean measured concentration ±3 times the standard deviation of measured concentrations)
2) Not more than three consecutive results exceed the warning level (mean measured concentration ±2 times the standard deviation of measured concentrations)
3) Not more than ten consecutive results fall on the same side of the mean

All four QC levels were further investigated regarding substantial changes due to revalidations that were conducted and regarding their variability across all concentration levels established.

Incurred sample reanalysis (ISR)

ISR is meant to determine the robustness of an assay based on re-measurement of study samples. In the LENA project, ISR had two purposes. Regarding assay development for paediatric application, the evaluation of a paediatric matrix on assay performance during validation was inevitable owing to ethical and technical reasons. Ethical approvals for blood withdrawal from healthy or diseased children for the validation of an assay are very restricted. Moreover, the paediatric population matures quickly and therefore the peptide and protein compositions alter among different age groups. As it cannot be ruled out that this maturation affects accurate and precise determination due to changes in protein-binding, unknown metabolites, or comedication, each single age group requires investigation. ISR is a suitable tool to assess these effects.

The performance of ISR and the extent of its usefulness in a clinical setting remain under discussion by experts. Although it is currently advised by the international regulatory authorities (e.g. EMA), its specifications are designed for pharmacokinetic studies in adults.

The EMA and FDA guidelines concerning bioanalytical method development recommend reanalysing 10% of study samples if the number of samples is less than 1000, and 5% if the number of study samples exceeds 1000. Additionally, study samples near the concentration maximum and minimum (elimination phase) are preferable, according to the recommendations (European Medicines Agency Committee for Medicinal Products for Human Use, 2012; U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) Center for Veterinary Medicine, 2018). ISR within the LENA project, however, was customised to the specific paediatric studies and is fit to evaluate pharmacodynamic parameters of interest. In contrast to the guidelines, reanalysis of 5% of actual study samples was attempted, regardless of the total number of samples. As the bioanalysis was conducted

blind, all samples were selected randomly and were not chosen for possessing concentrations at the maximum or minimum levels.

Study samples that had been previously analysed were repeatedly measured and the nominal concentration of the first valid result of a study sample (original) and the result of repeated measurement of the same study sample (repeat) were compared to ensure comparability and reproducibility through the continuous bioanalysis of aldosterone. For ISR, at least 67% of the incurred samples should be within ±30% of the mean of the original and reanalysed value, as calculated by Equation 3-4 and 3-5 (Rudzki et al., 2017; U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) Center for Veterinary Medicine, 2018).

$$Difference\ [\%] = \left(\frac{repeat\ value \left[\frac{pg}{mL}\right] - inital\ value\ \left[\frac{pg}{mL}\right]}{mean\ value\ \left[\frac{pg}{mL}\right]} \right) * 100\%$$

Equation 3-4. Deviation of a pair of samples during Incurred Sample Reanalysis

$$ISR\ [\%] = \left(\frac{Number\ of\ ISR\ pairs\ with\ |\%difference| \leq 30\%}{Total\ number\ of\ ISR\ pairs} \right) * 100\%$$

Equation 3-5. Percentage of Incurred Sample Reanalysis (ISR) pairs meeting the guideline criteria

Statistics and Software

Normal distribution was assessed using the Shapiro-Wilk test, and a paired-sample Wilcoxon signed-rank test was conducted to evaluate if the data from the original and repeated measurements differed significantly. For both tests, the level of significance was set to 0.05, and all reported p-values were corrected according to the Bonferroni correction (Ranstam, 2016). Graphical and statistical evaluation was conducted with OriginPro® 2018b (OriginPro 2018b [64-bit] b9.5.5.409 [Lehre]). Analysis of OD was conducted with the Magellan™ software Tracker V 7.0 (Tecan). The corresponding evaluated raw data were rechecked following the dual control principle. Finally, a validated in-house Microsoft® Excel

tool was used to check for standard operating procedure and guideline compliance in each bioanalytical run.

3.3. Results

Seventy bioanalytical runs were conducted between February 2016 and July 2018, using different lots of custom-made immunoassay kits over the 29-month study period. Within those runs, the aldosterone concentrations of 957 paediatric study samples were successfully determined. The developed and validated assay procedure underwent two revalidations to optimise the assay performance during its application for the analysis of the LENA study. The first revalidation was conducted in July 2017, and the second from December 2017 to January 2018. Before the first revalidation, thirteen of 40 runs were specified as invalid (33%). After the first revalidation, none of the 13 conducted runs was invalid. After the second revalidation, two of 17 runs were invalid (12%).

The CSs and QCs were used to evaluate the validity of the analytical runs. If both the calibration curve and the QCs were in accordance with the specifications given in the EMA guidelines on bioanalytical method validation, a run was defined as valid. The validity of the run was evaluated directly after its conduction and confirmed by an independent member of the bioanalytical laboratory. A summary of the course of action and the corresponding results are presented in **Figure 3-1**.

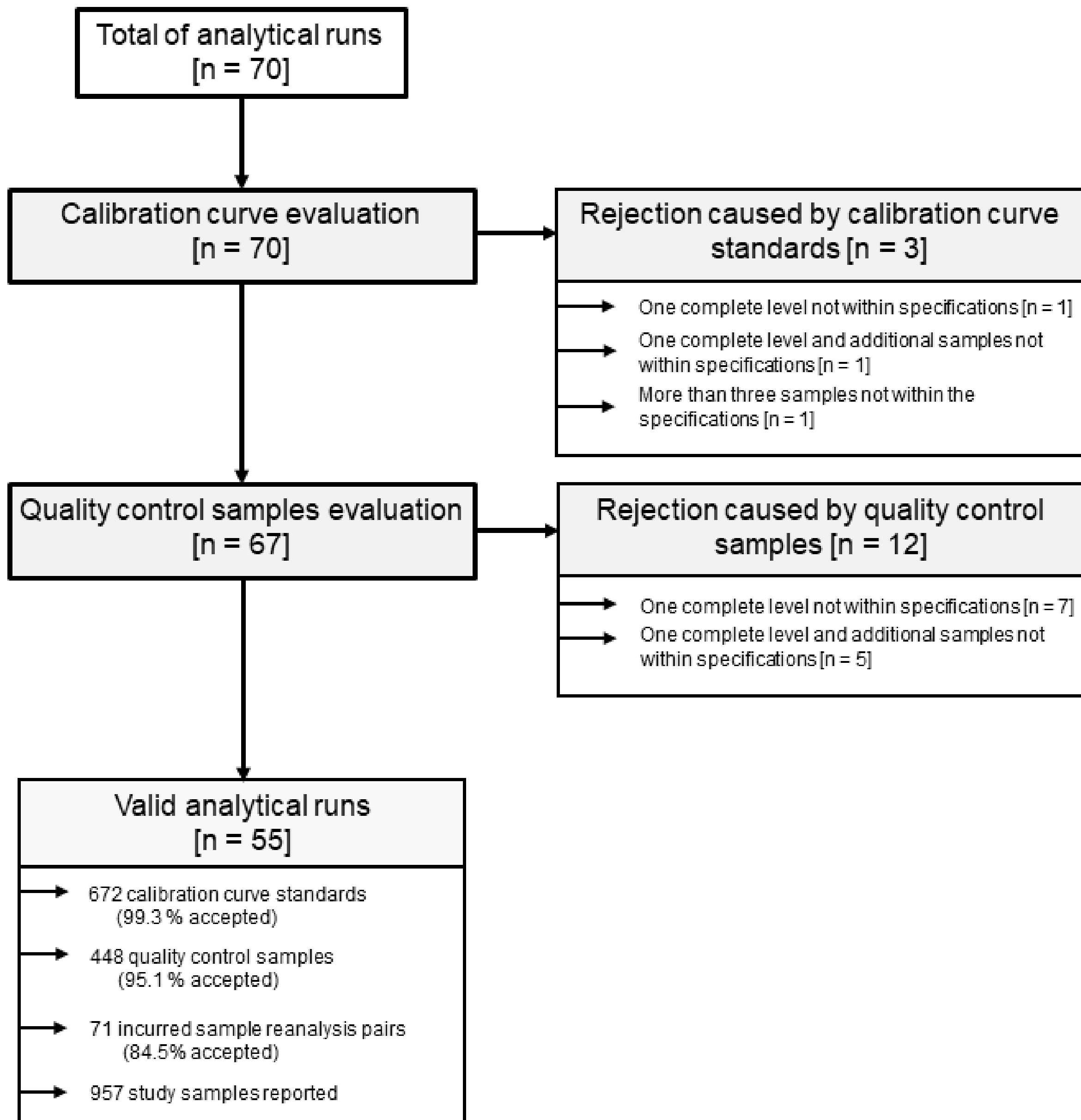

Figure 3-1. Summary of the quality assurance results for all runs conducted.

Calibration curve standards and quality control samples

Performance qualification (PQ)

The established alizarin PQ approach ensured instrument functionality prior to all aldosterone bioanalytical runs. PQ for determination of unknown aldosterone concentrations within the LENA paediatric studies was successfully conducted on each day of measurement. Performances during validation and revalidation runs were likewise ensured.

Calibration curve standards (CSs)

Of the 70 analytical runs conducted, three runs were classified as invalid due to the CSs. These were non-consecutive runs and, in each case, different calibration curve levels were rejected owing to inaccuracy regarding the specifications of the quality assurance system and hence also the international guidelines. In all valid runs, 660 CSs were measured, of which 99% were in accordance with the predefined, guideline-conforming accuracy requirements of ±20% (±25% at the LLOQ). No pattern was observed in the CSs excluded.

Quality control samples (QCs)

Following the compliance evaluation of the calibration standards, the bioanalytical runs were investigated regarding the outcome of QCs in the remaining 67 runs. Twelve of the remaining 67 runs were rejected because of QC non-compliance with the guideline specifications. In all cases, the cause of invalidity was the rejection of a complete QC level. The most often rejected QC level was the medium-high level (250 pg/mL), which was rejected in six runs. The medium-low (125 pg/mL) and low levels (83.3 pg/mL) were rejected in three and five runs, respectively. In all 55 valid runs, 95% of the 440 QCs distributed over four concentration levels were in accordance with the predefined requirements of a within-run accuracy of ±20% (see **Figure 3-2**). Thus, 22 QCs (5%) showed a relative error greater than the ±20% demanded in the EMA guidelines. These accurate and precise findings prove the suitability of the established QCs and the assay performance within EMA guideline specifications.

Retrospective investigation of the between-run accuracy showed compliance with two of the derived Westgard rules from van Bruijnsvoort (2017). In accordance with the first rule, in no concentration level did two subsequent QC values exceeded the alert limit (mean measured concentration ±3 times the SD of measured concentrations). Additionally, at no QC level did four consecutive values surpass the warning level (mean measured concentration ±2 times the SD of measured concentrations). At one of the four QC levels, however, 11 instead of the acceptable 10 values fell on one side of the overall mean. The affected QC level (250 pg/mL) determined a middle concentration level within the calibration range and therefore indicated a warning. For the other three QC levels, fewer

than eleven subsequent values fell on the same side of the mean (see **Figure 3-2**). The evaluation of between-run accuracy was conducted as a 'final' evaluation and not in subsequent steps (e.g. after the first 20+40 values and every following 40 values).

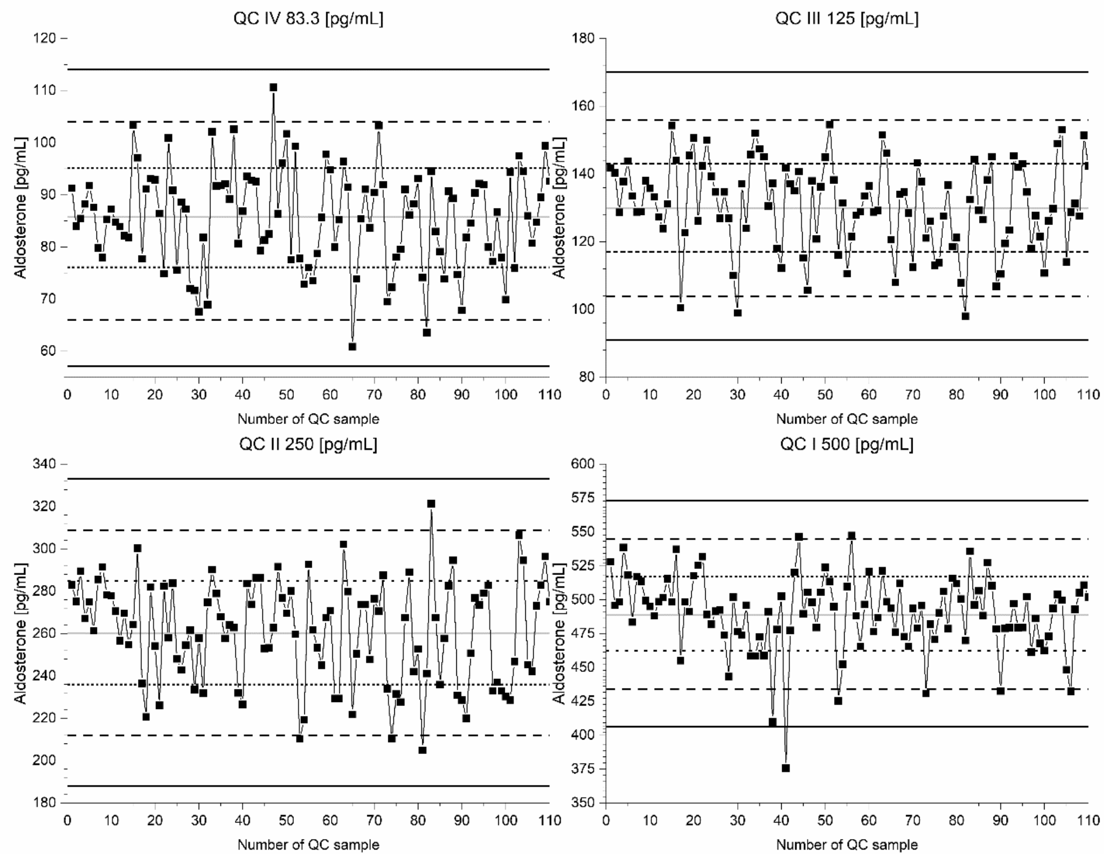

Figure 3-2. Evaluation of the consecutive quality control samples (QCs) of valid runs presented, according to the different QC concentration levels. The solid grey line indicates the overall mean of QCs of the same level. The black lines indicate control limits – mean ±1 standard deviation (black dotted line); the warning limit of mean ±2 standard deviations (black dashed line); the alert limit of mean ±3 standard deviations (solid black line).

In addition, the Shapiro-Wilk test confirmed normal distribution for the low (n = 110; p = 0.849), medium-low (n = 110; p = 0.07923), and medium-high (n = 110; p = 0.07848) QC levels. For the high QC, normal distribution of the measured concentrations was rejected (n = 110; p = 0.000747) due to the observed outliers (see **Figure 3-3**). All reported p-values were corrected according to the Bonferroni correction (Ranstam, 2016).

