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After another decade: LC–MS/MS became routine in clinical diagnostics

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ABSTRACT

Tandem mass spectrometry – especially in combination with liquid chromatography (LC–MS/MS) – is applied in a multitude of important diagnostic niches of laboratory medicine. It is unquestioned in its routine use and is often unreplaceable by alternative technologies. This overview illustrates the development in the past decade (2009–2019) and intends to provide insight into the current standing and future directions of the field. The instrumentation matured significantly, the applications are well understood, and the in vitro diagnostics (IVD) industry is shaping the market by providing assay kits, certified instruments, and the first laboratory automated LC–MS/MS instruments as an analytical core. In many settings the application of LC–MS/MS is still burdensome with locally lab developed test (LDT) designs relying on highly specialized staff. The current routine applications cover a wide range of analytes in therapeutic drug monitoring, endocrinology including newborn screening, and toxicology. The tasks that remain to be mastered are, for example, the quantification of proteins by means of LC–MS/MS and the transition from targeted to untargeted omics approaches relying on pattern recognition/pattern discrimination as a key technology for the establishment of diagnostic decisions.

1. Introduction: tandem mass spectrometry – a technology in transition

In 2008, one of the authors (CS) of this overview reflected on a decade of innovation and change in clinical mass spectrometry and speculated about the years to come [1]. Since this author was still active in this science field in 2019, the decision was made to present an updated commentary on the current standing of the field. Similar to the first review, this commentary will be limited to the application of tandem mass spectrometry combined with liquid chromatography (LC–MS/MS). Hence, MALDI (Matrix Assisted Laser Desorption Ionization) based mass spectrometry applications in clinical microbiology [2], genetics [3,4] or GC–MS (gas chromatography-mass spectrometry) applications, which are still of importance in some routine settings [5–7], will not be discussed in depth.

We still see that LC–MS/MS in laboratory medicine is in a transition process from the pioneering stage we described a decade ago to routine layouts alike automated analyzer solutions frequently observed in modern day laboratory environment [8]. We believe that in certain areas, significant progress has been made, whereas in other areas, astonishingly minimal advancement has been observed.

Threads and chances characterize any transition process, especially in technology driven environments such as clinical laboratories [9]. If starting from research environments, the endpoint of technology transition is usually routine application. Such transformations do not only occur in scientific niches open to only few specialists, but with technical objects of our daily life. To cope with the technological complexity of our daily environment, e.g. if using a cellular phone or driving a vehicle, we need customized user interfaces which allow us to “forget” about the technological complexity of the used equipment. Hence, to allow utilization, we treat complex technical equipment as a “black box”. Be it in the setting of everyday life or in a professional context; e.g. in a clinical laboratory where we operate highly complex analytical instruments with the sole aid of instruction manuals, application specialist tutoring and user-friendly (graphical) interfaces. As a limiting consequence we are frequently not in the position to use the presented technology at the peak capacity of its design. For example, if operating FDA cleared or IVD-CE certified laboratory automates enabling the immunoassay analysis of several dozens of parameters in parallel the timing of the individual reactions (e.g. one assay on one channel every 20 min) may impair assay performance but increases the overall application throughput [10]. The convenience of automated assays includes the availability of prepacked reagents (with undisclosed formulation) and calibrator/control materials stabilized in surrogate matrices.

It can be summarized, that frequently findings of basic research

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used in a pioneering stage of technology development to design a procedure, kit, or measurement platform, tend to become "forgotten" in transition to routine. In this respect immunoassays have developed far from the research settings of early immunoassay technology discovery and adoption [11] and tandem mass spectrometry in clinical routine will certainly follow this example.

Usually technology limitations discovered in research are not communicated by the assay vendors to customers of automated ("black box") routine measurement applications. For example, in clinical LC–MS/MS application this is true for the well known effect of ion yield attenuations ("ion suppression"). Whoever is designing a laboratory-developed test (LDT) understands that an assay under consideration must be thoroughly investigated for such effects. However, package inserts and instruction manuals of IVD-CE certified LC–MS/MS kit solutions do usually not comment on "ion suppression" effects [12]. In addition, these effects are often not checked during end-user site assay verification measurement conducted by the assay provider. Furthermore, no information is provided to the customer how to handle individual patient samples showing signs of strong ion yield loss in the ion trace of the stable isotope labelled internal standard [13].

