Case Study:
Aldosterone measurement outside the qualified diagnostic Context Of Use in a phase-I trial
❖ challenges to make the grade ❖

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Clinical development of an Aldostrone Synthase Inhibitor (Asi)

At a first sight Aldosterone measurement in a clinical study seems to be straight forward and can be analyzed with “standard assays”

☺ ! That‘s easy

- A lot of assays are commercially available (mostly competitive ELISAs)
- Clinical laboratories offer Aldosterone analysis mostly based on LC-MS/MS
- partly controlled by round robin testing
- Aldosterone analysis can easily be outsourced
  - e.g. to the same CRO that is used for PK analysis
  - to commercial clinical chemistry laboratories, hospital laboratories

Really? Let‘s have a closer look and exchange information

Biomarker translation from Research / Conception to Clinical Operation

- **Importance of information exchange**

…it will always be necessary to ensure that agreed **lines of communication** are established between the laboratory and the sponsor…

EMA reflection paper; section 6.4. Trial conduct (2012)

…the importance of integrating all scientific aspects, from **purely analytical aspects**, all the way to understanding the **biology and effects of the biomarker**, prior to embarking on method establishment or sample analysis, cannot be underestimated. Close and **iterative interactions with the teams requesting the data** is imperative to develop a bioanalytical strategy that combines science, analytical performance and regulations…

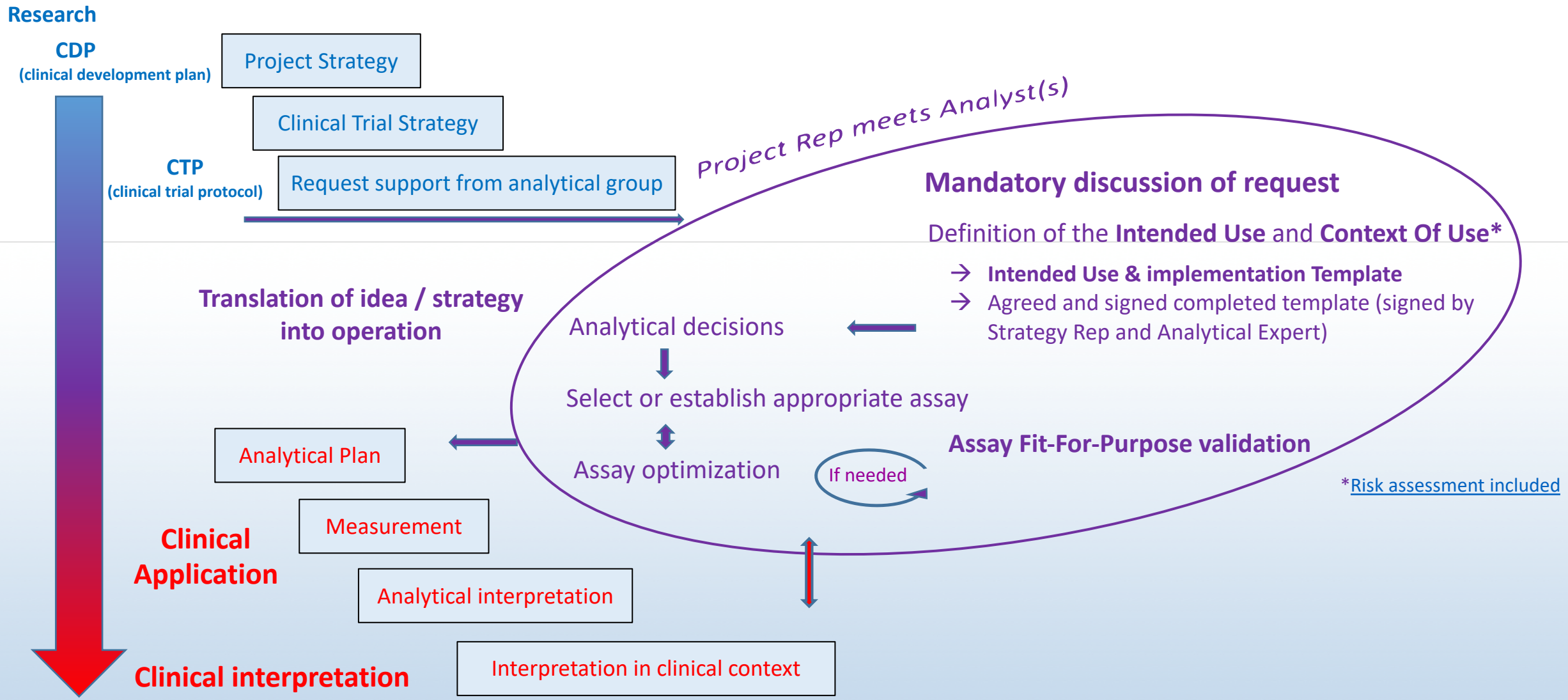
Timmerman et al, Bioanalysis (2012) 4(15), 1883-1894