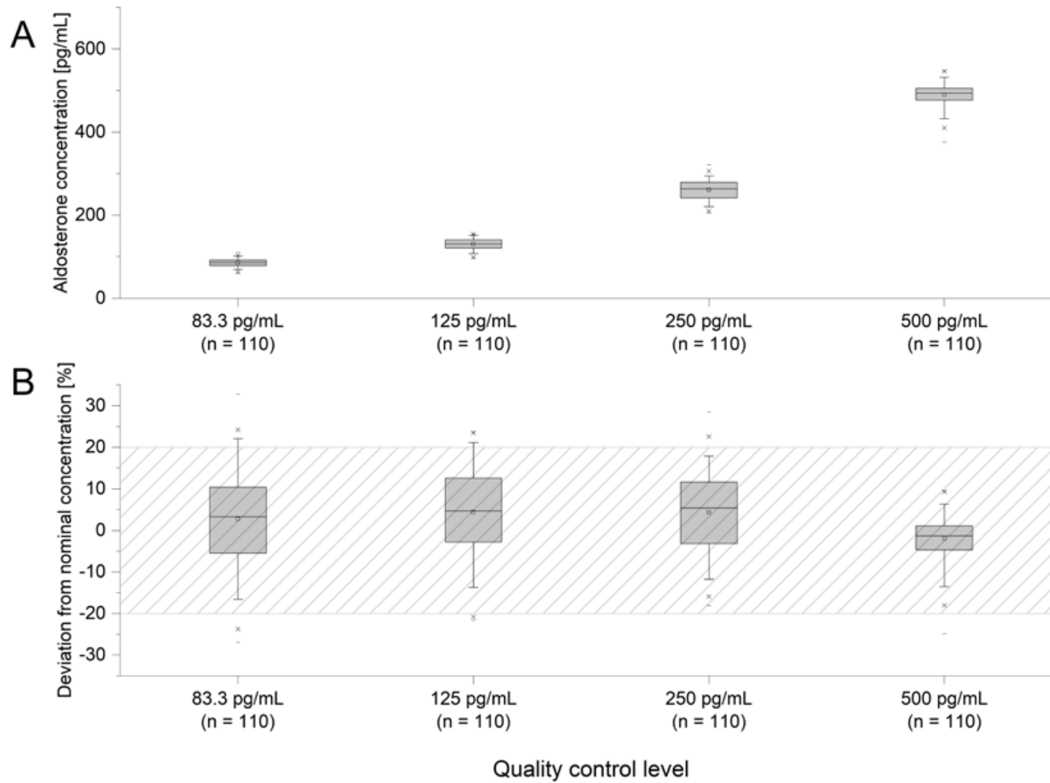

Figure 3-3. Comparison of the different quality control sample (QC) level concentrations. A: Comparison of the distributions of the values in the concentration levels. B: Deviation from nominal concentration shown for all four QC levels. According to the Mann-Whitney test, at a level of significant of 0.05 the difference between the 500 pg/mL QC level and the 83.3 pg/mL (p = 0.00093), 125 pg/mL (p = 0.00000031), and 250 pg/mL (p = 0.000000786) levels is highly significant. No significant difference was found between the other concentration levels. The grey patterned area indicates the European Medicine Agency guideline limit on the within-run accuracy of ±20%. * indicates a significant difference from 83.3/125/250 pg/mL according to the Mann-Whitney test, with a corrected p-value <0.05. ■ = 25-75%; T = 5%; ⊥ = 95%; — = Median; ▫ = Mean; x = 1%/99%; - = Minimum/Maximum value).

Incurred Sample Reanalysis (ISR)

In total, 71 ISR samples (repeats) were measured. Hence, 7.4% of the study samples were repeatedly measured.

In accordance with the EMA guidelines, a total of 60 ISR pairs (84.5%) met the specifications of a deviation of less than ±30% between the original value and the repeat. Eleven of the 71 ISRs showed a greater deviation (see **Figure 3-4**). These overall results confirm the robustness of the assay with actual study samples and confirm the reliability of the aldosterone data generated within the LENA project. A substantial impact on the paediatric matrix due to, for example, unknown metabolites, protein-binding, or comedication was not detected.

The mean difference between ISR pairs was used to investigate the influence of the different revalidations on the overall comparability of study results. Within all revalidations, the determined variability was within the predefined acceptance limits and confirmed the robustness of the assay. Moreover, a positive alteration across the different validations was visible and can be attributed to the conducted optimisations. For the first 21 ISR pairs measured after the original validation, the mean difference was 12%. This difference was reduced to 2% by the first revalidation in July 2017. After the second revalidation in December 2017/January 2018, the mean difference between the ISR original and repeat was –4% (see **Figure 3-4** and **Figure 3-5**). A high rate of 84.5% of successfully reanalysed samples using the different modified methods complied with the recommendations of the EMA guidelines (67% required; **Figure 3-4**).

Samples at the maximum and minimum concentrations could not be implemented due to the blind analysis approach. The randomised sampling, however, resulted in an ISR concentration range between 43 and 958 pg/mL aldosterone, and therefore covered the whole assay calibration range (31.3 – 1000 pg/mL). The median concentration of the mean concentration of the ISR pairs was 132 pg/mL (interquartile range: 86 – 178 pg/mL). Thus, over 85% of the ISR samples fell in the lower quartile of the calibration range. Moreover, there was no substantial difference between the ISR analysed with the originally-validated assay and the first or second revalidations. This accounts for the absolute concentrations, as well as the corresponding variability. The paired-sample Wilcoxon signed-rank

test at a level of significance of 0.05 was used to analyse the comparability over the 29-month study period. This statistical analysis of the ISR data pairs showed no significant difference between originals and repeats (n = 71; exact p = 0.84; asymptotic p = 0.84) over the entire study period (**Figure 3-5**).

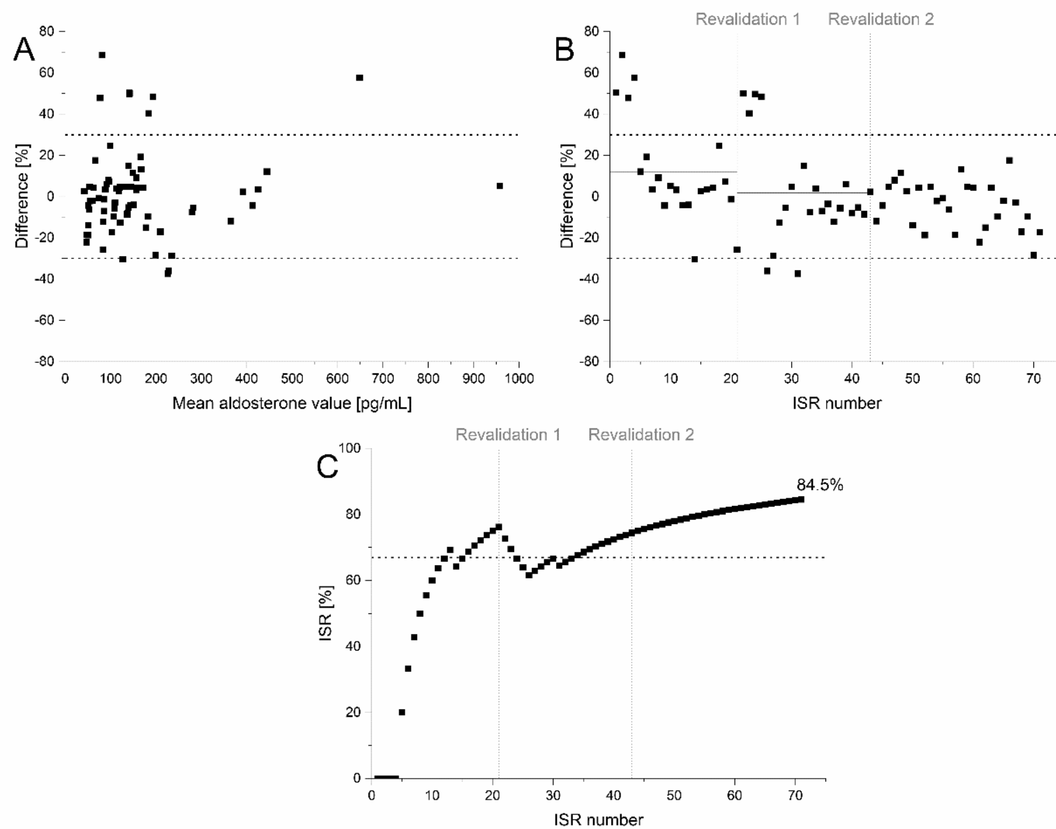

Figure 3-4. Incurred sample reanalysis (ISR) results presented as recommended by Rudzki et al. (2017). A: Difference between the ISR pairs distributed over the concentration range. The dotted lines indicate the ±30% thresholds given by the European Medicine Agency (EMA) and US Food and Drug Agency (FDA). B: The distribution of the difference between the ISR pairs over time. The solid black lines indicate the mean difference of ISR for each data set. The dotted lines indicate the thresholds given by the EMA and FDA of ±30% deviation C: Cumulative ISR samples indicating the overall ISR performance against the 67% regulatory limit (dotted line).

As different ELISA kits and modified methods have been utilised over the 29-month study period the comparability of results over this period was further investigated. The paired-sample Wilcoxon signed rank test at a level of significance of 0.05 was used to analyse the comparability. This statistical analysis of the ISR data pairs, showed no significant difference between originals

and repeats (n = 71; exact p = 0.84; asymptotic p = 0.84) over the whole study period, as shown in **Figure 3-5**.

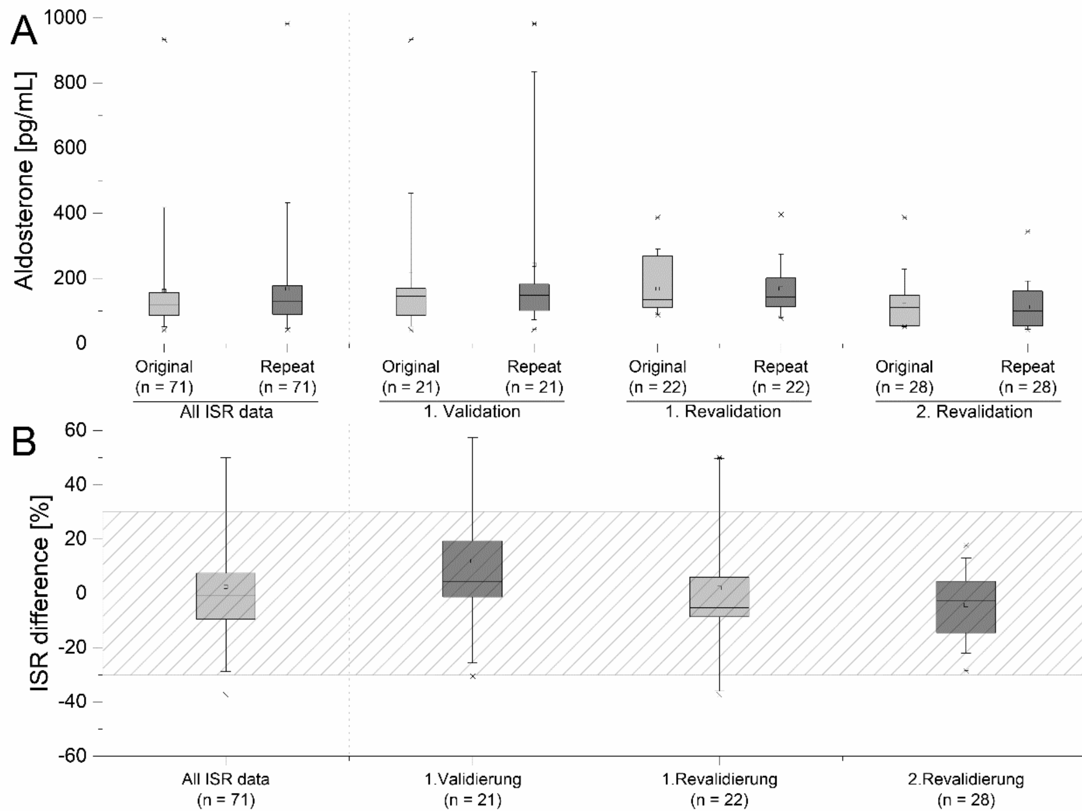

Figure 3-5. A: **Overall comparison of the original and repeatedly measured values for incurred sample reanalysis, and comparison between before and after revalidations**. For the complete ISR set (p-value = >1), as well as for the first validation (p-value = 0.167), and first (p-value = >1) and second revalidations (p-value = >1) separately, no significant differences were found between the original and repeat values using the paired-sample Wilcoxon signed-rank test at a level of significance of 0.05 and with Bonferroni correction. B: Comparison of the ISR differences among the different ISR data sets. The grey patterned area indicates the European Medicine Agency and US Food and Drug Agency guideline limit of an ISR difference of ±30%. No significant differences in the differences between ISR pairs were found among the different data sets using the Mann-Whitney test at a level of significance of 0.05 and with Bonferroni correction. ■ = Original value in pg/mL; ■ = Repeat value in pg/mL; ■ = ISR difference in %; ■ = 25 – 75%; T = 5%; ⊥ = 95%; — = Median; ▫ = Mean; x = Outlier; - = Minimum/Maximum value.

External quality assurance by participation in ring tests

For external verification of accuracy, the assay took part in three ring tests, which are the common quality assurance demanded by the German Medical Association for quality assurance of laboratory medicine (Rili-BÄK) in Germany. Samples with two unknown concentration levels (A and B sample) were successfully analysed in duplicate in 2017 and 2018. While in January 2017 198 laboratories participated in the ring test, 200 and 202 laboratories evaluated the accurate determination in July 2017 and January 2018, respectively. Mean values were reported to the Reference Institute for Bioanalytics (Bonn, Germany). In January 2017, the deviation to the official reference value varied by -13% for the A sample (154 pg/mL vs. 134 pg/mL [Reference vs bioanalytical laboratory at the Institute of Clinical Pharmacy]) and by -11.4% for the B sample (263 pg/mL vs. 223 pg/mL). In July 2017, the difference for the A sample was -2.3% (263 pg/mL vs. 257 pg/mL) and -24.8% for the B sample (480 pg/mL vs. 361 pg/mL). The A sample differed by -26.9% in January 2018 (480 pg/mL vs. 351 pg/mL), while the B sample varied by -9.1% (263 pg/mL vs. 239 pg/mL). All results were well within the specifications of the ring tests. Evaluation of reported values was conducted against an LC-MS/MS method, which served as the reference method. In direct comparison to other laboratories, the difference to the obtained median was 3.1%/-9.7% (A-/B sample) in January 2017, 0%/7.9% (A-/B sample) in July 2017 and 1.7%/0% (A-/B sample) in January 2018. These results confirm the comparability of the developed low-volume aldosterone immunoassay against commercially available kits.

3.4. Discussion

A fit-for-purpose quality assurance system taking the special demands of paediatric research into account was successfully established. This quality assurance system used multiple monitoring steps to ensure all aldosterone bioanalytical runs conducted for the pharmacodynamic parameter aldosterone in the context of the LENA project followed international guidelines and relevant recommendations. The system monitored all 70 bioanalytical runs conducted for the determination of aldosterone content and identified 15 bioanalytical runs that did not meet the customised specifications of this quality assurance system. These specifications established within the assurance system were further suitable to ensure high-quality data generation for continuous bioanalysis over the entire 29-month LENA study period. The acquired bioanalytical data for study samples can be classified as reliable and will enable valuable insight into the maturing RAAS of children suffering from heart failure.

Validation according to current bioanalytical guidelines supports the reliability of the established method. Typically, validation is only conducted once if the method is not changed intentionally. During the application of a method in a long-term clinical study like LENA, however, the performance can alter unintentionally (e.g. effects of consumables, new lots of chemicals, changing seasons, etc.) and subsequently impact the reliability and comparability of the generated data. Validation should therefore only be considered the first step to ensuring the quality of acquired data (van Bruijnsvoort et al., 2017; Kadian et al., 2016; Rozet et al., 2011) and should be accompanied by further permanent quality measures. Thus, a quality assurance system is required to systematically and objectively evaluate these permanent quality measures and thereby ensure data reliability. This system must facilitate not only the monitoring of single runs but also the observation of long-term reliability. When it comes to a continuous determination of unknown study samples over a long period of time, the latter is of particular importance. Within the LENA project, continuous bioanalysis of unknown samples was necessary due to the limited stability of aldosterone in the serum samples.

Unfortunately, no ready-to-use quality assurance system was available to address the special demands of bioanalytics within paediatric clinical studies. A

customised quality assurance system was therefore developed based on relevant advice from different sources (e.g. recommendations of regulatory authorities, guidelines, and current opinions of ongoing scientific discussion) to fit the system's purpose of ensuring high data quality in paediatric research. This system encompassed the monitoring of PQ, CSs, QCs, and ISR, and is therefore as comprehensive as required by EMA guidelines for adult research. This system based on special paediatric demands differs in its extent and specification, however, from the approaches for clinical studies in adults.

Although the specifications for CSs and QCs within one bioanalytical run are clearly outlined in the EMA and FDA guidelines and thus allow the classification of within-run accuracy and precision, no recommendations have been given for between-run performance. Between-run accuracy and precision are as important as within-run accuracy and precision, however, especially if the data are generated over a long period of time. They are a crucial tool for evaluating the comparability of all study samples, which contributes to an optimised drug therapy within all age groups of the vulnerable paediatric population.