Taken together it is evident that in technology driven businesses, if products are not developed and designed in a way that safe use is possible and residual risks are clearly communicated, their routine application might fail spectacularly. This has happened for a multitude of innovative initiatives in the past decade; even in laboratory medicine [14].

We never must forget to "*be clear what your technology can do today – not what you hope it can do someday*" [15]. We are convinced that LC–MS/MS application in clinical routine will not follow this disturbing trend in the culture of modern day technology transfer. Limitations and quality issues known in the present to scientists worldwide will be sought to be solved in near future. In the following chapters we will describe what we have achieved so far and which problems might be successfully addressable in the years to come.

2. LC–MS/MS application in diagnostics

From a global perspective, mass spectrometry is a niche technology in routine laboratory medicine and its very likely that it will stay a niche technology. Although meta-data from proficiency testing and the literature suggest that the application of LC–MS has significantly increased in the past ten years, valid data about the extent and dynamics of worldwide application are not available. There is no kind of instrumentation registry available, and systematic data from insurance or healthcare organizations are also missing. Nevertheless, the overall prevalence of the application of LC–MS is low, with probably far less than one percent of analyses performed in worldwide laboratory diagnostics based on MS. In some application fields, such as therapeutic drug monitoring (TDM) or toxicology, relative numbers are much higher due to a lack of alternative technologies. Taking immunosuppressive drug TDM (ISD-TDM) as a prominent example, up to 70% of laboratories participating in proficiency testing utilize LC–MS/MS for result generation [16,17].

In other TDM fields, e.g., the application of psychoactive drugs, where ligand-binding assays are not available for most compounds, liquid or gas chromatography combined with different detectors was the only option to address this diagnostic need. In this context, mass spectrometry swiftly replaced other detection modes, and LDTs were frequently used [18]. This is one of the major changes in clinical mass spectrometry in the past decade; more than 200 individual drugs, including ISDs, can be quantified with IVD-CE-certified kits made available by specialized IVD industry partners (e.g. Chromsystems™, Recipe™, Waters™). These kits were designed with a building block system with different calibrator sets combined with one common stationary/mobile phase combination. If such a system is installed on a conventional mass spectrometer, an expansion of application to analytes not covered by the vendor is possible in LDT mode [19]. This approach allows an individual laboratory to meet certain local routine research needs with high analytical quality, e.g., if novel drugs shall be monitored in preclinical or clinical trials.

In clinical toxicology, LDTs are still dominating LC–MS/MS use. However, based on the lessons learned in TDM assay development, commercial kit development initiatives have also begun to target this market. The current setup for clinical toxicology is a "screening procedure" relying on automated immunological analyte group testing available 24/7 that is performed in urine followed by "confirmative testing" based on chromatographical methods, if deemed necessary. This setting did remain almost unchanged for at least three decades, although the limitations of utilizing ligand-binding assays for initial testing were well understood from the beginning: Analyte cross-reactivity depends on analyte structures and varies significantly even within a single substance class (e.g., benzodiazepines) if structural heterogeneity is present [20]; frequent false positive testing for widely used drugs such as trazodone [21] occurs and the inability to identify via ligand binding is common [22]. Furthermore, a complete diagnostic concept failure occurred in the modern recreational drug consumer environment backed by highly flexible drug producers using organic synthesis skills to increase the number of congeners (e.g., JWH type of synthetic cannabinoids) available in the market since the production and evaluation of diagnostic antibodies is a time-consuming business [23]. Maintaining ligand-binding assays as a major diagnostic strategy in emergency medicine in light of such methodological flaws not only illustrates the pressing clinical need for such analytical services but also substantiates the lack of trust in the early adoption of mass spectrometry for reasons including doubts regarding operational failure risks and complicated "experts only" data interpretation, making a 24/7 operation impossible for most scientists. Consequently, only a very limited number of laboratories were ever in the position to wave the immunoassay screening to offer 24/7 mass spectrometry in a routine clinical setting, although a plethora of different attempts involving the complete armamentarium of modern-day mass spectrometry including GC–MS, LC–MS/MS, ion-trap mass spectrometry and high mass resolution LC–MS/MS were undertaken [24–27].