…In order to ensure that Fit-For-Purpose BM Assay Validation fully supports the **intended use of the data** in a clinical setting, it is crucial to understand and **define the biology, the reasons for investigating that biomarker, the hypothesis is for its use, the COU**, and then design the analytical validation and required evaluations to achieve the most optimal assay for the intended purpose…

Gupta Shalini. et al. Bioanalysis (2017) 9(24), 1967–1996

Biomarker translation from Research/Conception to Clinical Operation

- **A process to find a common unifying spirit**

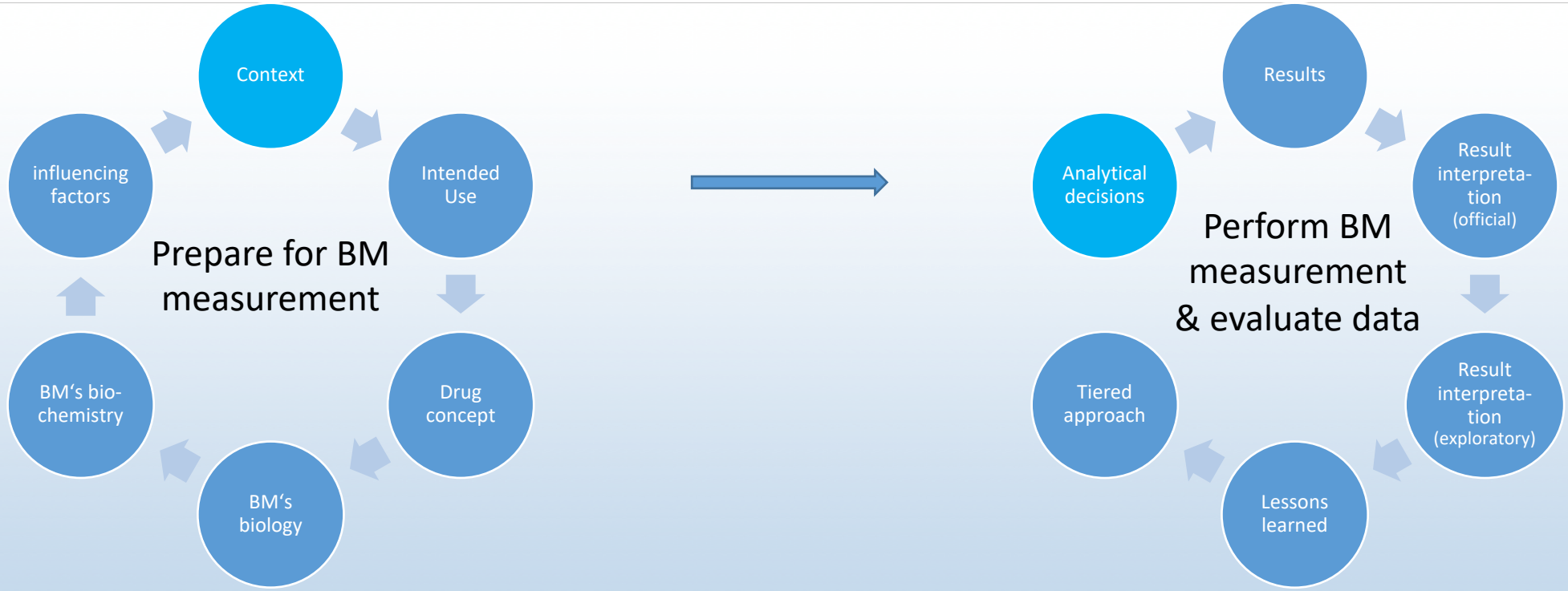


Case Study:

Project: Aldosterone Sythase Inhibitor

Study: Clinical Single Rising dose (SRD) in healthy subjects

Biomarker: Plasma Aldosterone



Context of Adosterone measurements

diagnostic use **versus** **application in SRD – trial**

Analytical measurement range is different !

Aldosterone measurement in human plasma is a **qualified medical diagnostics** in which elevated levels of the hormone indicate:

- primary aldosteronism (eg. adrenal adenoma/ carcinoma and adrenal cortical hyperplasia)
- secondary aldosteronism (e.g. renovascular disease, salt depletion, potassium loading, cardiac failure with ascites)

The diagnostic purpose is to measure **increased** Aldosterone concentraions in suspected **ill patients above the "normal range upper limit"** as an indication of **RAAS* activation** to **trigger medical treatment decisions** by the physicians.

*Renin-Angiotensin-Aldosteron System

In the context of the clinical development (Phase-I in healthy volunteers) of an exploratory Aldosterone Synthase inhibitor, Aldostreone is measured as an **indicator (Biomarker)** of:

- Target Engagment / Inhibition
- PK/PD relationship

The "scientific" purpose is to demonstrate **dose-dependent reduction** of Aldosrerone concentrations in **healthy subjects below the normal concentration range** which is considered as a response to drug induced **Aldosterone Synthase inhibition** to support further drug development decisions.