To evaluate the long-term performance and the comparability of the results from the first up to the last paediatric sample analysed, suitable specifications derived from the Westgard rules were implemented for the evaluation of the QCs. The rules were selected based on recommendations given by van Bruijnsvoort (2017), who condensed the comprehensive Westgard rules to the most suitable ones for bioanalytical research. In contrast to van Bruijnsvoort, a final retrospective evaluation of the QC data was conducted, instead of intermediate performance analysis using decision points after the first 60 and every further 40 QC values. This evaluation showed that two out of three rules were fulfilled ('No two consecutive results exceed the alert level'; 'Not more than three consecutive results exceed the warning level'). The third rule, regarding no more than ten values falling on the same side of the mean, was not met. In one of the four QC levels, eleven values fell on one side of the overall mean. The retrospective analysis revealed that this result would not have been found in the recommended intermediate performance analysis of the data, using the decision points. The other three QC levels did not show such a distribution, and no further warning or

alert was triggered by the acquired QC data. Hence, this deviation was classified minor, with no substantial impact on the quality of the study data.

During the initial validation of the aldosterone assay, the matrix effect was investigated using the serum of healthy adult volunteers, as the use of serum from neither healthy nor diseased children was ethically justifiable. Changes in protein-binding, the impact of unknown metabolites, or concomitant medication in the actual matrix could affect the accurate determination of aldosterone concentration in the LENA study samples, however. As the paediatric organism differs strongly from the adult one and undergoes a substantial change throughout its maturation (Cock et al., 2011; Barker et al., 2018), the maturating matrix was considered an essential effect on the bioanalysis and was monitored to evaluate data comparability. ISR of actual study samples was therefore implemented into the quality assurance system. Although the low-volume study samples marked a substantial challenge for reanalysis, the comprehensive investigation of accuracy in the actual study matrix was necessary for assessing the comparability and robustness of the results. In total, it was possible to perform ISR on 7.4% of all paediatric LENA aldosterone samples. Recommendations regarding the amount of ISR samples have been a topic of debate for over a decade, and a final consistent recommendation has not yet been proposed (Vazvaei, 2018; Fluhler, 2018). Currently, the EMA and FDA recommend that ISR be performed for 10% of study samples when using a sample size of <1000 and 5% when the sample size is >1000 (European Medicines Agency Committee for Medicinal Products for Human Use, 2012; U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) Center for Veterinary Medicine, 2018). Some, however, have questioned why 5% of the sample size is a sufficient number for QCs to judge the quality, but not enough for ISR to be useful. Thus, a compromise – reanalysing 5% of the study samples with an absolute minimum of 20 study samples – is advised by Fluhler (Fluhler, 2018). Other working groups reason that the use of an absolute number of 100 study samples is cost-effective and a means of reducing inordinate efforts (Vazvaei, 2018). This approach may be applicable and beneficial in large studies conducted with adults, but is considered critical for paediatric studies. As all these considerations do not take the particular circumstances that investigators face in the performance of paediatric research into consideration,

the total number of 7.4% of samples undergoing ISR within LENA were considered appropriate.

In addition to ongoing scientific discussions concerning the appropriate amount of ISR in relationship to the total amount of study samples, the recommendations regarding the concentration of the samples that should be enclosed for ISR still do not sufficiently cover the specific situation of investigating humoral parameters like aldosterone. The FDA recommends that ISR should cover concentrations close to the maximal concentration and the elimination phase (minimum) (European Medicines Agency Committee for Medicinal Products for Human Use, 2012; U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) Center for Veterinary Medicine, 2018). The bioanalysis in this work was conducted blind, however, and all ISR samples were picked randomly. Further, the humoral parameter aldosterone is characterised by a circadian rhythm, and its impact on paediatric aldosterone values has not yet been clarified. It was therefore assumed that an appropriate number of ISR would automatically result in a comprehensive set of aldosterone concentrations covering high and low concentrations, as required by the guidelines. This assumption was supported by the retrospective analysis, indicating that ISR with aldosterone concentrations across the whole concentration range, consequently including physiological high and low values, were enclosed in the evaluation. The global bioanalysis consortium harmonisation team, however, advises the exclusion of ISR with concentrations of less than three-times the LLOQ and more than 80% of the upper limit of quantification (ULOQ) from the statistical evaluation (Fluhler et al., 2014). This recommendation has been considered, but as the low and high concentrations represented the most error-prone ranges, they were considered valuable for the evaluation of the quality of data acquisition and the robustness of the assay. Additionally, over 85% of the ISR samples fell in the lower quartile of the calibration range (less than 250 pg/mL). All randomly selected ISR data were therefore evaluated to support the comparability and robustness of the assay at the outer range of the method as well.

Finally, the EMA and FDA recommend a threshold of 67% successfully determined ISR, which can range ±30% of the original concentration(European

Medicines Agency Committee for Medicinal Products for Human Use, 2012; U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) Center for Veterinary Medicine, 2018). In the aldosterone investigation, 84.5% of the ISR showed compliance with this specification. This is especially remarkable as the whole concentration range was used for the evaluation, instead of the more robust range of three-times the LLOQ up to 80% of the ULOQ, as suggested by the global bioanalysis consortium harmonisation team (Fluhler et al., 2014).

The quality assurance system presented here, developed for the purposes of the LENA project, was successfully implemented and used as a helpful tool for the evaluation of data quality. It can be consulted as a foundation for decision-making to ensure the quality of bioanalytical data.

The newly developed and implemented quality assurance system presented here enabled seamless evaluation of the quality of data collected during bioanalysis of aldosterone in the context of the LENA project. With the implementation of this fit-for-purpose multi-step quality assurance approach, reliable acquisition of aldosterone data from children suffering from heart failure was achieved, taking into account the special demands of paediatric research. Accordance with the recommendations given by EMA guidelines reinforced the usefulness and efficacy of the implementation of such a tailored quality assurance system. The success of the presented QA system should encourage its use in other academic settings to further strengthen high-quality research based in an academic framework.

4. Development of a low-volume LC-HRMS assay for the simultaneous determination of aldosterone, its precursor and main metabolite

4.1. Introduction

As part of the RAAS, aldosterone is key to the pathology of cardiovascular and renal diseases, leading to end-organ damage and cardiovascular death (Brown, 2005). However, as much is known about the RAAS in adults, as much is still to be learned in the paediatric population. It has become evident that the paediatric organism differs strongly from the adult one. Over time, the body composition, metabolic pathways, and liver, as well as renal functions, alter (Cock et al., 2011). Because of the fundamental differences, not only in the body composition and metabolism but also in the pathophysiology of diseases, an extrapolation of adult data into the vulnerable paediatric population is not rational (Barker et al., 2018; Ginsberg, 2002). While recent research in paediatrics mainly investigates the pharmacokinetic behaviour of commonly administered drugs, their pharmacodynamic effect on the maturing organism (e.g. on humoral parameters like aldosterone) is mostly unknown. To complement the missing links between pharmacokinetic and pharmacodynamic knowledge in paediatrics, the investigation of such a biomarker in parallel to precursors and metabolites is necessary.

Concerning the biomarker aldosterone, the direct precursor and its main metabolite are 18-hydroxycorticosterone and tetrahydroaldosterone, respectively. The direct precursor, 18-hydroxycorticosterone, is converted exclusively via the 18-oxidase activity of aldosterone synthase (CYP11B2) to aldosterone (Peter et al., 1999; Bureik et al., 2002). The corresponding enzyme activity of aldosterone synthases can, therefore, be derived from aldosterone and 18-hydroxycorticosterone concentrations (Roumen et al., 2011; Hattangady et al., 2012) and subsequently facilitates the investigation of the maturation of this metabolic pathway. Elevated levels of aldosterone and 18-hydroxycorticosterone in children suggest that the RAAS is considerably different to adults (Fiselier et al., 1984; Travers et al., 2018a; Riepe et al., 2003), which highlights the need for comprehensive investigation of the paediatric RAAS and its internal pathways.

Moreover, data in adults show that tetrahydroaldosterone is mainly renally eliminated after glucuronidation (Abdelhamid, 2003). In children, however, the impact of glucuronidation differs from that in the adult organism and matures over time. In the first year of life, the primary metabolic pathway is sulfation while the glucuronidation and the CYP metabolism underlie maturation (Cock et al., 2011).Together, sophisticated investigations in the paediatric population allow for further insights into the ontogeny of the not fully matured paediatric (renal) metabolism and the aldosterone metabolism.

For investigations of aldosterone in the diseased paediatric population, LC-MS/MS methods with small sample volumes are necessary. Currently, LC-MS/MS methods for steroid determination including aldosterone require 150 to 1200 µL of the sample (Häkkinen et al., 2018; Mayo Clinic Laboratories). These volumes equate to about 12.5 to 100% of the blood volume drawable from a neonate of 3 kg at one sample time point (Committee for Medicinal Products for Human Use, Ad-hoc group for the development of implementing guidelines for Directive 2001/20/EC, 2008). These sample volumes are neither acceptable when multiple sampling time points are needed, for the determination of various parameters, nor the application in very small and severely diseased children.

The LENA aims to investigate pharmacokinetics and pharmacodynamics in children suffering from heart failure. Over 70% of the study population is below one year of age and severely diseased. For a comprehensive investigation of this population in parallel to routine care, the blood volume for the determination of RAAS parameters (aldosterone, angiotensin I, plasma-renin activity, renin) is strongly limited. A low-volume liquid LC–HRMS assay for the simultaneous determination of aldosterone, 18-hydroxycorticosterone, and tetrahydroaldosterone was developed to gain valuable new insights into the maturing of the RAAS of the LENA population and subsequently aldosterone in cardiovascular-diseased children.

4.2. Methods

Chemicals and consumables

Certified reference solutions of aldosterone (97.9% purity, LC-MS) and 18-hydroxycorticosterone (98.0% purity, LC-MS) in primary measurement standard quality were obtained from Cerilliant (Darmstadt, Germany). d4-Aldosterone (98% purity, HPLC), 3α,5β-tetrahydroaldosterone (98.6% purity, HPLC), and dextran-coated charcoal (64–76 kDa) were purchased from Sigma Aldrich (Darmstadt, Germany). Water CHROMASOLV™ LC-MS and methanol CHROMASOLV® for HPLC (≥99.9%) were supplied by Honeywell (Seelze, Germany). Methanol (Optima® LC-MS), water (HPLC gradient grade), and ammonium acetate (analytical reagent grade) were provided by Fisher Scientific (Loughborough, UK). Formic acid (98% p.a.) was obtained from AppliChem (Darmstadt, Germany). For the sample clean-up, Oasis® PRiME HLB 96-well plates (10 mg sorbent per well) by Waters (Eschborn, Germany) were used. A CORTECS® C18+ (2.1 mm × 150 mm, 2.7 µm) analytical column connected to a corresponding CORTECS® C18+ VanGuard® guard column (2.1 mm × 5 mm, 2.7 µm) by Waters (Eschborn, Germany) was facilitated.

Serum was donated by healthy volunteers. The approval of the ethics committee of the University of Duesseldorf was granted in October 2017 (study number: 6099). All participants gave their written informed consent.

Stock and standard solutions

Stock solutions of aldosterone, 18-hydroxycorticosterone, and tetrahydroaldosterone were prepared in methanol and diluted with methanol/water (10/90 [v/v]) to generate an analyte working solution (1 µg/mL). The internal standard (IS) d4-aldosterone was separately diluted with methanol to a 100 ng/mL IS-working solution.

Generation of blank serum

A purified blank standard based on human blood, which is usually supplied in a commercial aldosterone immunoassay kit (Aldosterone ELISA, DRG Instruments GmbH, Marburg, Germany), was evaluated for its applicability during method development. The residual amount of aldosterone was too high to enable a reasonable LLOQ for a high sensitivity analytical method.

Alternative approaches like bovine serum, lyophilisates of human serum, buffer, or serum from steroid-depleted patients have been evaluated. Because the matrix used for calibration curves and QCs (quality control samples) should be as comparable to the study matrix as possible, artificial matrices like bovine serum or buffer solutions were considered inappropriate. The use of serum from steroid-depleted patients (e.g. after a bilateral adrenalectomy) could have been seen as an alternative, but as these are very particular patients, they would not be approachable in many research settings. From an ethical point of view, this approach was also considered awkward because the patients would have had to donate blood after surgery and before any interfering medication (e.g. MRAs) was introduced (Vilela and Almeida, 2017; Galati, 2015; Espiner et al., 2003). Thus, this already limited specimen would have been rather hard to collect, especially if one does not work in a hospital setting. Human serum lyophilisates were also not considered suitable because it often remains unclear how the lyophilisates may have been purified so that the comparability to the sample matrix was not assessable (DRG Instruments GmbH; IBL International GmbH 2015). Further, it could not be guaranteed that the residual steroid concentrations were acceptable for the demands of a highly sensitive analytical method and constant over different batches, similar to the already evaluated blank standard by DRG. Hence, to be able to use a blank matrix as similar to the investigated matrix as possible, still allowing the investigation with highly sensitive bioanalytical methods and offering the chance of implementation in many research settings, steroid-stripped human serum was considered as the most suitable approach. Thus, an in-house protocol for the cost-effective and easy-to-implement preparation of a suitable human blank matrix by steroid stripping was established.

To deplete the endogenous analytes of interest in native human serum, and thus create an analyte-free blank serum, dextran-coated charcoal was used to strip

the hormones from the native serum. Adapted from Green and Leake, an in-house stripping protocol, consisting of three consecutive stripping steps of 24 h, was developed (Green and Leake, 1987). In each stripping step, the native human serum was added to dextran-coated charcoal (200 mg dextran-coated charcoal per 10 mL native human serum) in a 50 mL falcon tube. The serum mix was kept at 4 °C and under constant movement at 5 rpm in an Intelli-Mixer by ELMI Ltd. (Riga, Latvia). At the end of each step, the serum mix was centrifuged at 3220 *g* for 15 min at 4 °C. The supernatant was transferred to the next stripping step. Finally, the supernatant was filtered by a combination of round filter plus syringe filter (0.2 µm) to obtain the blank serum.

Calibration curve and quality control samples

The calibration curve and QCs were freshly prepared by spiking blank serum with the analyte working solution (1 µg/mL).

The 10 ng/mL steroid mix in serum was serially diluted in nine steps of 1:2 dilutions down to a concentration of 19.53 pg/mL to set up the calibration curve. According to preliminary data, different calibration ranges were chosen. For aldosterone, the calibration range was 19.53 to 2500 pg/mL, which was achieved with eight calibration levels. For 18-hydroxycorticosterone, one less calibration level was utilised to cover its calibration range of 39.06 to 2500 pg/mL. The calibration range of 78.13 to 2500 pg/mL of tetrahydroaldosterone was maintained with six calibration levels.

The QCs were prepared independently by serially diluting the 10 ng/mL steroid mix in serum to a concentration of 39.06 pg/mL. Four concentration levels (39.06 pg/mL, 156. 25 pg/mL, 312.5 pg/mL and 2500 pg/mL) were used as QCs for aldosterone and 18-hydroxycorticosterone. For tetrahydroaldosterone, three concentration levels (156.25 pg/mL, 312.5 pg/mL and 2500 pg/mL) were assessed.

Sample preparation

Sample processing was done with an individualised solid phase extraction protocol. First, 50 µL of the serum samples were added to 500 µL methanol/water (10/70 [v/v]) and 1% formic acid, containing the IS. This sample mix was vortexed for 90 s at 600 rpm and directly transferred onto an Oasis® PRiME HLB 96-well plate (10 mg). After 5 min of rest at atmospheric pressure, positive pressure (1 psi) was applied carefully to the sample with a Waters Positive Pressure-96 Processor. Positive pressure was maintained throughout the SPE process. Next, a first washing step, consisting of 500 µL methanol/water (30/70 [v/v]) and 1% formic acid, followed by 500 µL pure methanol/water (30/70 [v/v]) was conducted. Finally, the elution was performed in two steps, both consisting of 250 µL methanol/water (60/40 [v/v]). The evaporation to dryness was conducted under nitrogen in a Thermomixer by Eppendorf (Hamburg, Germany) at 50 °C and 550 rpm. The samples were reconstituted in 50 µL methanol/water (10/90 [v/v]).