In one of the innovative approaches launched by a mass spectrometer producer, a low-resolution ion-trap mass spectrometer in combination with liquid chromatography (Bruker™ Toxtyper™ concept) was utilized. The analytical readout – MS/MS spectra and retention times under controlled chromatographic conditions – was interpreted by comparison with model spectra from a database to allow (unequivocal) analyte assignment for qualitative confirmative drug analysis. It was shown that this approach possessed the potential to simplify clinical workflows [28]. Its utilization was, however, limited – certainly (at least in Europe) due to a lack of IVD clearance of instrumentation and application notes and a lack of commercial kit components. A recent joint venture of the instrument producer with a key player in the TDM IVD industry (Recipe™) increases hope that these disadvantages can be overcome in the near future. Another approach pursued by an IVD kit provider (Chromsystems™) relies on classic MS/MS technology operating in the conventional target-oriented multiple reaction monitoring (MRM) mode. The developed kit-based solution (MassTox® Drugs of Abuse Testing in Urine) is dedicated to the qualitative or quantitative confirmatory analysis of more than one hundred drugs and their metabolites.

Whether one of these novel approaches will have the potential to replace immunological screening in 24/7 routine laboratory settings serving emergency wards remains doubtful. In particular ligand-binding assays do not require time-consuming glucuronide hydrolysis prior to analysis. However, in other settings, it is feasible to assume that immunological drug screening assays will be outdated within the next few years.

Clinical toxicology, with its shifting analytical targets, its demanding short turnaround times and its impact on clinical decision

making, provides a very good opportunity for the application of the newest technologies and the development of new analytical approaches. Other clinical application fields requiring a multitude (dozens to hundreds) of analytes to be monitored, e.g., metabolomics, lipidomics or steroid metabolome quantification, will certainly profit from the pioneering work in clinical toxicology [29], especially considering that the differentiation of TDM and (clinical) toxicology into distinctively different scientific fields is highly artificial [30].

Another major LC–MS/MS application field with a long-standing tradition is clinical endocrinology. Currently, two substance classes can be identified as major targets: endogenous steroids with different biological modes of action ("steroid hormones") [31] and biogenic amines (catecholamines, metanephrines) utilized as markers for different neuroendocrine and cardiovascular disorders [32]. LC–MS/MS assays for other endocrinologically active small-molecule analyte classes play a minor role in the clinical routine. In the field of thyroid hormone analysis (T3, T4, fT3, fT4), ligand-binding assays are still a key technology, although analytical solutions have been proposed more than a decade ago [33]; however, for 1,25-dihydroxy-vitamin D analysis, some LC–MS/MS sample-preparation strategies, including immunoaffinity enrichment strategies, seem promising [34]. Large-molecule analysis (peptides and proteins) in endocrinology is still limited to a few highly specialized laboratories [35,36]. In this field, a special emphasis and long-standing history of assay development is associated with thyroglobulin (TG) quantification [37] and apolipoprotein multiplex testing [38,39]. Mass spectrometry based TG measurements might allow to overcome the problem of anti-TG masking of ligand binding based assay approaches. Apolipoprotein quantification has the potential to mature to an alternative rational approach to conventional lipoprotein quantification by physicochemical fractionation followed by cholesterol quantification in the sub-fractions. Since the "standard lipid profile" based on this challenging technology is the cornerstone of cardiovascular disease diagnostics, mass spectrometry based apolipoprotein quantification is the key application to bring targeted proteomics into clinical routine [40–42].

In the field of pheochromocytoma and paraganglioma diagnosis, the past decade saw dramatic changes – mainly due to the establishment of robust, precise and sensitive LC–MS/MS assays devoted to the analysis of metanephrines and catecholamines in routine clinical settings [43]. The detection of biogenic amines (catecholamines, metanephrines) in chromatographic separation assays has always been challenging. Prior to the development of very sensitive tandem mass spectrometers, electrochemical detection was the method of choice. However, due to their low concentration in serum, measurement of "free" (unmetabolized/unconjugated) metanephrines in healthy subjects was hardly possible, even with the most delicate detector settings [44,45], thus limiting the application of these markers in diagnostic screening. Immunochemical analysis was limited to radioimmunoassays (RIAs) and ELISAs with limited analytical performance. Consequently, the clinical guidance literature in the first decade of the 21st century that already overcame the need to measure catecholamines saw "plasma-free metanephrines" and "urinary fractionated metanephrines," with their respective pros and cons, in a diagnostically equivalent position, provided, of course, that preanalytical and analytical phase conditions are strictly observed [46,47]. This situation has changed substantially with the recent finalization of the prospective multicenter "Pheo PMT" (prospective monoamine-producing tumor) study [43,48]. The authors did prove beyond doubt that LC–MS/MS-based analysis [49,50] of metanephrine and normetanephrine is superior to urinary metanephrine analysis. Clinical decision limits were established based on the study cohort. The novel clinical guidance document devoted to the management of pheochromocytoma or paraganglioma stated that correct sampling provided measurement results exceeding the established reference intervals 2-fold and "indicate a high probability of PCC/PGL even at low pretest prevalence of disease" [51]. Overall, the LC–MS/MS-based analysis of serum metanephrines is definitively a success story in this diagnostic niche. Its availability in the routine clinical setting results in a significant improvement and simplification in the differential diagnosis of endocrine hypertension [52–54].