Aldosterone Intended Use description

Analysis is performed outside the (qualified) diagnostic context

- no medical decisions
  - no risk or harm for study patients (besides sample taking)

Analytical results don‘t contribute to the primary study endpoints, Safety and PK

The biomarker Aldosterone provides supportive information by demonstrating Target Engagement

- Complementary to PK
- the treatment induced decline of Aldosterone concentrations indicates inhibition of Aldosterone Synthase activity

Analytical results may influence business decisions

- the demonstration of Target Engagement will support the continuation of the NBE project
  - demonstration of a change (decline) of concentrations post-treatment is the primary goal (no influence on Cortisol levels)
    - A ≥ 80% reduction of Aldosterone levels is desirable

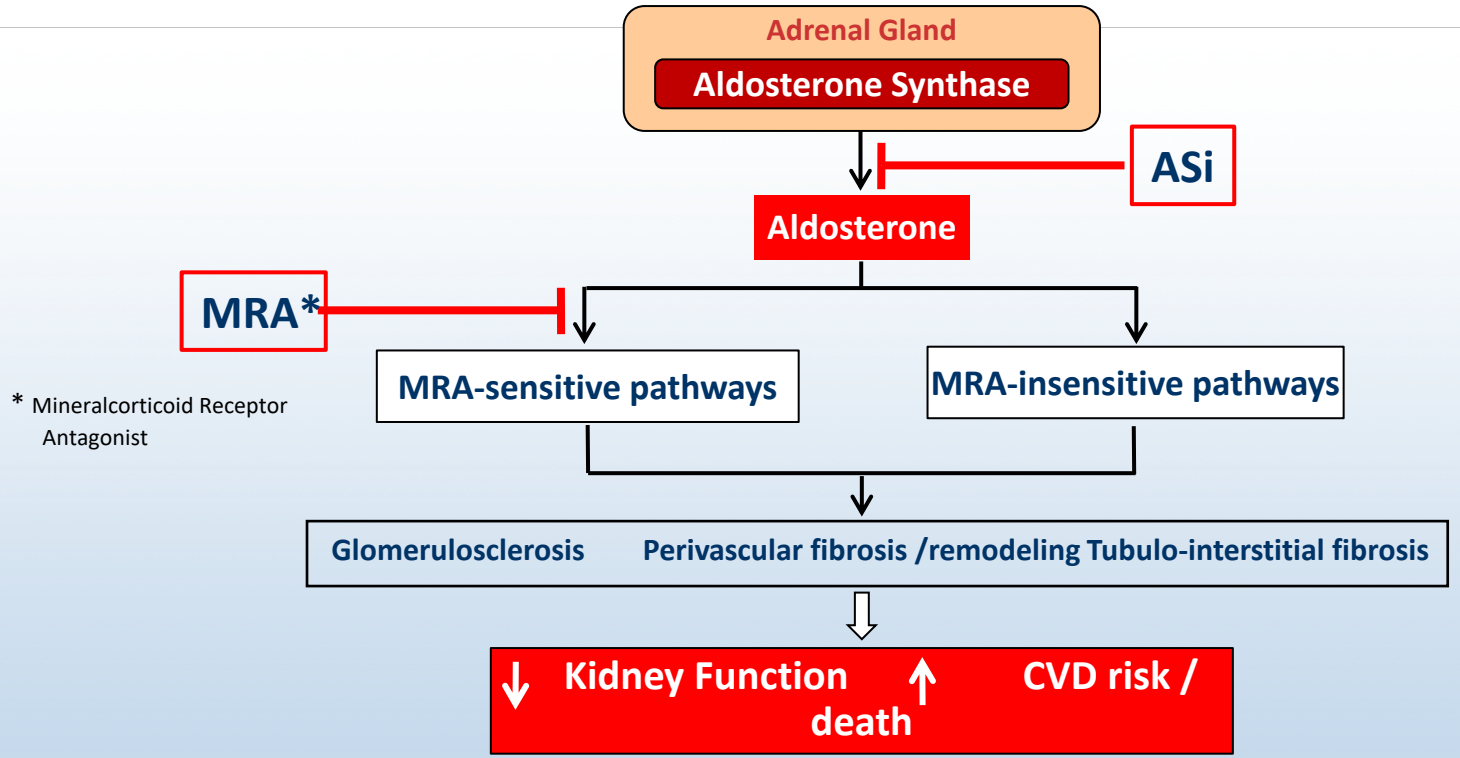
Aldosterone analysis will support data driven hypotheses generation useful for planning the subsequent MRD trials

- If a dose-dependency can be demonstrated based on relative-quantitative data, a PK/PD analysis may be performed
  - possible the data may serve as basis for modelling approaches

Aldosterone is considered as “low-risk“ explorative Biomarker in the SRD phase-I study context.