LC-HRMS equipment and conditions

The measurement was conducted using a Shimadzu (Duisburg, Germany) Nexera HPLC (High-performance liquid chromatography) system including two LC-20AD XR pumps, a SIL-30AC autosampler, CTO-20AC column oven, and a CBM-20A communication bus module.

The column oven was maintained at 50 °C and the autosampler at 15 °C. The injection volume was 40 µL. Separation was achieved on a Waters CORTECS® C18+ (2.1 mm × 150 mm, 2.7 µm) analytical column connected to a corresponding CORTECS® C18+ VanGuard® guard column (2.1 mm × 5 mm, 2.7 µm). The mobile phase consisted of 2 mM ammonium acetate in water (solvent A) and 2 mM ammonium acetate in methanol (solvent B), respectively. Gradient conditions were applied (see **Table 4-1**), with a run time of 8 min plus one min equilibration, at a constant flow rate of 0.6 mL/min.

Table 4-1. High-performance liquid chromatography gradient

Time [Minutes]	B Concentration [%]
0.00	10
0.50	10
1.00	33
2.50	38
2.75	45
3.50	45
3.75	50
4.25	50
4.30	55
4.60	55
6.00	98
7.50	10
8.00	Stop

The HPLC system was coupled to an AB Sciex TripleTOF 6600 mass spectrometer (Concord, Canada). Detection was performed in negative ion mode using electrospray ionisation with a Turbo V® source. Compressed air was used as nebuliser (gas 1), heater (gas 2), and collision gas. Nitrogen was used as the

curtain gas. The following settings were applied: Gas 1 and 2 each 50 psi, curtain gas 35 psi, source temperature 550 °C, and Ion Spray Voltage Floating –4500 V. For each run, four product ion scans in high sensitivity mode with enhancement of the different fragments and one TOF–MS scan were conducted. The individualised settings for each experiment can be found in **Table 4-2**. Data acquisition and processing were performed using Analyst® (Version TF 1.7.1) and MultiQuant® (Version 3.0.2).

Table 4-2. MS experiments and corresponding parameters

	Aldosterone	18-Hydroxycorticosterone	Tetrahydroaldosterone	d4-Aldosterone (IS)	TOF MS Scan
Precursor mass [m/z]	359.21	361.21	363.22	363.23	n.a.
Product Ion [m/z]	189.092 Enhanced	331.200 Enhanced	193.140 Enhanced	190.110 Enhanced	n.a.
Declustering Potential [V]	-110	-111	-96	-90	-75
Collision Energy [V]	-24 .5	-29	-24	-20	-10
Collision energy spread [V]	0	0	0	0	n.a.
Ion Release Delay [ms]	47	63	48	48	n.a.
Ion Release Width [ms]	17	23	17	17	n.a.

Method validation

Aldosterone validation was carried out according to the current EMA and FDA guidelines on bioanalytical method validation (European Medicines Agency Committee for Medicinal Products for Human Use, 2012; U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) Center for Veterinary Medicine, 2018). The precursor and metabolite validation were conducted for semi-quantitative analysis. The guideline parameters assessed were carry-over, the LLOQ, linearity, accuracy, precision, matrix effect, recovery, and stability.

Linearity

Linearity was required to be confirmed in three runs. It was assessed utilizing eight calibration curve levels of aldosterone, seven levels of 18-hydroxycorticosterone, and six levels of tetrahydroaldosterone. The calibration curves were measured in duplicate. A calibration curve standard had to be rejected if its deviation from the nominal concentration was greater than ±15% (±20% at the LLOQ). 75% of each calibration curve standards or at least six concentration levels of the calibration curve had to fulfil these criteria.

Accuracy, precision, and total error

Within-run and between-run accuracy and precision were investigated in at least three runs, conducted on different days. Each run consisted of a calibration curve, measured in duplicate, against which five replicates of QCs with known concentrations (19.53 pg/mL, 39.06 pg/mL, 156.25 pg/mL, and 2500 pg/mL) were measured.

The accuracy was assessed by the deviation of the mean of five replicates of a quality control level from its expected nominal concentration. The mean accuracy was allowed to vary by ±15% (±20% at the LLOQ). The same specification applied to the between-run accuracy.

The within- and between-run precision was evaluated by the mean coefficient of variation (CV) of the QCs. It was allowed to vary by ±15% (±20% at the LLOQ). These specifications also applied for between-run precision.

The total error was calculated from the mean (n = 5) deviation of nominal concentration and CV values according to Equation 4-1.

$$Total\ error\ [\%] = |Deviation\ from\ nominal\ concentration\ [\%]| + |Coefficient\ of\ variation\ [\%]|$$

Equation 4-1. Total error

The lower limit of quantification

To ensure the robustness of the determination at low concentration samples, accuracy and precision at the LLOQ (n = 5) of aldosterone (19.53 pg/mL) and 18-hydroxycorticosterone (39.06 pg/mL) were investigated in three runs conducted on three different days, and in one separate run for tetrahydroaldosterone (78.13 pg/mL). To confirm the accuracy and precision of the LLOQ, the mean deviation from nominal concentration and its CV had to be within a range of ±20%.

The signal-to-noise ratio (S/N) was assessed by comparing the mean intensity of the signal of the blank samples (at least n = 2) to the mean intensity of the signal at the LLOQ (n = 5) at the retention time of the corresponding analyte (see Equation 4-2). Acceptance value of the S/N was ≥5:1. The points across the baseline were taken into account for the analyte peaks and should range between 10 and 20 (Kromidas, 1997) to ensure the ability of the analytical method to reflect the analyte concentrations robustly.

$$\text{Signal} - \text{to} - \text{noise ratio} = \frac{\text{Mean intensity at LLOQ [cps]}}{\text{Mean intensity of the blank sample [cps]}}$$

Equation 4-2. Signal-to-noise ratio

Carry-over

The concentrations of aldosterone, 18-hydroxycorticosterone, and tetrahydroaldosterone, and IS were determined in three runs in the blank sample measured after a fivefold injection of the 2500 pg/mL high calibration standard. The residual analyte that was found in the blank had to be less than 20% of the analyte concentration of the LLOQ and less than 5% of the IS.

Matrix effect and recovery

The matrix effect was determined to ensure that no alteration of the results was caused by interfering components in the sample. The serum of six different human sources was extracted and then postspiked with a high (1250 pg/mL), a mid (156.25 pg/mL), and a low (39.06 pg/mL) concentration of aldosterone, 18-hydroxycorticosterone, and tetrahydroaldosterone. Blank correction was conducted for the postspiked values based on the mean concentration of the analyte in the native serum (n = 5). The matrix effect and IS-normalized matrix effect were calculated according to Equation 4-3 and Equation 4-4 for all concentration levels from the blank corrected postspiked samples (n = 5). The CV of the IS-normalised matrix factor calculated from the six sources of matrix had to be less than 15%.

$$Matrix\ factor\ [\%] = \left(\left(\frac{peak\ area\ in\ presence\ of\ the\ matrix\ [cps]}{peak\ area\ in\ absence\ of\ the\ matrix\ [cps]}\right) \times 100\right)$$

Equation 4-3. Matrix factor

$$\text{IS-}normalised\ matrix\ factor\ [\%] = \left(\left(\frac{matrix\ factor\ of\ the\ analyte}{matrix\ factor\ of\ the\ IS}\right) \times 100\right) - 100$$

Equation 4-4. IS-normalised matrix factor

The recovery of the analytes was determined in six sources to analyse the loss of analyte during the preparation and SPE. For the determination of the recovery, additional to the postspiked samples, the serum of the six sources was prespiked with a high (1250 pg/mL), a mid (156.25 pg/mL), and a low (39.06 pg/mL) concentration of aldosterone, 18-hydroxycorticosterone, and tetrahydroaldosterone and then extracted via SPE (n = 5). The recovery was calculated based on the mean concentration of the analytes (n = 5) found in the native serum of the corresponding source (see Equation 4-5), after blank correction of the pre- and postspiked values. The mean recovery was regarded as acceptable if at least 50% of the analytes were recovered.

$$Recovery\ [\%] = \left(\frac{Prespiked\ concentration\ \left[\frac{pg}{mL}\right]}{Postspiked\ concentration\left[\frac{pg}{mL}\right]}\right) \times 100$$

Equation 4-5. Recovery

Stability

The autosampler stability was determined to ensure the stability of the analytes in the autosampler throughout the analysis. Therefore, a 16.5-h break was included between the measurement of the calibration curve and QCs (n = 5). The calibration curve and QCs were kept in the autosampler at 15 °C for the whole experiment, while the calibration curve was measured directly after sample processing. The QCs were determined after a break of 16.5 h. The mean accuracy and mean precision, calculated as the deviation from the nominal concentration and the CV, respectively, were allowed to vary by ±15% (±20% at the LLOQ) to confirm the stability of the analytes.

4.3. Results

Linearity

Calibration curves were constructed by plotting the mean of the IS-normalised areas against the IS-normalised analyte concentrations. A calibration range of 19.53 to 2500 pg/mL was successfully established for aldosterone. Ranges of 39.06 to 2500 pg/mL and 78.13 to 2500 pg/mL were determined for 18-hydroxycorticosterone and tetrahydroaldosterone, respectively. For each analyte, more than 75% of the calibration standards at six or more concentration levels showed less than 15% (20% at the LLOQ) deviation from the nominal concentration. Linearity was demonstrated with a coefficient of determination ≥0.995 for all analytes of interest (n = 3). The best fit of the linear regression was achieved with $1/x^2$ weighting. Example chromatograms of each analyte for the ULOQ, the LLOQ, and the blank are presented in **Figure 4-1**. In the example chromatogram, the peak widths at half height at the LLOQ were 0.04 min for aldosterone, 0.03 min for 18-hydroxycorticosterone, and 0.04 min for tetrahydroaldosterone. At the ULOQ, peak widths at half height were 0.05 min for all three analytes. These peak widths at half height were in accordance with the overall results observed for all peaks acquired in the accuracy and precision runs (n = 525) with a median of 0.05 min and an interquartile range of 0.04 to 0.06 min. These peak widths and variations were considered acceptable.

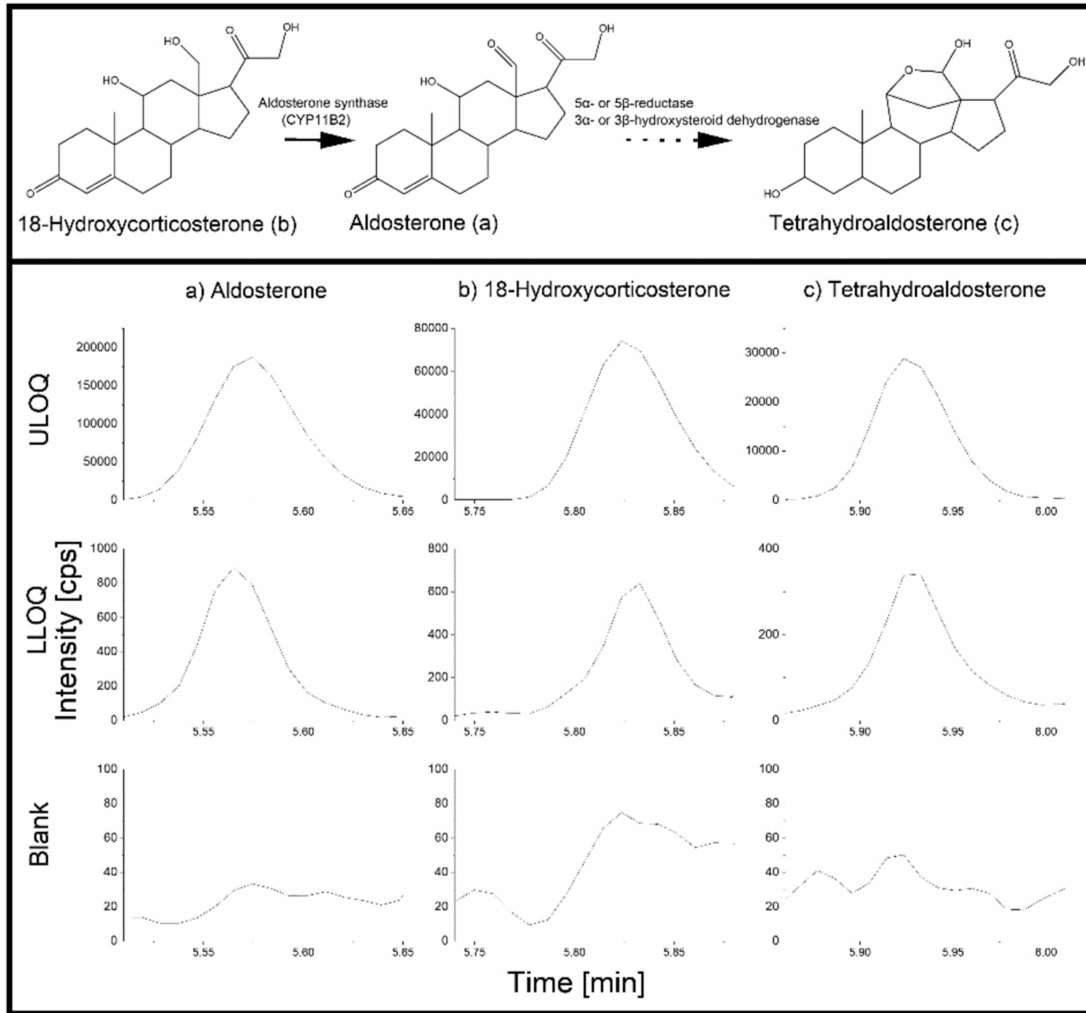

Figure 4-1 The direct conversion of 18-hydroxycorticosterone to aldosterone and the subsequent multistep (indicated with the dotted arrow) **formation of tetrahydroaldosterone and the detailed example chromatograms** for the upper limit of quantification (ULOQ), the lower limit of quantification (LLOQ), and the blank for aldosterone (ULOQ: 2500 pg/mL; LLOQ: 19.53 pg/mL), 18-hydroxycorticosterone (ULOQ: 2500 pg/mL; LLOQ: 39.06 pg/mL), and tetrahydroaldosterone (ULOQ: 2500 pg/mL; LLOQ: 78.13 pg/mL) (Hattangady et al., 2012; Roumen et al., 2011).

Accuracy, precision, and total error

The values obtained in human steroid-depleted serum for the within-run accuracy, precision, and total error (n = 5) runs were evaluated by four QC levels for aldosterone, three for 18-hydroxycorticosterone, and two for tetrahydroaldosterone.

The within-run accuracies observed were in accordance with the EMA and FDA guideline recommendations because the mean deviation from the nominal concentration of aldosterone, 18-hydroxycorticosterone, and

tetrahydroaldosterone ranged between 6.30 and 12.69%, 2.02 and –1.99%, and 0.68 and 8.91%, respectively. Results for the between-run accuracy (n = 3) were acquired in three runs conducted on three non-consecutive days. For aldosterone and tetrahydroaldosterone, the results were also in accordance with the EMA and FDA guidelines. The mean deviation from nominal concentration ranged between –1.21 and –6.99%, and 2.99 and –3.43% for aldosterone and tetrahydroaldosterone, respectively (European Medicines Agency Committee for Medicinal Products for Human Use, 2012; U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) Center for Veterinary Medicine, 2018). For 18-hydroxycorticosterone, a range of 4.66 to –21.99% in the between-run accuracy was found.

The within-run precision showed guideline-conforming variations between 2.05 and 11.20% for aldosterone and variations between 3.94 and 17.14% and 7.74 and 23.09% for 18-hydroxycorticosterone and tetrahydroaldosterone, respectively. Values obtained for the between-run precision (n = 3) of aldosterone, 18-hydroxycorticosterone, and tetrahydroaldosterone were 2.07 and –10.22%, 7.66 and 13.38%, and 2.23 and 7.49%, which complied with the guideline limits of EMA and FDA as well .