Within the past decade, steroid analysis has matured significantly – especially due to technological advancements such as improvements in mass spectrometer sensitivity and chromatographic resolution. The innovation progress started more than a decade ago and was triggered by the marked inferiority of some ligand-binding assays still on the market at the turn of the century [55,56]. Mass spectrometry was envisioned as an alternative analytical approach. However, lessons learned from failing 25-OH vitamin D analyses [57] quickly urged the scientific community to seek traceability concepts [58] safeguarding these novel technologies [59]. The center piece of this undertaking was the establishment of a measurement standardization project for steroid hormones established at the CDC a decade ago [60]. Testosterone was the first analyte assessed starting in 2010, with 25-OH vitamin D and estradiol following in 2012 and 2014, respectively. In an overview published some years after the establishment of the program, Hubert Vesper and coworkers nicely illustrated how participating in the different rounds of the "CDC HoST" (CDC Hormone Standardization) program improves the assay accuracy ("method bias assessment") of the individual participants over time [61].

Passing the program leads to certification, with 14 laboratories outside the CDC currently holding a certificate [62]. Running a measurement service certified by the CDC HoST program has its advantages. The latest Endocrine Society Clinical Practice Guidelines on testosterone therapy in men with hypogonadism clearly state that the lower limit of the normal total testosterone (TT) – a key element in the clinical decision-making process – "harmonized to the CDC standard in healthy nonobese young men is 264 ng/dL (9.2 nmol/L)". The guidelines further state that "this limit could be used for TT assays that are CDC certified" and the authors finally warn the readers that "for laboratories that are not CDC certified and do not participate in an accuracy-based quality control program, the reference range may vary considerably depending on the assay and reference population used" [63]. However, if inter-assay bias terms are reduced due to the availability of IVD-CE-certified kit solutions and/or IVD-CE-certified calibrator/control materials including higher order certified reference materials, modern testosterone assay comparability is quite high [64–66].

With this said, it must be clear to the readership that this analyte holds a flagship position in endocrinological mass spectrometry as ISD monitoring does in TDM. Although estradiol is also part of the CDC HoST program, the analytical goals are not as well met as those of testosterone. The other commonly measured steroid hormones still lack such standardization efforts among individual laboratories. This said, it must not be overlooked that the JCTLM lists reference methods and reference measurement services for aldosterone, cortisol, estradiol, progesterone and testosterone dedicated to supporting the IVD industry in terms of the reference material value [67].

The fact that mass spectrometry matured into a reliable analytical alternative to immunoassays for steroid hormone measurement is mirrored by the fact that years ago, one of the most important worldwide proficiency testing schemes (UK NEQAS for Steroid Hormones [68] operated by the EQA provider Birmingham Quality [69]) changed their strategy of target value assignment from ALTM (all laboratories trimmed mean) to the mean of the mass spectrometry group for most analytes. Currently, only estradiol (low and high values separated) and progesterone have maintained ALTM-based target values (Table 1). The PT scheme results also allow insight into the acceptance of LC–MS/MS measurements as the basis of clinical decision making. From January 2011 to November 2019 (analyzed PT distributions: 371 and 469), the absolute and relative number of participants in the LC–MS/MS group did increase significantly for all analytes (Table 1).