Aldosterone Synthase (CYP11B2) Inhibitor - Drug Concept for CKD / DN

A selective aldosterone synthase (AS; CYP11B2) inhibitor will lower kidney exposure to aldosterone reducing glomerular, tubular, and vascular damage in the kidney and slowing progression of diabetic nephropathy, while leaving cortisol synthesis unaffected.



* Mineralcorticoid Receptor Antagonist

Below the concentrations that will cause blood pressure effects!

⇩

Possibly only small changes of concentrations

Possibly need to measure small increments of concentration changes

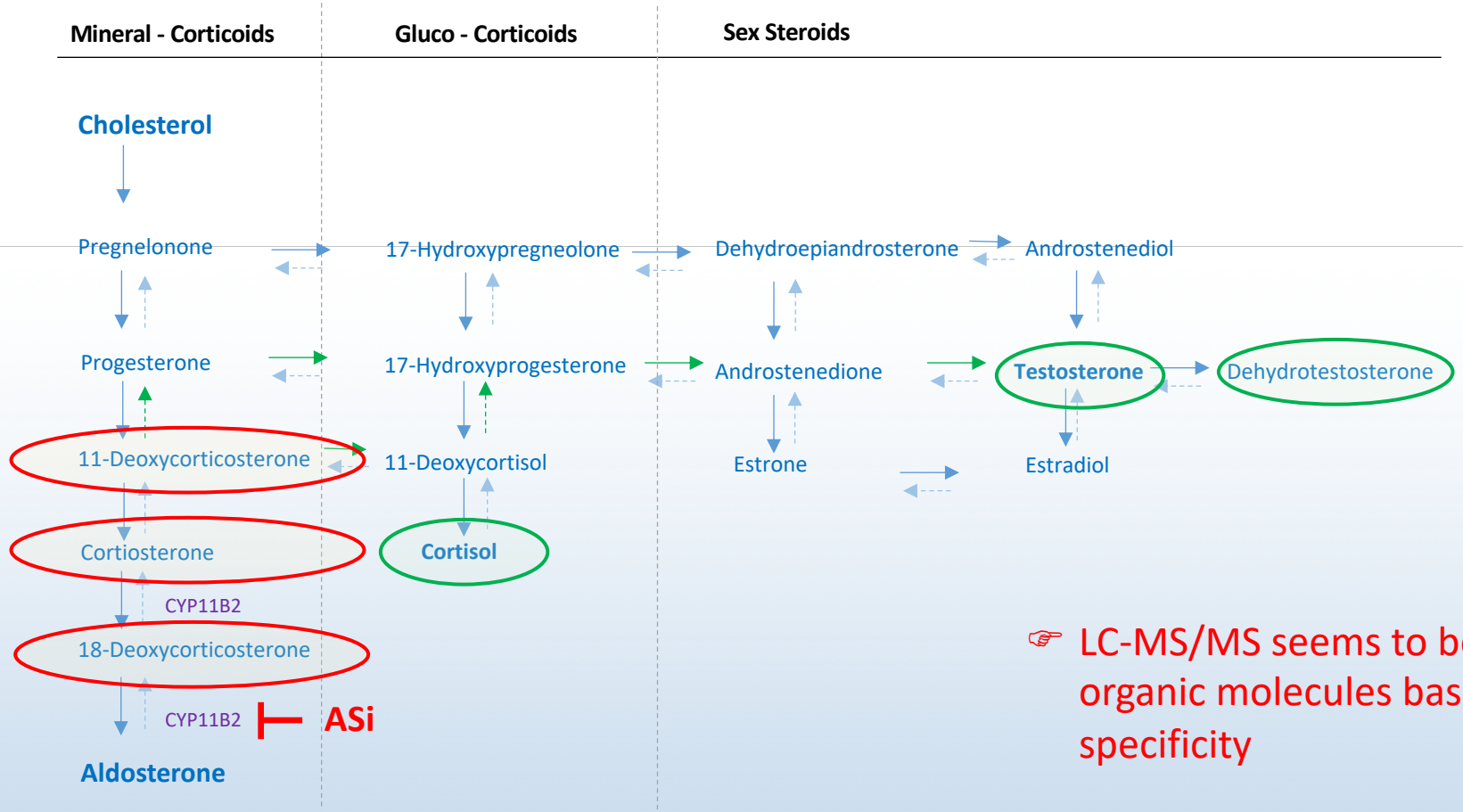
Relative quantitative assay with appropriate precision and no cross-reactivity to Cortisol!

Boehringer Ingelheim

Aldosterone Biosynthesis – Possible consequences of AS inhibition

Steroid pathways

accumulation ⇨ Shunting into other pathway



Many organic molecules with comparable structures

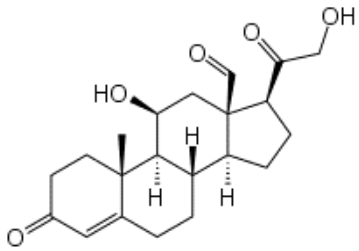
Chemical variability !