The values calculated for the within-run total error of aldosterone, 18-hydroxycorticosterone, and tetrahydroaldosterone varied between 6.48 and 20.16% for aldosterone, between 9.84 and 35.37% for 18-hydroxycorticosterone, and between 9.79 and 24.35% for tetrahydroaldosterone. Total errors between three runs ranged between 5.34 and 11.43%, 12.32 and 35.37%, and 5.66 and 10.48% for aldosterone, 18-hydroxycorticosterone, and tetrahydroaldosterone, respectively. In **Figure 4-2**, results for the between-run accuracy, precision, and total error for all three analytes are shown in detail.

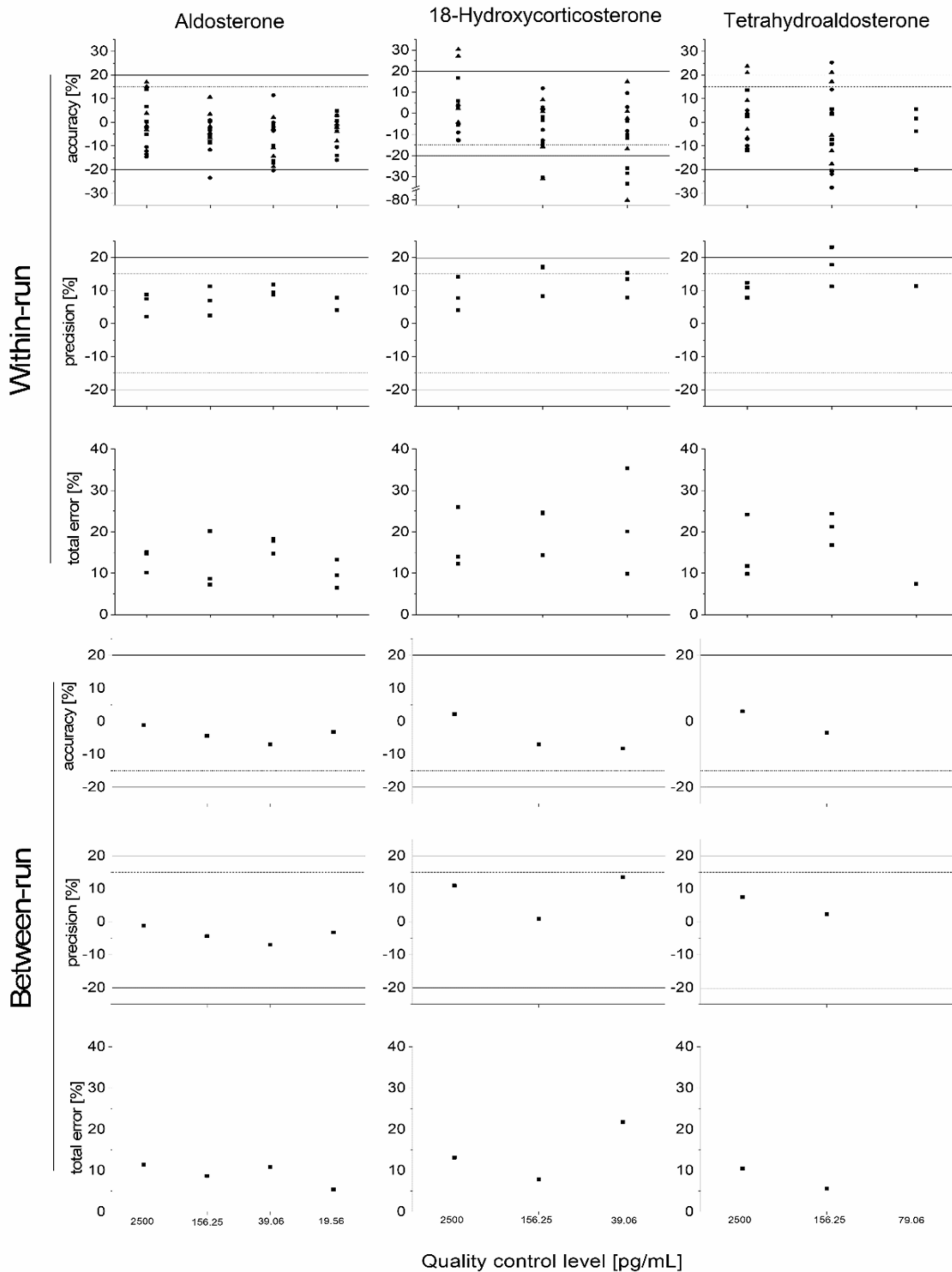

Figure 4-2. Within-run and between-run accuracy, precision and total error results. Presented are the within-run accuracy results for run 1 (■) run 2 (●) run 3 (▲), the mean (n = 5) within-run precision, total error results, and between-run (n = 3) accuracy, precision, and total error for aldosterone, 18-hydroxycorticosterone, and tetrahydroaldosterone. The maximum variation of ±15% is indicated by the dotted line applying for all quality controls except the lower limit of quantification, which is allowed to vary by 20% (solid line), according to the EMA and FDA guidelines. Run 1: ■; run 2: ●; run 3: ▲.

<u>The lower limit of quantification</u>

At the LLOQ of aldosterone, investigated in three runs, values ranging between –5.56 and –1.88% for the deviation from nominal concentration, 4.11 and 7.63% for the CV, and 6.48 and 13.30% for the total error were observed. At the LLOQ of tetrahydroaldosterone, investigated in one run, the deviation from nominal concentration was 7.36%, the CV was 11.23%, and a total error of 18.59% has been observed. These results of the investigation at the LLOQ of aldosterone and tetrahydroaldosterone varied in a ≤20% range and thus complied with the guideline specifications recommended by the EMA (European Medicines Agency Committee for Medicinal Products for Human Use, 2012). For 18-hydroxycorticosterone, ranges of 2.02 to –21.99%, 7.82 to 15.23%, and 9.84 to 35.37% were obtained in three runs for the deviation from the nominal concentration, the CV, and total error, respectively. The results for the accuracy and precision at the LLOQ of aldosterone and 18-hydroxycorticosterone are displayed in **Figure 4-2**.

The S/N was calculated as the ratio of the mean intensity (n = 5) of the signal at the LLOQ and the mean intensity of the blank and zero at the same retention time as the corresponding analyte. For aldosterone, 18-hydroxycorticosterone, and tetrahydroaldosterone, the S/Ns at the LLOQ were ≥8:1, ≥13:1, and ≥27:1 times the intensity of the blank samples, respectively. Hence, the results complied with the guideline specifications of the EMA and FDA on bioanalytical method development (European Medicines Agency Committee for Medicinal Products for Human Use, 2012; U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) Center for Veterinary Medicine, 2018), which demanded S/N ≥ 5:1 at the LLOQ. Available data points across the baseline were in compliance with the given recommendations of 10–20 points per peak. For aldosterone, 18-hydroxycorticosterone, and tetrahydroaldosterone, the median of points across the baseline at the LLOQ were 17, 13, and 14, respectively.

<u>Carry-over</u>

No peak for aldosterone, 18-hydroxycorticosterone, tetrahydroaldosterone, or d4-aldosterone was found in the analyte-free solvent sample after five consecutive

injections of the 2500 pg/mL high standard. Thus, no carry-over was present. The blank after five consecutive injections of the standard IS concentration of 500 pg/mL likewise revealed no peak.

Matrix effect and recovery

The matrix effect and recovery were successfully evaluated in six different adult sources, of which four were female, and two were male. High (1250 pg/mL), mid (156.25 pg/mL), and low (39.06 pg/mL) spiked samples were utilised.

The CVs for the IS-normalised matrix effect for aldosterone for the high, mid, and low concentrations were 10.44%, 5.25%, and 9.29%, respectively. For 18-hydroxycorticosterone, the CVs obtained for the high-, mid-, and low-spiked samples were 28.22%,14.28%, and 11.29%, respectively. CV values of 5.48% and 5.26% were obtained for the high- and the mid-spiked concentration of tetrahydroaldosterone. All of these results complied with the recommendations given in the EMA guideline, except for the CV observed at the low concentration of 18-hydroxycorticosterone (European Medicines Agency Committee for Medicinal Products for Human Use, 2012). Detailed results are presented in **Figure 4-3**.

The mean recovery of analyte achieved in all six sources in the recovery investigation for aldosterone at the low, mid, and high concentrations were 68%, 63%, and 62%, respectively. For 18-hydroxycorticosterone, mean recoveries of 76%, 66%, and 62% were observed. For the mid- and high-spiked concentrations investigated for tetrahydroaldosterone, mean recoveries of 62%, and 66% were found, respectively. The results were in accordance with the specification. The overall CV calculated with the mean recoveries of the concentration levels and analytes is 7.42%, which underlined the consistency of the results. Details are shown in **Figure 4-3**.

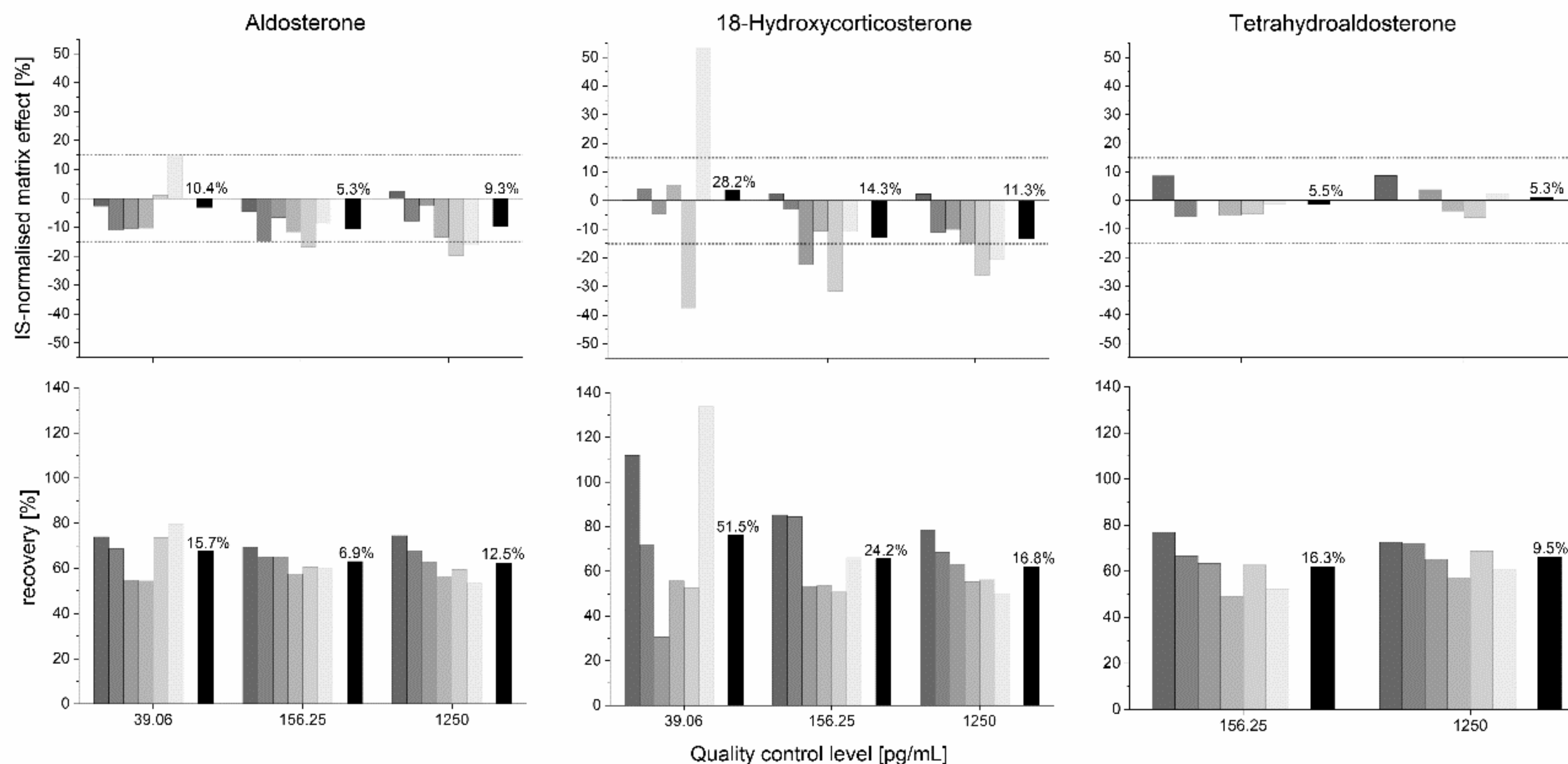

Figure 4-3. Internal standard (IS)-normalised matrix effect and recovery results for aldosterone, 18-hydroxycorticosterone, and tetrahydroaldosterone. The different patterns represent the six different sources each measured in a fivefold approach (■ Source 1, ■ source 2; ■ source 3 ■ source 4 ■ source 5; ■ source 6). The solid black bar(■) gives the mean value over all sources. Given above the mean column is the coefficient of variation for the six different sources per concentration. The maximum variation of ±15% advised by the EMA guideline is indicated by the dotted line applying for the mean IS-normalised matrix effect.

Stability

The autosampler stability was successfully established for 16.5 h for aldosterone. All obtained values were within the limits of ±15% (±20% at LLOQ). Thus, degradation of the analyte was not present. Long-term storage data at –80 °C was derived from previous investigations by Schaefer et al. (2017a).

4.4. Discussion

With a child-appropriate sample volume of 50 µL serum, aldosterone, its direct precursor 18-hydroxycorticosterone, and its main metabolite tetrahydroaldosterone can be determined simultaneously with the established LC–HRMS method. The method allows for the reliable determination of aldosterone according to the EMA and FDA guidelines on bioanalytical method validation, and it enables, applied in the LENA project, data generation in a paediatric population suffering from heart failure. Further, the determination of 18-hydroxycorticosterone and tetrahydroaldosterone provides the possibility to gain new knowledge about the paediatric metabolic system and its maturation in newborns up to adolescents.

As an effector of the RAAS, aldosterone can provide information on the activation status of this important humoral system. In combination with other parameters like renin, angiotensin, and plasma renin activity, it can also give insight on feedback mechanism and functionality of the whole RAAS. Reference values for aldosterone were reported between 50 and 442 pg/mL for healthy adults (Hannemann et al., 2010; Kerstens et al., 2011; O'Shea et al., 2016). For children, some working groups observed higher aldosterone values than the ones found in adults, especially in very young children. Aldosterone values around 2000 pg/mL have been reported in the first months of life (Fiselier et al., 1984; Gjuric et al., 1982). In addition to the alteration of aldosterone levels in very young children, it became apparent that children with heart failure seem to have higher aldosterone values than healthy children (Saiki et al., 2016; Masutani et al., 2013; Francois et al., 2009), which can be explained by the activation of the RAAS as a means to compensate for the oxygen deficiency caused by the pathophysiology of the disease. To be able to cover the calibration range needed for a comprehensive investigation in this population, the emphasis was put on a broad calibration range rather than a very low LLOQ. With the established calibration range of 19.53 to 2500 pg/mL, this aim was successfully addressed without dilution of samples.

Moreover, in addition to aldosterone, its direct precursor and main metabolite, 18-hydroxycorticosterone and tetrahydroaldosterone, respectively, can simultaneously be semi-quantitatively determined in the paediatric population

with the here-presented method. The combination of 18-hydroxycorticosterone and aldosterone allows the investigation of aldosterone synthase activity as it converts 18-hydroxycorticosterone to aldosterone in the last step of the multistep aldosterone synthesis. Further, information on the maturing of this metabolic pathway and the possibly involved ontogeny can be assessed. The application in the LENA study can thus supply unique data on the behaviour of aldosterone, 18-hydroxycorticosterone, and tetrahydroaldosterone in a young paediatric population suffering from heart failure.