The significant analyte-specific discrepancies in the acceptance of LC–MS/MS measurements in the clinical setting, with progesterone,

Table 1
Comparison of relative and absolute numbers of UK NEQAS steroid hormone proficiency testing scheme [68] participants from 2011 and 2019, demonstrating a strong increase in LC–MS/MS installations in clinical practice. Consequently, in this decade, for most analytes, the target value assignment was changed from all laboratories trimmed mean (ALTM) to mass spectrometry (LCMS).

Analyte	PT distribution 371 (1/2011)			PT distribution 469 (11/2019)		
	LC–MS/MS participants (%)	Overall participants (n)	Target value	LC–MS/MS participants (%)	Overall participants (n)	Target value
Testosterone (female)	6	264	ALTM	25	218	LCMS
Testosterone (male)	4	268	ALTM	17	219	LCMS
Estradiol (low)	0	298	ALTM	7	234	ALTM
Estradiol (high)	0	127	ALTM	4	112	ALTM
Progesterone	0	295	ALTM	2	253	ALTM
Cortisol (serum)	0	278	ALTM	6	256	LCMS
Cortisol (urine)	19	91	ALTM	67	82	LCMS
17-OH-Progesterone	12	52	ALTM	71	69	LCMS
Androstenedione	12	73	ALTM	57	82	LCMS
Aldosterone	0	131	ALTM	14	149	LCMS
DHEAS	0	68	ALTM	20	81	LCMS

estradiol and serum cortisol lagging significantly behind other congeners, reflects the ability of the scientific community to carefully balance the pros and cons of immunoassays and mass spectrometry-based assays for their individual laboratory services [70]. If diagnostic antibodies are tailored such that cross-reactivities and other maladies accompanying some assay realizations are minimized to a clinically insignificant level, the ease of use and 24/7 availability of an automated immunoassay solution still outweigh the reported advantages of LC–MS/MS installations with their well-understood limitations [12,71,72].

In summary, it can be stated that in the past decade, whenever LC–MS/MS was in the position to meet the qualitative requirements of a specific diagnostic problem, it was successfully established and (if commercially feasible) a transformation from “lab-developed tests” to regulated IVD-CE-certified test systems was initiated. However, in any case where the major advantages of ligand-binding assays, e.g., the possibility of high-throughput automation in an industrial setting, outbalanced the selectivity and/or sensitivity advantages of mass spectrometry assays, these assays were not replaced. To put it into the words of Brian Keevil, “MS is roughly at the same stage in its clinical development that IA was 30 years ago when labour-intensive manual testing changed within several years to high throughput testing on large analyser platforms” [70]. Another limitation of mass spectrometry lies in its own analysis design and limitations in analytical sample workup possibilities. Whenever endogenous analyte classes such as steroid hormones are addressed [73], it is evident that the class specific submetabolome is much richer than the limited number of analytes targeted [74]. Consequently, and especially in nonhealthy individuals with altered metabolic pathway regulation, the possibility of interference from chromatographically coeluting isobaric congeners is a threat to assay accuracy [13]. It is very likely, that only technological solutions relying on high resolution mass spectrometry based will be able to address these issues in a scientifically satisfactory manner [75]. If such measures are needed for routine applications remains an open question to be solved in future.

Whenever mass spectrometry is applied to protein quantification, a plethora of questions ranging from topics such as analyte enrichment via immune-affinity based procedures [36] over analyte workup, including digestion to peptides, and the selection of unequivocal proteotypic signature peptides arise [76]. As of now, most applications are found in pharmaceutical production or research. Only a few assays are available to the diagnostic public via laboratory services, with providers predominately located in North America. Harmonization between these services are still to be improved. Although often addressed in theory and practice in recent decades [77–81], recent interlaboratory comparison studies have shown that, at least in the case of thyroglobulin, some lessons remain to be learned before considering such measurement services as true global clinical routine [82,83].

3. LC–MS/MS technology: fit for industrialized laboratory processes?

The past decade has seen significant improvements in the development of mass spectrometers and chromatographic frontends. In low-resolution tandem MS instrumentation, the sensitivity gain was 10–20-fold due to design optimization, and linear ion trap technologies evolved into valuable additional mass selectors. In the field of high-resolution mass spectrometry, the success of orbitrap technology increased, and a novel time-of-flight instrument with increased sensitivity and robustness was launched [84]. In chromatography high resolution stationary phases including “sub-2 μm” particle or core shell particle packings were made available leading to a trend to ultra-high-performance LC settings with significantly improved chromatographic resolution [74,85]. Care must be taken to ensure that technological innovations are not applied careless, but are subjected to a longer-term and critical evaluation before they are used in routine [14,15].