- ☞ LC-MS/MS seems to be the most suitable method for this organic molecules based on its advatages regarding specificity
- ☞ Analytical goal: simultaneous analysis Aldosterone, Cortisol, Corticosterone, 11-Deoxycortisol and 11-Deoxycorticosterone

Aldosterone Biosynthesis – cognate molecules and their in vivo concentrations

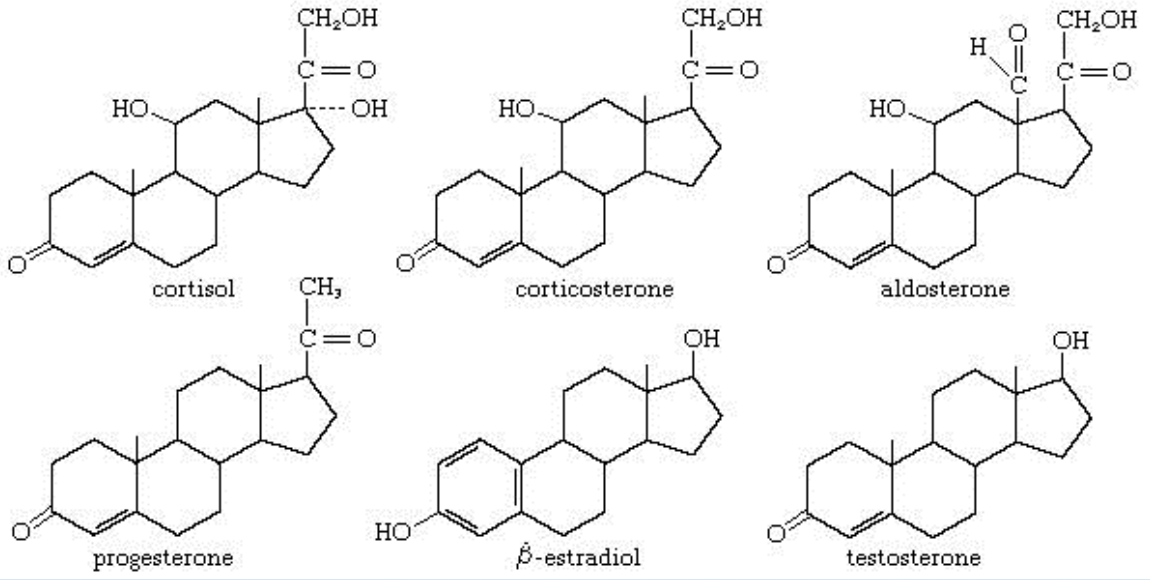
Conc in serum	mg/dL
Cholesterol	200
Cortisol	0.02
Corticosterone	0.00015
Aldosterone	0.00002

If there is a 0.01% cross-reactivity of an antibody in an Aldo-ELISA to Cortisol, 10% of the assay read out would be based on Cortisol!



Cholesterol

the starting point of steroid synthesis



Highly sensitive competitive ELISA methods too critical !

Analytical requirements:

- appropriate sensitivity
- **high specificity**

☞ LC-MS/MS seems to be the most suitable method for this organic molecule

Aldosterone physiology

The renin-angiotensin-aldosterone system (RAAS*) is the primary regulator of the synthesis and secretion of aldosterone.

Aldosterone is important in the maintenance of blood pressure, extracellular blood volume and perfusion of kidneys.

<u>Aldosterone concentration are influenced by:</u>

- Increased concentrations of potassium in the plasma which may directly stimulate adrenal production of the hormone.
- Sodium – concentrations in blood
- RAAS* (Renin activity , Angiotensin-II),
- ACTH (adrenocorticotrope Hormone)
- Vasopressin, Endothelin-1

→ *Biological variability !*
Patho-physiological variability !
Ethnic variability ?

*Renin-Angiotensin-Aldosteron System

Multiple factors influencing the concentrations of Aldosterone in human subjects

Influencing variables	Biologic characteristic	
Diurnal variations	Aldosterone levels are highest in the morning and lowest in the late afternoon and evening (~ 30% lower)	
Physical activity **Body posture**	stress causes 2-4-fold increase in levels of renin → influencing Aldosterone levels sitting: ~25% lower as compared to upright supine: ~50% lower as compared to upright	
Dietary influences	variability based on intake of electrolytes (NaCl, Potassium) take care of sufficient/appropriate NaCl intake in period before blood withdrawal	
Drug influences	diuretics, corticoids, anti-depressants, contraceptives, potassium medications, antibiotics, laxatives Spironolacton/Eplerenone (Aldosterone antagonists) All ACE inhibitors will absolutely interfere with interpretation of Aldosterone results • e.g. Captopril stimulates renin and inhibits aldosterone production	

Aldosterone Analytical Decisions

Analytical determinations tailored to the Intended Use

Aldosterone will be measured with an in-house established LC-MS/MS method

- a LLOQ of 50 pM (200µL sample) is considered applicable
- assuming an Aldosterone concentration of ~250 pM of helathy subjects (in supine position) a ≤ 80% reduction can be analyzed (on a relative quantitative basis)
- some BLQ samples would be acceptable
- change vs baseline is considered as appropriate “reportable result“
- Parallel analysis of other steroids for efficiency reasons