For tetrahydroaldosterone, the main metabolite of aldosterone, a concentration range of 78.13 to 2500 pg/mL was established in serum. For adults, tetrahydroaldosterone is commonly analysed in urine because it is mainly renally eliminated after glucuronidation. Even if a serum application may not be necessary for adults, it still is interesting for use in children because after birth sulfation is the main metabolic pathway as the glucuronidation and the CYP metabolism are not yet distinct (Rodieux et al., 2015; Wildt et al., 1999; Hines, 2008). Thus, the tetrahydroaldosterone values may underlie an ontogeny which can be detectable in serum. Especially for severely diseased children, like the LENA population, receiving pharmacotherapy that is mainly renally eliminated, which additionally influences the metabolic system, a comprehensive investigation of this process is of particular interest. To the authors' knowledge, no serum tetrahydroaldosterone values in the paediatric population have been reported so far.

Obstacles like the available amount of blood have to be taken into consideration to enable research in children, especially in very young and diseased children. When withdrawing blood from children, it is not advised to sample more than 1% of the blood volume at a single sampling time point and not more than 3% in four weeks. This equates to around 800 µL of whole blood per kilogram drawable from a child at a single time point (Committee for Medicinal Products for Human Use, Ad-hoc group for the development of implementing guidelines for Directive 2001/20/EC, 2008). Available assays for the determination of aldosterone utilise 40 to 1200 µL of sample, whereby LC-MS/MS assays need 150 to 1200 µL of sample (Häkkinen et al., 2018; Mayo Clinic Laboratories; Schaefer et al., 2017a). Travers et al., who reported aldosterone/18-hydroxycorticosterone ratios in

newborn infants, utilised 250 µL serum for their LC-MS/MS determination (Travers et al., 2017; Travers et al., 2018b). This volume equates to about 500 to 600 µL whole blood. For healthy children, this volume seems to be appropriate for the determination at one sample time point without the need for further extensive routine care. However, for sick children requiring additional routine care or for the additional determination of other RAAS parameters, and in very small children, this amount of blood is not appropriate. With the developed method, it is possible to determine aldosterone, 18-hydroxycorticosterone, and tetrahydroaldosterone regularly without infringing ethical considerations on blood volume. To the authors' knowledge, no other LC-MS/MS method utilised 50 µL or less sample for the simultaneous determination of the three compounds.

For the LENA project, it was necessary that blood sampling for several time points during the course of one day and for up to eight common RAAS parameters at one sample time point could be conducted, even in neonates suffering from a severe disease like heart failure. The required sample volume of 50 µL serum facilitated by this method enables the investigation of the aldosterone system in the context of this project. Because the assay also ensures the semi-quantitative detection of the direct precursor and metabolite of aldosterone, it additionally supports new insights into the behaviour and development of the RAAS in very young children with heart failure.

5. Overall conclusion and perspective

In summary, a literature review on aldosterone data in healthy and diseased children was successfully conducted indicating the age dependency of aldosterone values and overall elevated aldosterone values in children suffering from heart failure in comparison to the healthy paediatric population. The lack of data for children under the age of one year became appeared and underlined the need for further high-quality research.

Further, a quality assurance system approach fit for the use in bioanalytics in pivotal paediatric trials was successfully developed and its utilisation in the LENA study has illustrated its applicability. The system supported the generation of high-quality data meeting the requirements of guidelines provided by authorities like the EMA and FDA, while being able to handle low volume samples, which are one of the major hurdles in paediatric research.

The development of a LC-HRMS method for the determination of aldosterone, its precursor 18-hydroxycorticosterone, and its main metabolite tetrahydroaldosterone was successful. A validation according to international guidelines enables the use in Good Clinical Laboratory Practice (GCLP)-conform environments. Further, the low sample volume of 50 µL offers the possibility of the use in paediatric research. The simultaneous determination of the three analytes allows for the generation of comprehensive data enabling insight into the maturation of the paediatric metabolic system and, further, the maturating of the paediatric RAAS.

By means of the developed low-volume LC-HRMS method for the determination of aldosterone, 18-hydroxycorticosterone and tetrahydroaldosterone, in combination with the established quality assurance system, available serum samples of the LENA population can be reanalysed. The advanced bioanalytical method allows for the acquisition of new additional data on the precursor and main metabolite of aldosterone and the cross-check of aldosterone data acquired with the ELISA method. Further the simultaneous determination of the three analytes enables new insight into the maturating metabolism due to derivations of enzyme activities. A comparison with the data acquired with the established

ELISA method allows to investigate into the comparability of results and may reveal influencing factors possibly leading to differences of assay results. The novel data can subsequently be put into context utilising the data compiled with the conducted literature search. This facilitates an overall comparison with already reported data.

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7. References

AB Sciex. TripleTOF® 6600 System, https://sciex.com/Documents/manuals/6600-system-user-guide-en.pdf; 2018 [accessed May 18, 2019].

Abd-Allah NM, Hassan FH, Esmat AY, Hammad SA. Age dependence of the levels of plasma norepinephrine, aldosterone, renin activity and urinary vanillylmandelic acid in normal and essential hypertensives. Biol Res 2004; 37(1): 95–106.

Abdelhamid S. Urinary tetrahydroaldosterone as a screening method for primary aldosteronism: a comparative study. Am J Hypertens 2003; 16(7): 522–30.

Alpert BS, Bain HH, Balfe JW, Kidd BS, Olley PM. Role of the renin-angiotensin-aldosterone system in hypertensive children with coarctation of the aorta. Am J Cardiol 1979; 43(4): 828–34.

Alvarez Kindelan A, Pérez Navero JL, La Ibarra de Rosa I, Concha Ruiz M, Montilla López P, Romanos Lezcano A. Relationship between hemodynamic changes and blood hormone concentrations after cardiac surgery in children with congenital heart disease. Crit Care Med 1994; 22(11): 1754–61.

Anand KJ, Hansen DD, Hickey PR. Hormonal-metabolic stress responses in neonates undergoing cardiac surgery. Anesthesiology 1990; 73(4): 661–70.

Ationu A, Singer DR, Smith A, Elliott M, Burch M, Carter ND. Studies of cardiopulmonary bypass in children: implications for the regulation of brain natriuretic peptide. Cardiovasc Res 1993; 27(8): 1538–41.

Bajcetic M, Wildt SN de, Dalinghaus M, Breitkreutz J, Klingmann I, Lagler FB, et al. Orodispersible minitablets of enalapril for use in children with heart failure (LENA): Rationale and protocol for a multicentre pharmacokinetic bridging study and follow-up safety study. Contemp Clin Trials Commun 2019; 15: 100393.

Barker CIS, Standing JF, Kelly LE, Hanly Faught L, Needham AC, Rieder MJ, et al. Pharmacokinetic studies in children: recommendations for practice and research. Arch Dis Child 2018; 103(7): 695–702.

Baylen BG, Johnson G, Tsang R, Srivastava L, Kaplan S. The occurrence of hyperaldosteronism in infants with congestive heart failure. Am J Cardiol 1980; 45(2): 305–10.

Briet M, Schiffrin EL. Vascular actions of aldosterone. J Vasc Res 2013; 50(2): 89–99.

Briones AM, Nguyen Dinh Cat A, Callera GE, Yogi A, Burger D, He Y, et al. Adipocytes produce aldosterone through calcineurin-dependent signaling pathways: implications in diabetes mellitus-associated obesity and vascular dysfunction. Hypertension 2012; 59(5): 1069–78.

Brown NJ. Aldosterone and end-organ damage. Current Opinion in Nephrology and Hypertension 2005; 14(3): 235–41.

Brown NJ. Contribution of aldosterone to cardiovascular and renal inflammation and fibrosis. Nat Rev Nephrol 2013; 9(8): 459–69.

Brown NJ, Vaughan DE. Angiotensin-Converting Enzyme Inhibitors. Circulation 1998; 97(14): 1411–20.

Bureik M, Lisurek M, Bernhardt R. The human steroid hydroxylases CYP1B1 and CYP11B2. Biol Chem 2002; 383(10): 1537–51.

Burrello J, Monticone S, Buffolo F, Lucchiari M, Tetti M, Rabbia F, et al. Diagnostic accuracy of aldosterone and renin measurement by chemiluminescent immunoassay and radioimmunoassay in primary aldosteronism. J Hypertens 2016; 34(5): 920–7.

Caldwell PHY, Murphy SB, Butow PN, Craig JC. Clinical trials in children. The Lancet 2004; 364(9436): 803–11.

Campino C, Martinez-Aguayo A, Baudrand R, Carvajal CA, Aglony M, Garcia H, et al. Age-related changes in 11beta-hydroxysteroid dehydrogenase type 2 activity in normotensive subjects. Am J Hypertens 2013; 26(4): 481–7.

Capelli I, Gasperoni L, Ruggeri M, Donati G, Baraldi O, Sorrenti G, et al. New mineralocorticoid receptor antagonists: update on their use in chronic kidney disease and heart failure. J Nephrol 2019.

Carvajal CA, Tapia-Castillo A, Valdivia CP, Allende F, Solari S, Lagos CF, et al. Serum Cortisol and Cortisone as Potential Biomarkers of Partial 11beta-Hydroxysteroid Dehydrogenase Type 2 Deficiency. Am J Hypertens 2018; 31(8): 910–8.

Chang RK, Chen AY, Klitzner TS. Factors associated with age at operation for children with congenital heart disease. Pediatrics 2000; 105(5): 1073–81.

Ciplea AM, Laeer S, Bengt Burckhardt B. A feasibility study prior to an international multicentre paediatric study to assess pharmacokinetic/pharmacodynamic sampling and sample preparation procedures, logistics and bioanalysis. Contemp Clin Trials Commun 2018; 12: 32–9.

Cock RFW de, Piana C, Krekels EHJ, Danhof M, Allegaert K, Knibbe CAJ. The role of population PK-PD modelling in paediatric clinical research. Eur J Clin Pharmacol 2011; 67 Suppl 1: 5–16.

Committee for Medicinal Products for Human Use, Ad-hoc group for the development of implementing guidelines for Directive 2001/20/EC. ETHICAL CONSIDERATIONS FOR CLINICAL TRIALS ON MEDICINAL PRODUCTS CONDUCTED WITH THE PAEDIATRIC POPULATION, https://ec.europa.eu/health/sites/health/files/files/eudralex/vol-10/ethical_considerations_en.pdf; 2008 [accessed February 22, 2019].

Cugini P, Scavo D, Halberg F, Sothern RB, Cornelissen G, Meucci T, et al. Ageing and circadian rhythm of plasma renin and aldosterone. Maturitas 1981; 3(2): 173–82.

Dillon MJ, Ryness JM. Plasma renin activity and aldosterone concentration in children. Br Med J 1975; 4(5992): 316–9.

Doi M, Takahashi Y, Komatsu R, Yamazaki F, Yamada H, Haraguchi S, et al. Salt-sensitive hypertension in circadian clock–deficient Cry-null mice involves dysregulated adrenal Hsd3b6. Nat Med 2010; 16(1): 67–74.

Dotsch J, Hohenberger I, Riepe FG, Sippell WG, Dorr HG. Serum cortisol and cortisone levels in newborns with congenital adrenal hyperplasia before the start of therapy. J Endocrinol Invest 2005; 28(5): 413–6.

El-Raziky MS, Gohar N, El-Raziky M. Study of substance P, renin and aldosterone in chronic liver disease in Egyptian children. J Trop Pediatr 2005; 51(5): 320–3.

Espiner EA, Ross DG, Yandle TG, Richards AM, Hunt PJ. Predicting surgically remedial primary aldosteronism: role of adrenal scanning, posture testing, and adrenal vein sampling. J Clin Endocrinol Metab 2003; 88(8): 3637–44.

European Medicines Agency Committee for Medicinal Products for Human Use. Guideline on bioanalytical method validation 2012.

Fiselier T, Monnens L, van Munster P, Jansen M, Peer P, Lijnen P. The renin-angiotensin-aldosterone system in infancy and childhood in basal conditions and after stimulation. Eur J Pediatr 1984; 143(1): 18–24.

Fiselier TJ, Lijnen P, Monnens L, van Munster P, Jansen M, Peer P. Levels of renin, angiotensin I and II, angiotensin-converting enzyme and aldosterone in infancy and childhood. Eur J Pediatr 1983; 141(1): 3–7.

Fluhler E, Vazvaei F, Singhal P, Vinck P, Li W, Bhatt J, et al. Repeat analysis and incurred sample reanalysis: recommendation for best practices and harmonization from the global bioanalysis consortium harmonization team. AAPS J 2014; 16(6): 1167–74.

Fluhler EN. Incurred sample reanalysis: 'too much of a good thing?'. Bioanalysis 2018; 10(21): 1719–21.

Francois K, Bove T, Groote K de, Panzer J, Vandekerckhove K, Suys B, et al. Pleural effusions, water balance mediators and the influence of lisinopril after completion Fontan procedures. Eur J Cardiothorac Surg 2009; 36(1): 57–62.

Freel EM, Connell JMC. Mechanisms of hypertension: the expanding role of aldosterone. Journal of the American Society of Nephrology 2004; 15(8): 1993–2001.

Funder JW, Carey RM, Mantero F, Murad MH, Reincke M, Shibata H, et al. The Management of Primary Aldosteronism: Case Detection, Diagnosis, and Treatment: An Endocrine Society Clinical Practice Guideline. J Clin Endocrinol Metab 2016; 101(5): 1889–916.

Galati S-J. Primary aldosteronism: challenges in diagnosis and management. Endocrinol Metab Clin North Am 2015; 44(2): 355–69.

Garcia del Rio C, Acuna D, Bustamante M, Soler A, Garcia-Torres L, Osorio C. Increased activity of the renin-angiotensin-aldosterone system during the perinatal period. Rev Esp Fisiol 1982; 38(2): 171–6.

Gemelli M, Mami C, Luca F de, Stelitano L, Bonaccorsi P, Martino F. Atrial natriuretic peptide and renin-aldosterone relationship in healthy newborn infants. Acta Paediatr Scand 1991; 80(12): 1128–33.

Ginsberg G. Evaluation of Child/Adult Pharmacokinetic Differences from a Database Derived from the Therapeutic Drug Literature. Toxicological Sciences 2002; 66(2): 185–200.

Gjuric G, Mardesic D, Plavsic V, Dumic M. Plasma renin activity and plasma aldosterone concentration in infants with various potassium intakes. Eur J Pediatr 1982; 139(2): 139–41.

Glinicki P, Jeske W, Gietka-Czernel M, Bednarek-Papierska L, Kruszyńska A, Słowińska-Srzednicka J, et al. The effect of blood collection procedure on plasma renin activity (PRA) and concentrations of direct renin (DRC) and aldosterone. J Renin Angiotensin Aldosterone Syst 2015; 16(2): 339–43.

Green B, Leake RE. Steroid hormones. Oxford: IRL Press, 1987. 261 p.

Gross JH. Massenspektrometrie. Berlin, Heidelberg: Springer Berlin Heidelberg, 2013. 820 p.

Häkkinen MR, Heinosalo T, Saarinen N, Linnanen T, Voutilainen R, Lakka T, et al. Analysis by LC-MS/MS of endogenous steroids from human serum, plasma, endometrium and endometriotic tissue. J Pharm Biomed Anal 2018; 152: 165–72.

Hannemann A, Friedrich N, Lüdemann J, Völzke H, Rettig R, Peters J, et al. Reference intervals for aldosterone, renin, and the aldosterone-to-renin ratio in the population-based Study of Health in Pomerania (SHIP-1). Horm Metab Res 2010; 42(6): 392–9.

Harshfield GA, Alpert BS, Pulliam DA. Renin-angiotensin-aldosterone system in healthy subjects aged ten to eighteen years. J Pediatr 1993; 122(4): 563–7.

Hattangady NG, Olala LO, Bollag WB, Rainey WE. Acute and chronic regulation of aldosterone production. Mol Cell Endocrinol 2012; 350(2): 151–62.

He BJ, Anderson ME. Aldosterone and cardiovascular disease: the heart of the matter. Trends Endocrinol Metab 2013; 24(1): 21–30.

Hinchliffe E, Carter S, Owen LJ, Keevil BG. Quantitation of aldosterone in human plasma by ultra high performance liquid chromatography tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2013; 913-914: 19–23.

Hines RN. The ontogeny of drug metabolism enzymes and implications for adverse drug events. Pharmacol Ther 2008; 118(2): 250–67.

Hjortdal VE, Stenbog EV, Ravn HB, Emmertsen K, Jensen KT, Pedersen EB, et al. Neurohormonal activation late after cavopulmonary connection. Heart 2000; 83(4): 439–43.