Mass spectrometers and chromatographic equipment are still typical instrumental analysis items found predominately in research settings and industrial environments. In such settings, the broad application range is a key feature and hard- and software-based instrument control is often quite complicated in their use to support this need. The choice of LC–MS/MS equipment and stationary phases is based more on the needs of the application and less on the needs of the operators. Consequently, industrial LC–MS/MS instrumentation is much less user friendly than user interfaces commonly encountered in clinical laboratory environments. Hence, the training effort for LC–MS/MS laboratory personnel is much higher than that in the clinical setting, even if instrument care is not included in the workplace profile. From the regulatory point of view, modern LC–MS/MS setups generally fit to be operated in a GLP/GMP environment, audit trials, logs, and user management in agreement with 21 CFR Part 11 regulation are currently taken for granted. However, regulations for in vitro diagnostics are more demanding, with, for example, IVD-CE certification and FDA assay/instrumentation clearance as major hurdles in the global market. Here, the past decade saw promising initiatives from several key players in the mass spectrometry industry, including a successful FDA “De Novo Classification Request” (DCR) for a “Total 25-hydroxyvitamin D Mass Spectrometry Test System” (DEN170019) issued by Sciex™ [86] or IVD-CE certified instruments (e.g. as provided by Sciex™ or Waters™). This translational process undertaken by research or industry oriented instrument vendors without longstanding experience in in vitro diagnostics is cumbersome since the needs of the clinical market are often not very easy to understand.

Aside from these initiatives, which are unfortunately restricted to a

very limited number of analytes, laboratories were, until recently, forced to purchase general laboratory equipment to be established on site by appropriate measures in accordance with local regulatory requirements (e.g., accreditation in accordance with ISO 15189 or ISO 17025) [87,88]. Such measures usually include at least instrument qualification and assay performance verification in the case of IVD-CE-certified solutions. If de novo development and validation of a procedure – a lab developed test (LDT) – is undertaken, it must not be overlooked that running an LDT might be burdensome and expensive in daily practice [12,89]. Hence, the increasing availability of IVD-CE-certified kit solutions in the past decade resulted in significant relief. Currently, several hundred analytes from TDM, toxicology, and endocrinology are covered by at least one, if not more, commercial solutions. For any of these kit solutions – either FDA cleared or IVD-CE certified – care must be taken, that safe application is warranted and that the provider supports the customer in the application of the assay in routine (e.g. by defining minimal analytical requirements). This statement is of course not limited to LC–MS/MS but applies to the whole field of laboratory medicine, e.g. certified ELISA type immunoassays operated in lab developed automation solutions [90].

Only recently the first “all-in-one” IVD-CE-certified clinical mass analyzer, the “Cascadion™ SM Clinical Analyzer”, was introduced to the scientific public by Thermo-Fisher™. This analyzer combines LC and MS/MS in one instrument; primary tubes are used for the specimen as in routine laboratory tests. Typical chromatography-related working process steps as hardware handling (changing stationary phases) or manual chromatographic peak review are eased or waived, respectively. The analyzer operates under full automation with bidirectional access to the LIS, a feature missing for most conventional instrumentation setups [91]. As of now, this breakthrough in laboratory automation is equipped only with one IVD-CE-cleared application (total 25-hydroxyvitamin D), which is well comparable to other 25-hydroxyvitamin D assays on the market [92]. Since the Cascadion™ starts to establish well in the field, other assays will be added soon. Two other globally acting industry enterprises, one devoted to liquid chromatography and mass spectrometry (Shimadzu™) [93,94] and the other one a prominent healthcare provider (Roche Diagnostics™) [95], are currently showing efforts to bring interesting instrumental solutions to the market.