Reference intervals for Aldosterone for healthy subjects taken from a Text book :

Adults in supine position	81-402 pM
In an upright position	180- 790 pM

L. Thomas; Clinical Laboratory Diagnostics (1998)

Assay validation focus – core validation

- needs of a single site trial
  - quantitative comparability of analytical results between different studies is not needed
  - stability evaluation can be adjusted to the timelines of the SRD trial
- all samples from a patient are analyzed together in one analytical batch

Acceptance criteria

- Run acceptance limits (dev.): STD (LLOQ): 25% STD: 25% QC: 25%
- Run acceptance limits (CV) : STD (LLOQ): 25% STD: 25% QC: 25%

- Tight timelines before and during study
- 2000 samples in total
- Analysis time: 15 min/sample

Results of Aldosterone measurement in a SRD trial with helathy male volunteers

Evaluation of explratory (extrapolated) results

Treatment group with 0.7 mg ASi | **Treatment group with 80 mg ASi**

Pre-treatment period

Post-treatment period

time	p103	p104	p105	p106	p107	p108	p703	p704	p705	p706	p707	p708
-24	84	31	174	60	95	160	80	24	56	73	236	211
-23	123	51	90	64	58	78	96	13	38	42	382	100
-22	119	47	44	57	68	65	123	25	42	53	380	69
-20	74	44	58	39	47	38	57	19	67	40	243	52
-16	105	48	227	56	25	26	73	13	51	72	84	88
-12	136	65	31	76	141	49	67	0	65	77	77	39
0	117	59	51	83	53	82	97	31	69	60	144	62
1	120	58	63	92	50	128	60	11	32	120	80	37
2	134	38	70	86	58	77	22	0	14	15	22	15
4	86	37	42	60	32	27	0	0	0	0	12	0
8	76	47	38	68	33	36	0	0	0	0	0	0
12	93	44	36	56	28	26	0	0	0	17	0	0
24	160	121	75	68	138	164	49	16	71	55	63	20

Values reported BLQ of the assay (50 pM)

Unexpected low Aldo concentration in target population of study (mean: <123 pM, [107 pM])

High inter-individual variability, even in healthy subjects (<50 – 236 pM)

The LC-MS/MS assay performed better as expected (CV, dev <15%)

Diagrams on following page with extrapolated results

Results of Aldosterone measurement in a SRD trial with helathy male volunteers

Evaluation of explratory (extrapolated) results

Aldosteron concentrations in healthy voluntees - 24 hours predose-profile and 24 hours postdose profile after treatment with 0.7 mg Asi

Aldosteron concentrations in healthy voluntees - 24 hours predose-profile and 24 hours postdose profile after treatment with 80 mg ASi

Drug effect (target engagement) is only demonstrated on a qualitative level

No quantitative overall interpretation possible due to too much values (44%) below LLOQ

Results cannot be used for quantitative PK/PD or modelling

Results of Aldosterone measurement in a SRD trial with healthy male subjects Evaluation of explratory (extrapolated) results

Don‘t ignore exploratory results, since they may have value for internal decisions:

Check plausibility of results

Increase confidence in drug

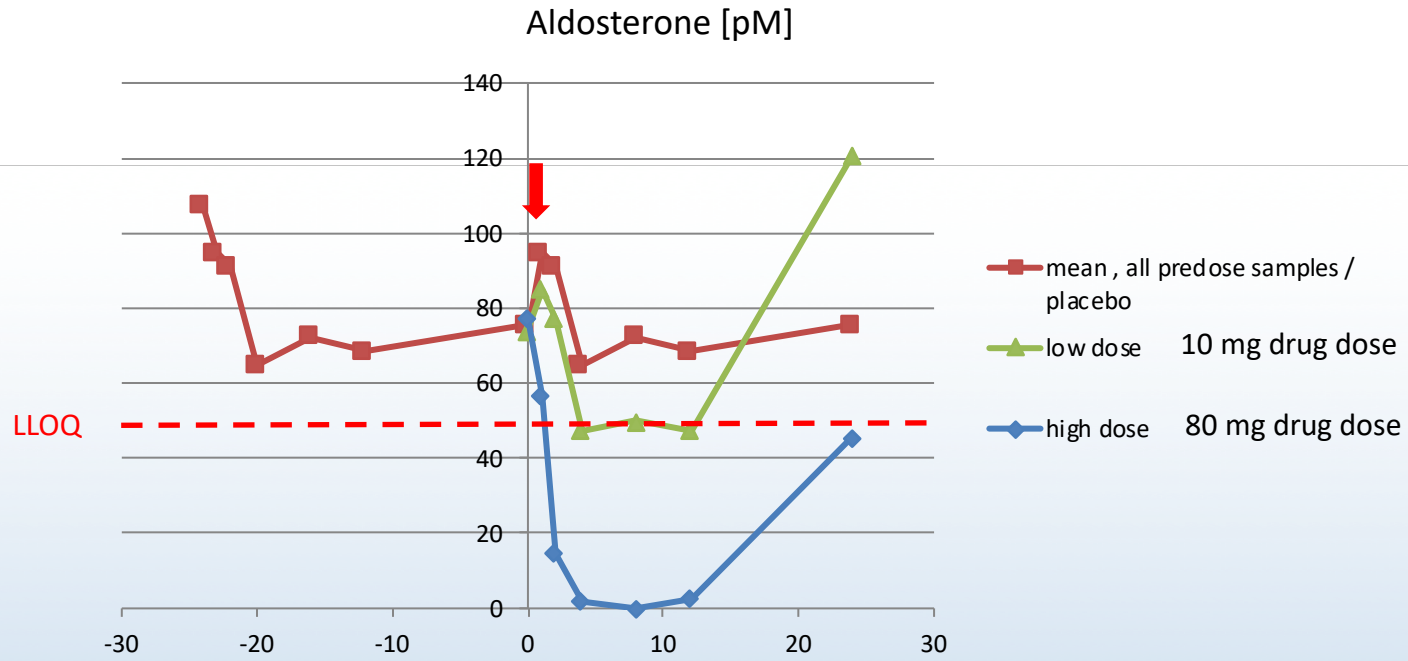
Increase confidence to proceed with the expoloratory drug to the next study