Hostetter TH. Aldosterone in Chronic Kidney and Cardiac Disease. Journal of the American Society of Nephrology 2003; 14(9): 2395–401.

Hsu DT, Pearson GD. Heart failure in children: part I: history, etiology, and pathophysiology. Circ Heart Fail 2009a; 2(1): 63–70.

Hsu DT, Pearson GD. Heart failure in children: part II: diagnosis, treatment, and future directions. Circ Heart Fail 2009b; 2(5): 490–8.

Hubl W, Lehmann GW, Buchner M, Dorner G. Plasma aldosterone in newborns and children. Endokrinologie 1978; 72(3): 367–70.

Kadian N, Raju KSR, Rashid M, Malik MY, Taneja I, Wahajuddin M. Comparative assessment of bioanalytical method validation guidelines for pharmaceutical industry. J Pharm Biomed Anal 2016; 126: 83–97.

Kearns GL, Abdel-Rahman SM, Alander SW, Blowey DL, Leeder JS, Kauffman RE. Developmental pharmacology--drug disposition, action, and therapy in infants and children. N Engl J Med 2003; 349(12): 1157–67.

Kerstens MN, Kobold ACM, Volmer M, Koerts J, Sluiter WJ, Dullaart RPF. Reference values for aldosterone-renin ratios in normotensive individuals and effect of changes in dietary sodium consumption. Clin Chem 2011; 57(11): 1607–11.

Kirk R, Dipchand AI, Rosenthal DN, Addonizio L, Burch M, Chrisant M, et al. The International Society for Heart and Lung Transplantation Guidelines for the management of pediatric heart failure: Executive summary. Corrected. J Heart Lung Transplant 2014; 33(9): 888–909.

Kolodziejczyk P, Gromotowicz-Poplawska A, Aleksiejczuk M, Chabielska E, Tutka P, Miltyk W. New Sides of Aldosterone Action in Cardiovascular System as Potential Targets for Therapeutic Intervention. Curr Drug Targets 2018; 19(16): 1968–79.

Kromidas S. HPLC-Tipps Band 1, 1997.

Lamarre-Cliche M, Champlain J de, Lacourcière Y, Poirier L, Karas M, Larochelle P. Effects of circadian rhythms, posture, and medication on renin-aldosterone interrelations in essential hypertensives. Am J Hypertens 2005; 18(1): 56–64.

Lashansky G, Saenger P, Dimartino-Nardi J, Gautier T, Mayes D, Berg G, et al. Normative data for the steroidogenic response of mineralocorticoids and their precursors to adrenocorticotropin in a healthy pediatric population. J Clin Endocrinol Metab 1992; 75(6): 1491–6.

Lee TM, Hsu DT, Kantor P, Towbin JA, Ware SM, Colan SD, et al. Pediatric Cardiomyopathies. Circ Res 2017; 121(7): 855–73.

Lee YH, Kim TY, Hong YM. Metalloproteinase-3 genotype as a predictor of cardiovascular risk in hypertensive adolescents. Korean Circ J 2009; 39(8): 328–34.

Lyngsø KS, Assersen K, Dalgaard EG, Skott O, Jensen BL, Hansen PBL. Does Aldosterone Play a Significant Role for Regulation of Vascular Tone? J Cardiovasc Pharmacol 2016; 68(1): 1–10.

Mainwaring RD, Lamberti JJ, Moore JW, Billman GF, Nelson JC. Comparison of the hormonal response after bidirectional Glenn and Fontan procedures. Ann Thorac Surg 1994; 57(1): 59-63; discussion 64.

Mangge H, Zelzer S, Puerstner P, Schnedl WJ, Reeves G, Postolache TT, et al. Uric acid best predicts metabolically unhealthy obesity with increased cardiovascular risk in youth and adults. Obesity (Silver Spring) 2013; 21(1): E71-7.

Martinez-Aguayo A, Aglony M, Campino C, Garcia H, Bancalari R, Bolte L, et al. Aldosterone, plasma Renin activity, and aldosterone/renin ratio in a normotensive healthy pediatric population. Hypertension 2010; 56(3): 391–6.

Masutani S, Saiki H, Kurishima C, Ishido H, Tamura M, Senzaki H. Heart Failure With Preserved Ejection Fraction in Children. Circ J 2013; 77(9): 2375–82.

Mayo Clinic Laboratories. Aldosterone, Serum, https://www.mayocliniclabs.com/test-catalog/Clinical+and+Interpretive/8557 [accessed May 18, 2019].

Moher D, Liberati A, Tetzlaff J, Altman DG. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. PLoS Med 2009; 6(7): e1000097.

Muñoz-Durango N, Fuentes CA, Castillo AE, González-Gómez LM, Vecchiola A, Fardella CE, et al. Role of the Renin-Angiotensin-Aldosterone System beyond Blood Pressure Regulation: Molecular and Cellular Mechanisms Involved in End-Organ Damage during Arterial Hypertension. Int J Mol Sci 2016; 17(7).

MVZ Labor Dr. Limbach Heidelberg. Aldosteron, https://lv.limbachgruppe.com/onlinelvz/11_de#/detailView/25 [accessed May 18, 2019].

Nalcacioglu H, Ozkaya O, Baysal K, Kafali HC, Avci B, Tekcan D, et al. The role of bioelectrical impedance analysis, NT-ProBNP and inferior vena cava sonography in the assessment of body fluid volume in children with nephrotic syndrome. Nefrologia 2018; 38(1): 48–56.

Nandi D, Rossano JW. Epidemiology and cost of heart failure in children. Cardiol Young 2015; 25(8): 1460–8.

Nguyen Dinh Cat A, Briones AM, Callera GE, Yogi A, He Y, Montezano AC, et al. Adipocyte-derived factors regulate vascular smooth muscle cells through mineralocorticoid and glucocorticoid receptors. Hypertension 2011; 58(3): 479–88.

Nicolaidou P, Georgouli H, Matsinos Y, Psychou F, Messaritaki A, Gourgiotis D, et al. Endothelin-1 in children with acute poststreptococcal glomerulonephritis and hypertension. Pediatr Int 2003; 45(1): 35–8.

O'Shea P, Brady JJ, Gallagher N, Dennedy MC, Fitzgibbon M. Establishment of reference intervals for aldosterone and renin in a Caucasian population using the newly developed Immunodiagnostic Systems specialty immunoassay automated system. Ann Clin Biochem 2016; 53(Pt 3): 390–8.

Parth K, Zimprich H, Brunel R. Determination of plasma aldosterone in children by thin layer chromatography and radioimmunoassay. Acta Endocrinol (Copenh) 1976; 81(2): 330–9.

Payne AH, Hales DB. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr Rev 2004; 25(6): 947–70.

Peter M, Dubuis JM, Sippell WG. Disorders of the aldosterone synthase and steroid 11beta-hydroxylase deficiencies. Horm Res 1999; 51(5): 211–22.

Pica N, Bourgeois F. Discontinuation and Nonpublication of Randomized Clinical Trials Conducted in Children. Pediatrics 2016; 138(3).

Pitt B, Remme W, Zannad F, Neaton J, Martinez F, Roniker B, et al. Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction. N Engl J Med 2003; 348(14): 1309–21.

Pitt B, Zannad F, Remme WJ, Cody R, Castaigne A, Perez A, et al. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. N Engl J Med 1999; 341(10): 709–17.

Pleil JD, Isaacs KK. High-resolution mass spectrometry: basic principles for using exact mass and mass defect for discovery analysis of organic molecules in blood, breath, urine and environmental media. J Breath Res 2016; 10(1): 12001.

Ponikowski P, Voors AA, Anker SD, Bueno H, Cleland JGF, Coats AJS, et al. 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: The Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC)Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. Eur Heart J 2016; 37(27): 2129–200.

Pratt JH, Ambrosius WT, Wagner MA, Maharry K. Molecular variations in the calcium-sensing receptor in relation to sodium balance and presence of hypertension in blacks and whites. Am J Hypertens 2000; 13(6 Pt 1): 654–8.

Ranstam J. Multiple P-values and Bonferroni correction. Osteoarthr Cartil 2016; 24(5): 763–4.

Riepe FG, Krone N, Peter M, Sippell WG, Partsch C-J. Chromatographic system for the simultaneous measurement of plasma 18-hydroxy-11-deoxycorticosterone and 18-hydroxycorticosterone by radioimmunoassay: reference data for neonates and infants and its application in aldosterone-synthase deficiency. J Chromatogr B Analyt Technol Biomed Life Sci 2003; 785(2): 293–301.

Rittig S. Neuroendocrine response to supine posture in healthy children and patients with nocturnal enuresis. Clin Endocrinol (Oxf) 2010; 72(6): 781–6.

Rittig S, Matthiesen TB, Pedersen EB, Djurhuus JC. Circadian Variation of Angiotensin II and Aldosterone in Nocturnal Enuresis: Relationship to Arterial Blood Pressure and Urine Output. The Journal of Urology 2006; 176(2): 774–80.

Rodieux F, Wilbaux M, van den Anker JN, Pfister M. Effect of Kidney Function on Drug Kinetics and Dosing in Neonates, Infants, and Children. Clin Pharmacokinet 2015; 54(12): 1183–204.

ROE Laboratorio Clinico. ALDOSTERONA SERICA, https://www.labroe.com/roe/roe_analisis_detalle_home.aspx?ca=zRGoTo2XvW8= [accessed December 5, 2018].

Rosenthal D, Chrisant MRK, Edens E, Mahony L, Canter C, Colan S, et al. International Society for Heart and Lung Transplantation: Practice

guidelines for management of heart failure in children. J Heart Lung Transplant 2004; 23(12): 1313–33.

Roumen L, van Hoof B, Pieterse K, Hilbers PAJ, Custers EMG, Plate R, et al. Application of a ligand-based theoretical approach to derive conversion paths and ligand conformations in CYP11B2-mediated aldosterone formation. J Comput Chem 2011; 32(11): 2441–8.

Rozet E, Marini RD, Ziemons E, Boulanger B, Hubert P. Advances in validation, risk and uncertainty assessment of bioanalytical methods. J Pharm Biomed Anal 2011; 55(4): 848–58.

Rudzki PJ, Biecek P, Kaza M. Comprehensive graphical presentation of data from incurred sample reanalysis. Bioanalysis 2017; 9(12): 947–56.

Saiki H, Kuwata S, Kurishima C, Masutani S, Senzaki H. Vulnerability of Coronary Circulation After Norwood Operation. Ann Thorac Surg 2016; 101(4): 1544–51.

Schaefer J, Burckhardt BB, Tins J, Bartel A, Laeer S. Validated low-volume aldosterone immunoassay tailored to GCLP-compliant investigations in small sample volumes. Pract Lab Med 2017a; 9: 28–38.

Schaefer J, Burckhardt BB, Tins J, Bartel A, Laeer S. Validated low-volume immunoassay for the reliable determination of direct renin especially valuable for pediatric investigations. J Immunoassay Immunochem 2017b; 38(6): 579–94.

Senzaki H, Kamiyama M, Masutani S, Ishido H, Taketazu M, Kobayashi T, et al. Efficacy and safety of torasemide in children with heart failure. Arch Dis Child 2008; 93(9): 768–71.

Siahanidou T, Nicolaidou P, Doudounakis S, Georgouli E, Papadimitriou A, Karpathios T. Plasma immunoreactive endothelin levels in children with cystic fibrosis. Acta Paediatr 2000; 89(8): 915–20.

Sigirci A, Hallac T, Akyncy A, Temel I, Gulcan H, Aslan M, et al. Renal interlobar artery parameters with duplex Doppler sonography and correlations with age, plasma renin, and aldosterone levels in healthy children. AJR Am J Roentgenol 2006; 186(3): 828–32.

SIMPSON SA, TAIT JF, Wettestein A, NEHER R, EUW J von, REICHSTEIN T. Isolierung eines neuen kristallisierten Hormons aus Nebennieren mit besonders hoher Wirksamkeit auf den Mineralstoffwechsel. Experientia 1953; 9(9): 333–5.

SIMPSON SA, TAIT JF, WETTSTEIN A, NEHER R, EUW J von, SCHINDLER O, et al. Konstitution des Aldosterons, des neuen Mineralocorticoids. Experientia 1954; 10(3): 132–3.

Smyth RL, Weindling AM. Research in children: ethical and scientific aspects. The Lancet 1999; 354: SII21-SII24.

Soldin OP, Sharma H, Husted L, Soldin SJ. Pediatric reference intervals for aldosterone, 17alpha-hydroxyprogesterone, dehydroepiandrosterone, testosterone and 25-hydroxy vitamin D3 using tandem mass spectrometry. Clin Biochem 2009; 42(9): 823–7.

Stern H, Weil J, Genz T, Vogt W, Buhlmeyer K. Captopril in children with dilated cardiomyopathy: acute and long-term effects in a prospective study of hemodynamic and hormonal effects. Pediatr Cardiol 1990; 11(1): 22–8.

Sulyok E, Nemeth M, Tenyi I, Csaba IF, Thurzo V, Hadnagy J. The possible role of prostaglandins in the hyperfunction of the renin-angiotensin-aldosterone system in the newborn. Br J Obstet Gynaecol 1979; 86(3): 205–9.

Sulyok E, Varga F, Csaba IF, Nemeth M, Tenyi I, Gyory E, et al. Function of the renin-angiotensin-aldosterone system in relation to electrolyte balance in the small-for-date neonate. Acta Paediatr Acad Sci Hung 1980; 21(2-3): 153–7.

Tiosano D, Schwartz Y, Braver Y, Hadash A, Gepstein V, Weisman Y, et al. The renin-angiotensin system, blood pressure, and heart structure in patients with hereditary vitamin D-resistance rickets (HVDRR). J Bone Miner Res 2011; 26(9): 2252–60.

Travers S, Martinerie L, Boileau P, Lombès M, Pussard E. Alterations of adrenal steroidomic profiles in preterm infants at birth. Arch Dis Child Fetal Neonatal Ed 2018a; 103(2): F143-F151.

Travers S, Martinerie L, Boileau P, Xue Q-Y, Lombès M, Pussard E. Comparative profiling of adrenal steroids in maternal and umbilical cord blood. J Steroid Biochem Mol Biol 2018b; 178: 127–34.

Travers S, Martinerie L, Bouvattier C, Boileau P, Lombès M, Pussard E. Multiplexed steroid profiling of gluco- and mineralocorticoids pathways using a liquid chromatography tandem mass spectrometry method. J Steroid Biochem Mol Biol 2017; 165(Pt B): 202–11.

Tu W, Eckert GJ, Decker BS, Howard Pratt J. Varying Influences of Aldosterone on the Plasma Potassium Concentration in Blacks and Whites. Am J Hypertens 2017; 30(5): 490–4.

Tu W, Eckert GJ, Hannon TS, Liu H, Pratt LM, Wagner MA, et al. Racial differences in sensitivity of blood pressure to aldosterone. Hypertension 2014; 63(6): 1212–8.

Turpeinen U, Hämäläinen E, Stenman U-H. Determination of aldosterone in serum by liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2008; 862(1-2): 113–8.

U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) Center for Veterinary Medicine. Bioanalytical Method Validation Guidance for Industry 2018.

Universitätsklinikum Leipzig Institut für Laboratoriumsmedizin, Klinische Chemie und Molekulare Diagnostik. Aldosteron im Serum, http://ilm.uniklinikum-leipzig.de/ilm.site,postext,laborkatalog.html?modus=detail&gruppe=ALST_S.1 [accessed December 5, 2018].

van Bruijnsvoort M, Meijer J, van den Beld C. The application of control charts in regulated bioanalysis for monitoring long-term reproducibility. Bioanalysis 2017; 9(24): 1955–65.

Vazvaei F. A look back at the incurred sample reanalysis. Bioanalysis 2018; 10(21): 1711–3.

Vilela LAP, Almeida MQ. Diagnosis and management of primary aldosteronism. Arch Endocrinol Metab 2017; 61(3): 305–12.

Vogt B, Burnier M. Aldosterone and cardiovascular risk. Current Science Inc 2009; 11(6): 450–5.