4. Need for and regulation of lab-developed tests

Tests developed locally by individual laboratories (“lab-developed tests”, LDTs) were always an important link in the chain of technology development from research to routine practice. Automated immunoassays, diagnosis of inborn error of metabolism, and MALDI-based germ identification in microbiology (e.g. Bruker™ Biotyper™, Biomerieux™ VITEK MS™) are unthinkable without local initiatives to set new standards for clinical diagnosis. Hence, LC–MS/MS-based LDTs are of the upmost importance for technology development and maturation. Only in the demanding environment of LDTs the limitations of LC–MS/MS were recognizable, and rulesets for assay design, validation, and application were worked out, defined and published (e.g., CLSI C62-A, for details see further below). From a diagnostic viewpoint, LC–MS/MS-based LDTs are of the upmost importance in tertiary care settings. A plethora of diagnostic valuable metabolites, drugs, medications and other xenobiotics are not covered by industrialized, FDA-cleared or IVD-CE certified assays. LDTs are still pivotal for diagnostic service in TDM, toxicology, workplace drug testing, and endocrinology [96].

With this pressing need and increasing technological possibilities, LDT implementation is booming. When producing and employing LDTs in a local setting, the producer has the responsibility to mitigate the associated risks for the recipient of testing and the testing personnel. With risks understood and communicated, an intended use is determined, and clinical decisions or reference/target ranges must be defined and answered. It is understandable that the overseeing authorities see a need to respond to this situation in a regulatory manner [97]. Consequently, the FDA issued a draft guidance document in 2014 [98] and added a discussion paper in 2017 [99]. The IVD regulation of the European Community issued in 1998 (98/79/EC) [100] did put the responsibility for LDTs in the hands of the individual member states. With the novel legislation issued in 2017 (EU regulation 2017/746) [101], LDT establishment is still allowed, as long as no products with similar performance quality are made available by the regulated IVD industry (kits or complete systems). The requirements for assay documentation and publication have been clarified and harmonized with requirements for the IVD-industry. Furthermore, the quality management system of the laboratory performing such tests must adhere to ISO15189. Overall, the establishment and use of LDTs is still allowed, at least to fulfill diagnostic needs arising locally that are not being covered by the IVD industry, which is economically not in the position to fill diagnostic needs of niche markets [102–104]. We clearly identified the need to deal with orphan diagnostics in a similar manner as medicine must deal with orphan diseases. Often, the needs go hand in hand, as orphan diagnostics are needed for orphan diseases. We also encountered certain patient groups not covered sufficiently by IVD-certified/FDA-cleared diagnostic tests in the market. For example, it is very well understood in the scientific community that none of the currently available assays for the quantification of estradiol in serum/plasma are sensitive enough to reliably quantify the changes in hormone levels during the sexual maturation of children [105,106]. IVD-CE-certified mass spectrometry assays are currently also not designed to be sensitive enough, leaving this assessment to highly specialized laboratories serving endocrinologists in pediatric settings to fulfil this need with an estradiol LDT.

5. The role of LC–MS/MS in reference method development

For several decades, the concept of measurement traceability has been a major cornerstone in the worldwide effort to standardize laboratory measurements [107,108]. In brief, the “chain of traceability” is an unbroken series of material comparisons with appropriate measurement procedures. At the top of the chain (the top/highest metrological order), a certified “primary reference standard” – neat material with certified purity and traceability to the SI unit – with a well characterized associated uncertainty is located. Starting from this material, a primary reference measurement procedure (in the top position, also referred to as the “highest order”) is applied to characterize a primary reference material produced from the neat standard, for example, by solving cortisol NIST SRM 921 in ethanol [58]. With another (or the same) measurement procedure, the production of a secondary reference standard (e.g., NIST SRM 921 in serum) can be performed. Subsequently, the chain can be taken further to routine measurements, which might utilize different measurement principles as the reference measurement procedures (e.g., ligand-binding assays for cortisol in serum) [71]. Due to its technological characteristics, mass spectrometry has always been a widely accepted instrumental analysis method for small-molecule analysis, and the concept of “definitive (absolute) reference methods” [109] was established utilizing GC–MS equipment and isotope-enriched internal standards not meeting the modern-day prerequisite of being “stable” since a radioactive ^{14}C-labeled material was employed [110]. Based on this concept and supported by the maturation of analytical instrumentation as well as the development of stable isotope labeling, important LC–MS/MS-based traceability chains were established for analytes not in the operation range of GC instruments. Significant progress has been made in steroid hormone analysis, especially the HoST program discussed further above, which must be understood as a major game-changing initiative in this context [60]. Aside from testosterone, estradiol and 25-OH vitamin D, some additional steroid hormones are traceable to the SI unit, e.g., cortisol, 17-OH progesterone or aldosterone. A complete up-to-date list of currently