Establish new dosing hypotheses

Possible contribution to business /portfolio decisions

Justification to spend resources for further method improvements

Mean Aldosterone concentrations (n=6) after treatment with 2 doses Asi



Lessons learned

Don’t rely on the information provided in text books

- It is not reported which methods were used to determine the reported healthy range
  - *e.g. Immunoassays deliver higher concentrations as compared to LC-MS/MS, possibly based on cross-reactivities or other selectivity issues*
- It is not reported which population was used to investigate the healthy range (given in text books)
  - *e.g. ratio male/female, age, etc.*

Consequence:

Exploratory pre-studies, which demonstrate the biomarker’s levels in the target population of the study at different pre-analytical conditions are considered important, especially when biomarker concentrations are expected to decline, below the lowest levels of the healthy reference range.

Analytical improvement: LLOQ reduction preferable for future studies

Performing orienting analyses in “target volunteers” could help a lot

- *e.g. variation of body posture, eventually consuming salted drinks, standardized movements, ….*

Aldosterone analysis: importance of COU on assay selection

Summary → theoretical forecast

Diagnostic application	Application in healthy subject within SRD / MRD trial context	Application in patients within MRD or phase-II trial context
Qualified diagnostic application is for secondary aldosteronism **→ known valid BM**	**Application for TE demonstration in an Aldosterone Synthase Inhibitor project** **→ exploratory BM**	
Identify / monitor disease **Qualified analytical scope/context:**	**Drug effect in in healthy subjects** **Exploratory analytical scope/context:**	
detect Aldo elevations in patients (clinical care) as compared **to** reference value in healthy subjects to diagnose disease (e.g. HT)	detect Aldo reductions in healthy volunteers (clinical study) even **below** the reference value in healthy subjects to demonstrate target engagement	
	The diagnostic assay is not appropriate (does not fit to the purpose) • levels to be quantified are below the range of the diagnostic purpose. The diagnostic assay is not validated for this range	

Short description of method - Tiered analytical & assay validation approach

Aldosterone, Cortisol, Corticosterone, 11-Deoxycortisol and 11-Deoxycorticosterone were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using adequate internal standards.

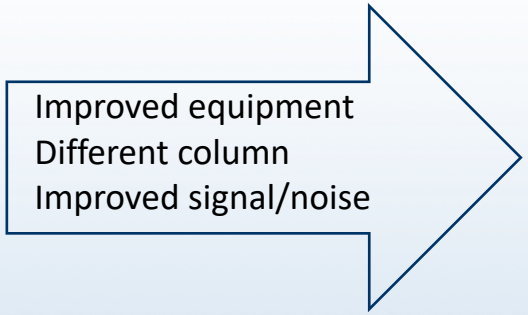
The samples were subjected to automated solid phase extraction (SPE) in the 96-well plate format followed by reversed-phase LC with gradient elution. The substances were detected and quantified by MS/MS using electrospray ionization in the positive and negative ion mode.