Wildt SN de, Kearns GL, Leeder JS, van den Anker JN. Glucuronidation in humans. Pharmacogenetic and developmental aspects. Clin Pharmacokinet 1999; 36(6): 439–52.

Wimmer M, Parth K, Holzer H. The effect of aldosterone antagonists in juvenile heart surgery. Acta Med Austriaca 1979; 6(1): 8–12.

Wyller VB, Evang JA, Godang K, Solhjell KK, Bollerslev J. Hormonal alterations in adolescent chronic fatigue syndrome. Acta Paediatr 2010; 99(5): 770–3.

Zeevi B, Gil-Ad I, Zabreski R, Berant M, Laron Z, Weizman A, et al. Interventional catheterization decreases plasma levels of atrial natriuretic peptide (ANP) in children with congenital heart defects. Cathet Cardiovasc Diagn 1998; 45(1): 27–32.

8. Appendix

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Appendix 1. Compiled data extracted from 33 full text articles for the healthy population (continued on the next two pages)

Age	Operator	n	Aldosterone [pg/mL]	Operator	Sampling procedure	Reference
5 – 10 years 11 – 15 years	Range	25 24	182.3±73.1 230.3±68.2	Mean±SD	Fasting, sitting, 30 min rest, serum, *	Abd-Allah et al., 2004
5- 15 years	Range	6	136±25	Mean±SD	Plasma, *	Alpert et al., 1979 (‡)
10.7 (8.7 – 12.6) years	Median (IQR)	93	68 (32 – 97)	Median (IQR)	Morning, fasting, sitting, 15 min rest, serum , *	Campino et al., 2013
12.5 (10.3 – 13.9) years	Median (IQR)	13	76.0 (42.5 – 121.5)	Median (IQR)	Morning, fasting, serum, *	Carvajal et al., 2018
1 – 11 months 1 – 4 years 5 – 9 years 10 – 15 years	Range	18 18 24 19	284 (59 – 1055) 106 (25 – 341) 53 (10 – 222) 76 (26 – 208)	Mean (Range)	Morning, supine, 2 h rest, plasma, *	Dillon and Ryness, 1975
7 (3 – 14) days	Median (Range)	28	340±90	Mean±SE	Morning, plasma, *	Dotsch et al., 2005
6.3±2.5 years	Mean±SD	10	81.6±38.6	Mean±SD	*	El-Raziky et al., 2005
1 – 12 weeks 3 – 12 months 1 – 4 years 4 – 8 years 8 – 13 years	Range	9 16 11 9 10	620 (300 – 2010) 180 (70 – 390) 150 (30 – 770) 110 (50 – 200) 80 (40 – 170)	Median (Range)	Morning, fasting, supine, 3 h/8.5 h rest, plasma, *	Fiselier et al., 1983
1 – 12 weeks 3 – 12 months 1 – 4 years 4 – 8 years 8 – 16 years	Range	16 32 20 17 20	660 (250 – 2130) 240 (70 – 1080) 160 (30 – 770) 140 (50 – 440) 100 (40 – 220)	GM (Range)	Morning, fasting, supine, 3 h/8.5 h rest, plasma, *	Fiselier et al., 1984
2 – 6 days 12 – 22 days	Range	15 10	749.2±68.4 617.5±98.8	Mean±SE	Morning, supine, plasma, *	Garcia del Rio et al., 1982
1 day 4 days	Time point	20 20	2685±1869 2982±1909	Mean±SD	Supine, 2 h rest, plasma, *	Gemelli et al., 1991

Age	Operator	n	Aldosterone [pg/mL]	Operator	Sampling procedure	Reference
4.12 (1 – 12) months	Mean (Range)	20	618 (207 – 1927)	GM (Range)	Morning, fasting, supine, plasma, *	Gjuric et al., 1982
13.4±2 years	Mean±SD	195	126±86	Mean±SD	Sitting, plasma, *	Harshfield et al., 1993
12 (6 – 16) years	Median (Range)	33	24 (20 – 42)	Median (IQR)	Fasting, plasma, *	Hjortdal et al., 2000
12 h 24 h 2 days 3 days 4 days 5 days 6 – 30 days 1 – 12 months 1 – 2 years 2 – 6 years 6 – 14 years	Time point /Range	20 12 11 13 9 11 16 11 9 10 12	795.3±226.8 633.3±213.0 637.7±224.7 500.7±222.7 501.9±261.7 441.8±182.9 453.3±142.9 312.5±114.3 279.0±84.4 156.4±77.4 88.1±35.8	Mean±SD	Plasma, *	Hubl et al., 1978
2 – 12 months 1 – 5 years 6 – 12 years	Range	♀: 8 ♂: 14 ♀: 8 ♂: 14 ♀: 8 ♂: 7	♀: 310±227 ♂: 328±414 ♀: 151±104 ♂: 101±86 ♀:133±58 ♂: 76±47	Mean±SD	Serum, #	Lashansky et al., 1992
16 – 17 years	Range	59	80.3±50.1	Mean±SD	Fasting, serum, *	Lee et al., 2009
14.0±3.0 years	Mean±SD	56	76.2 (65 – 114)	Median (IQR)	Morning, fasting, °	Mangge et al., 2013
11.1 (8.9 – 12.9) years	Median (IQR)	211	65 (34 – 94)	Median (IQR)	Morning, fasting, sitting, 15 min rest, serum, *	Martinez-Aguayo et al., 2010
5.98±2.84 years	Mean±SD	25	15.6 (0.77 – 210)	Median (Range)	Serum, *	Nalcacioglu et al., 2018
8.1±2.83 years	Mean±SE	14	191.2±101.7	Mean±SE	Fasting, supine, 30 min rest, serum, *	Nicolaidou et al., 2003

Age	Operator	n	Aldosterone [pg/mL]	Operator	Sampling procedure	Reference
2 – 6 years 6 – 10 years 10 – 14 years	Range	16 15 21	57±36 85±50 78±50	Mean±SD	Morning, supine, plasma, *	Parth et al., 1976
13.4±1.6 years 13.6±1.7 years	Mean±SD	282 201	149±77 91±81	Mean±SD	Plasma, *	Pratt et al., 2000
13.2±0.8 years	Mean±SE	10	13.9±2.8	Mean±SE	Upright, serum, *	Rittig, 2010
10.6±4.3 years	Mean±SD	28	147.7±107.8	Mean±SD	Fasting, supine, 30 min rest, serum, *	Siahanidou et al., 2000
5 days – 1 year 1 – 6 years 6 – 12 years 12 – 16 years	Range	34 48 50 37	248±138 167±112 181±100 146±98	Mean±SD	Morning, fasting, supine, 1 h rest, plasma, *	Sigirci et al., 2006
6 days	Time point	8	2110	Mean	Fasting, supine for 1 – 3h plasma, *	Sulyok et al., 1979 (†)
7 days	Time point	12	1440±720	Mean±SE	Morning, fasting, supine 1 – 3 h rest, plasma, *	Sulyok et al., 1980
7.5±2.1 years 14.2±2.2 years	Mean±SD	2 4	66±43 101±48	Mean±SD	Supine, 1 h rest, plasma, *	Tiosano et al., 2011 (†)
10.0 years 11.5 years	Mean	314 226	142 (133 – 152) 89 (80 – 97)	Mean (95% CI)	Morning, fasting, sitting, 15 min rest, plasma, *	Tu et al., 2014 (†)
14.06 years	Mean	302	95.8	Mean	Morning, fasting, sitting, 15 min rest, plasma, *	Tu et al., 2017
16 (13 – 18) years	Median (Range)	55	107	Median	Morning, fasting, supine, 15 min rest, serum, °	Wyller et al., 2010
14±11 days 6.8±2.5 years	Mean±SE	6 5	1145±242 120±29	Mean±SE	Plasma, *	Zeevi et al., 1998

CI: Confidence interval; GM: Geometric mean; h: hours IQR: Interquartile range; min: minutes; SD: standard deviation; SE: standard error; †: Data extracted from graph (mean of n=3); ‡: Individual values stated in publication, mean and standard deviation manually calculated; *: Aldosterone determination via radioimmunoassay; #: Aldosterone determination via Paper Chromatography; °: No information about assay method

Appendix 2. Compiled data extracted for children suffering from heart failure due to congenital heart defects or dilative cardiomyopathy (continued on the next three pages)

Age	Operator	n	Collective	Aldosterone [pg/mL]		Operator	Sampling procedure	Reference
A: 41.1±25.4 months B: 30.6±40.7 months	Mean ±SD	A: 27 27 B: 38 25	A: CHD correction surgery & normal pulmonary blood flow B: CHD correction surgery & increased pulmonary blood flow	A: Pre OP Post OP (48h) B: Pre OP Post OP (48 h)	766±829 494±476 784±894 1145±1066	Mean ±SD	Morning, supine, 15 min rest, serum, *	Alvarez Kindelan et al., 1994
5.3±0.8 days	Mean ±SE	15	Surgery for complex CHD	Pre OP Post OP (24 h)	1009±324 324±144	Mean ±SE	Plasma, *	Anand et al., 1990
4 (2 – 9) years	Mean (Range)	9	Underwent elective cardiac surgery for CHD correction	Pre OP Post OP (24 h)	434±26 495±47	Mean ±SE	Plasma, *	Ationu et al., 1993
4 – 9 months	Range	15 12	Moderate to severe congestive heart failure before and after the introduction of digoxin or diuretics	Pre Therapy Post Therapy (6 days):	1506 2605	Mean	Supine, serum, *	Baylen et al., 1980
A: 58±47 months B: 40±7 months	Mean ±SD	A: 12 B: 9	A: Cardiopulmonary bypass univentricular heart and chronic volume overload—36 h after surgery introduction of lisinopril. B: Cardiopulmonary bypass univentricular heart and chronic volume overload—no lisinopril introduction.	A: Pre OP Post OP (6 months) B: Pre OP Post OP (6 months)	1070±860 380±760 1090±760 270±280	Mean ±SD	Supine, plasma, °	Francois et al., 2009

Age	Operator	n	Collective	Aldosterone [pg/mL]		Operator	Sampling procedure	Reference
A: 36±10 months B: 13±5 months C: 25±8 months	Mean ±SE	A: 18 B: 15 C: 10	A: Fontan Procedure B: Bidirectional Glenn procedure C: Repair of ventricular septum defect	A: Pre OP Post OP (5 days) B: Pre OP Post OP (5 days) C: Pre OP Post OP (5 days)	220±40 290±40 280±40 270±40 150±30 210±30	Mean ±SE	Serum, *	Mainwaring et al., 1994
A: 1.1±0.8 years B: 8.4 ±6.3 years	Mean ±SD	A: 18 B: 22	A: Heart failure with preserved ejection fraction B: Systolic heart failure	A: B:	1375±1200 511±563	Mean ±SD	Serum, *	Masutani et al., 2013
A: 1.6±1.0 years B: 1.3±2.8 years C: 5.8±3.6 years	Mean ±SD	A: 29 B: 27 C: 30	A: Cardiac catheterisation (Norwood and Norwood/Glenn) B: Cardiac catheterisation (Aortopulmonary Shunt) C: Cardiac catheterisation (Biventricular circulation)	A: B: C:	2588±2186 1671±341 120±157	Mean ±SD	Catheter-isation, serum, °	Saiki et al., 2016)

Age	Operator	n	Collective	Aldosterone [pg/mL]		Operator	Sampling procedure	Reference
A: 0.46±0.78 years B: 5.5±5.4 years	Mean ±SD	A: 62 B: 40	A: Patients with chronic heart failure without diuretics B: Patients with chronic heart failure with diuretic different from torasemide	A: Pre Therapy Post Therapy (3 - 4 weeks) B: Pre Therapy Post Therapy (3 - 4 weeks)	42±22 88±50 228±415 327±528	Mean ±SD	Serum, *	Senzaki et al., 2008
5.8 (0.08 – 15) years	Median (Range)	12	Introduction of captopril to patients with dilated cardiomyopathy	Pre Therapy Post Therapy (3 months)	138 (34 – 602) 134.5 (64 – 380)	Median (95% CI)	Supine, 30 min rest, serum, *	Stern et al., 1990
1.42 – 14 years	Range	A: 11 B: 14	A: No introduction of spironolactone after CHD correction surgery B: Introduction of spironolactone after CHD correction surgery	A: Pre OP Post OP (7 days) B: Pre OP Post OP (7 days)	106.7 160 106.7 293	Mean	Morning, supine, Plasma, *	Wimmer et al., 1979 (†)
A: 6.8±6.4 years B: 8.5±5.5 days	Mean ±SE	A: 15 9 B: 5 4	A: CHD correction surgery in children 0.25 – 12 years B: CHD correction surgery in children 1 – 15 days	A: Pre OP Post OP (7 – 30 days) B: Pre OP Post OP (7 – 30 days)	113±35 78±20 1676±169 506±181	Mean±SE	Plasma, *	Zeevi et al., 1998

CI: Confidence interval; CHD: congenital heart defect; min: minutes; OP: operation; SD: standard deviation; SE: standard error; †: Data extracted from Graph (mean of n=3); *: Aldosterone determination via radioimmunoassay; °: No information about the assay method

9. Publications

Parts of this thesis have already been published in international journals or were previously presented at conferences:

Publications in peer-reviewed journals:

1. **Makowski N**, Ciplea AM, Ali M, Burdman I, Bartel A, Burckhardt BB. A customised quality assurance system for the continuous bioanalysis of aldosterone: Application in a European paediatric study. Manuscript submitted to Contemporary Clinical Trials 19. May 2019.
2. **Makowski N**, Burckhardt BB. Enabling insights into the maturation of the renin-angiotensin-aldosterone-system in children - Development of a low-volume LC-MS assay for the simultaneous determination of aldosterone, its precursor and main metabolite. Accepted 29. April 2019 in Steroids.
   Status: In press (https://doi.org/10.1016/j.steroids.2019.04.009)

3. Castro Díez C, Khalil F, **Makowski N**, Schwender H, Jovanovic I, Dalinghaus M, Walsh J, van der Meulen M, Bajcetic M, de Wildt SN, Läer S. Pharmacotherapy in paediatric heart failure: a Delphi process. Accepted 14. March 2019 in Cardiology in the Young. Status: In press

4. Castro Díez C, Khalil F, Schwender H, Dalinghaus M, Jovanovic I, **Makowski N**, Male C, Bajcetic M, van der Meulen M, de Wildt SN, Ablonczy L, Szatmári A, Klingmann I, Walsh J, Läer S. Pharmacotherapeutic management of paediatric heart failure and ACE-I use patterns: a European survey. BMJ Paediatrics Open (2019) 3(1), pp. 1-12

Regarding publication 1. and 2. listed above, the first author Nina Makowski was responsible for and substantially contributed to the conception and design as well as the analysis and interpretation of data, the drafting and the critical revision of the articles, and the final approval of the versions to be published.

Poster presentations:

1. **Makowski N**, Süßenbach FK, Burckhardt BB. A novel GCLP-compliant bioanalytical LC-HRMS method for the reliable determination of aldosterone, precursor and metabolite facilitating further insight into paediatric maturation of the renin-angiotensin-aldosterone-system. DPhG Annual Meeting. Hamburg, Germany (Oktober, 2018).

2. Süßenbach FK, **Makowski N**, Burckhardt BB. Evaluation of RAAS peptides in complex biological matrices in paediatric patients: Development and validation of a low-volume LC-HRMS Method. DPhG Annual Meeting. Hamburg, Germany (Oktober, 2018).

3. **Makowski N**, Castro Diez C, Dalinghaus M, DeWildt S, Van der Meulen M, Bajcetic M, Jovanovic I, Male C, Ablonczy L, Szatmári A, Schwender H, Läer S. Standard of care for children with heart failure in Europe: Results of a survey and a subsequent Delphi questionnaire. 16th Biannual Congress of the European Society for Developmental Perinatal and Pediatric Pharmacology (ESDPPP). Leuven, Belgium (June, 2017).

4. **Makowski N**, Castro Diéz C, Khalil F, Läer S. Reliability and validity of an European survey on the pharmacological management of paediatric heart failure. DPhG Annual Meeting. Düsseldorf, Germany (September, 2015)