No relevant interference of endogenous compounds was observed

1st generation method

Analyte	Matrix	LLOQ	ULOQ	unit	Volume (µL)
Aldosterone	plasma	50	5000	pM	200
Cortisol	plasma	50	5000	pM	200
Corticosterone	plasma	50	5000	pM	200
11-Deoxycortisol	plasma	50	5000	pM	200
11-Doexycorticostrone	plasma	50	5000	pM	200

Relative quantitative analysis



Improved 2nd generation method

Analyte	Matrix	LLOQ	ULOQ	unit	Volume (µL)
Aldosterone	plasma	25.0	5000	pM	200
Cortisol	plasma	2.50	500	pM	200
Corticosterone	plasma	125	25000	pM	200
11-Deoxycortisol	plasma	25.0	5000	pM	200
11-Doexycorticostrone	plasma	50.0	10000	pM	200

Absolute quantitative analysis

Thank you for your attention

Back-up

Short description of method

Analyte / BI code (Longname)	Aldosterone Cortisol 11-Deoxycortisol (11-DC) 11-Deoxycorticosterone (11-DOC) Corticosterone (CS)
Surrogate analyte	n/a
Matrix	Potassium EDTA plasma
Type of assay	Definitive quantitative LC-MS/MS with sample preparation
Validation Level	Level 3 (screening assay)
Calibration standard	biosynthetic material, identical with target analyte
Calibration range	• Aldosterone: 25.0 to 5000 pmol/L • Cortisol: 2.5 to 500 nmol/L • CS: 125 to 25000 pmol/L • 11-DC: 25 to 5000 pmol/L • 11-DOC: 50 to 10000 pmol/L
LLOQ	• Aldosterone: 25.0 pmol/L • Cortisol: 2.5 nmol/L • CS: 125 pmol/L • 11-DC: 25 pmol/L • 11-DOC: 50 pmol/L
ULOQ	• Aldosterone: 5000 pmol/L • Cortisol: 500 nmol/L • CS: 25000 pmol/L • 11-DC: 5000 pmol/L • 11-DOC: 10000 pmol/L
Internal standards	• $[D_7]$Aldosterone • $[D_5]$11-Deoxycortisol
Matrix of the calibration samples	HSS buffer as surrogate matrix
Fit function	Linear regression with 1/x2 weighting
Dependent replicates	two injection on liquid chromatography
Sample volume per determination	200 µL

Calibration samples are prepared by spiking of the calibration standard working solutions of all analytes in HBSS buffer as surrogate matrix. A calibration curve consisting of eight calibration concentration levels. The calibration samples STD.1, STD.2 and STD.8 are prepared in double

Aldosterone, Cortisol, Corticosterone, 11-Deoxycortisol and 11-Deoxycorticosterone were calibrated and quantified using the corresponding heavy isotope labelled molecule as internal standard. The calibration curves were fitted by the equation y = a + bx (weighting function 1/x2). All concentration data were calculated for the free base in undiluted plasma.

Intended Use & Implementation statement Questionnaire

BI drug code:	
Study type/phase:	
Study population:	
Number of subjects/patients:	
Number of sites/centres:	
Planned timelines	
CTP first draft:	
FPI:	
LPO:	
Topline results (if applicable):	
BM report (date):	
Interim analysis planned:	
If Yes, when	

Biomarker	BM Category	Purpose in intended population and decision point	Matrix	Method/Assay	BM clinical qualification status
BM1	PD Safety Prognositc etc,.	e.g. • Project go-nogo, • Dose determination for following studies, • mechnistical understanding • BM hypothsis generation • BM hypothsis generation • Etc. • Study endpoint	Sampling conditions (if known)		Known valid Qualified (which COU) Surrogate BM Probably valid BM Exploratory BM
BM2					
BM3					

In addition:

- quantifiction level expectation,
- expected change, change needed (based on internal or published data)
- evaluation plan (draft plan for statistical evaluation, needs for Pk/PD or modelling)

Intended Use & Implementation statement
Risk assessment

•BASIS FOR RISK ASSESSMENT

For any of the above metioned biomarkers please also consider the risk assessment aspects given below. Risk is defined as “Subject/Patient Risk”, Regulatory Risk” and “ BI Business Risk”. For further details regarding the risk-based quality concept see SOP xxxx. Please list all biomarkers where the respective question can be answered with “yes”.

	Question	Biomarker	Comment
1	Is the planned BM analysis used as surrogate endpoint for safety or efficacy measurements?		
2	Is it planned to use the analysis results for submissions to regulatory authorities?		
3	Are the analysis results used for patient selection and/or safety assessments like diagnostic or prognostic purposes (medical care decisions), safety decisions or prospective patient stratification decisions?		
4	Is it planned to qualify the biomarker in parallel to drug development? • realistic option for CDx assay development • need to compare results on a (rel-)quantitative level over longer time periods.		
5	Is the sampling procedure associated with a higher risk/invasiveness for the subject, e.g. lung biopsies?		
6	Is there a high risk of analysis failure due to lack of assay performance and validation data or an assay/method which does not fulfill the formal GCP requirements?		
7	Does the analysis result trigger or support any business-related (go/nogo; funding) decision?		

Any “yes” answer has to be addressed by TMCP and in the respective cross-functional teams (trial team, ECD team, etc.), as it might have influence on e.g. the analytical validation (validation level, timing), interaction with regulatory authorities, sampling, and archiving.