available reference measurement procedures, services, or materials is available from the "Joint Committee for Traceability in Laboratory Medicine" [111]. As stated previously, steroid hormone measurement is only a minor field of mass spectrometry application in clinical practice; the vast majority of analytes are associated with TDM and toxicology. However, whereas traceability chains were established decades ago in the field of endocrinology, exogenous analytes ("xenobiotics") have rarely been addressed. Currently, in TDM, only two measurement services for three analytes (digoxin, digitoxin and theophylline), 15 measurement procedures for 11 analytes, and 33 reference materials for 24 analytes (including metabolites) are JCTLM listed [111]. However, it must be stated that although these materials are listed, less than half of the reference materials for drugs are still produced and/or available for purchase [112–114]. Compared to the number of substances addressed in clinical routine TDM, this is a very small number. For example, the comprehensive "Consensus Guidelines for Therapeutic Drug Monitoring in Neuropsychopharmacology" holds 133 single analyte entries, of which 29 include metabolites [115]. From these, as of now, only 10 substances are covered by JCTLM entries with the limitations stated above. In the past decade, national metrological institutes (NMIs) have hardly added novel reference procedures, and the number of institutions providing reference measurement services in TDM is very low. Hence, the top-down approach with NMIs in the lead is only slowly filling the gap between intentionally agreed measurand definitions (e.g., cyclosporine in whole blood, reported as ng/ml) [116] and isolated (standalone) industry reference chains [117]. Consequently, IVD industry-based initiatives aiming to design and validate reference measurement procedures are an important opportunity to improve the overall situation [118,119]. For some important analytes such as antiepileptic drugs [120], antibiotic drugs [121,122] and immunosuppressive drugs [123], industry-based reference measurement procedures were recently published and listed in the JCTLM. The coming years will see increasing engagement of the IVD industry in this field, and reference material procurement will be aided by the novel concept of qNMR [124,125].

6. Educational initiatives in clinical mass spectrometry

Educational efforts play an important, if not pivotal, role in the establishment of any novel technology. Mass spectrometry and its application in laboratory medicine are not different in this respect. Consequently, within the past decade, a multitude of educational initiatives were established by major national and international societies as AACC [126], JSBMS [127], DGKL [128], IATDMCT, international conference series providers such as MSACL [129] or even vendors as Sciex™ or Thermo-Fisher™. All these activities aimed to improve the general technological understanding of LCMS, the LCMS-specific requirements for method design and method validation, and the applicability of LCMS in diagnostics. Aside from oral presentations, webinars, videos [120,130], tutorials [131], articles [129], reviews [132–134], book chapters, and books [135] serve the interested scientific public with valuable educational support. Of the vast variety of educational materials, the North American initiative that led to the CLSI guidance document C62-A "Liquid Chromatography-Mass Spectrometry" should be particularly emphasized [136]. This document, approved in 2014 and more or less replacing the pioneering document C50-A issued by CLSI in 2007 [137], is a very valuable starting point to responsibly establish LCMS as an LDT in a diagnostic laboratory [138,139]. In the still more research centered field of peptide and protein measurements in clinical routine, strong initiatives have been formed over the past years [79], which cumulated in the preparation of the novel CLSI guidance document C64 "Measurement of Proteins and Peptides by Mass Spectrometry" to be released to the process of public comment soon [140]. In summary, it can be said that the scientific societies of laboratory diagnostics have successfully emancipated themselves from the industry and are fulfilling their role of professional training responsibly.

7. Upcoming and developing fields for the application of LC–MS/MS

We are convinced that any mass spectrometry technology – especially in combination with sample preparation protocols enriching or selecting the analytes of interest from the complex biological background of human specimens (e.g., immunocapture techniques, liquid or gas chromatography, or PCR-based analyte enrichment) – will become an even more prominent key technology in many fields of laboratory medicine than they are currently. Tandem mass spectrometry (MS/MS) or high-resolution mass spectrometry (HR-MS) allow true analytical "multiplexing"; hence, parallel quantitative or qualitative analysis of dozens of analytes is possible from single sample aliquots. In addition to obvious "-omics" types of applications [141–145], which will become increasingly important if signal patterns can be reliably correlated with disease stratification or clinical outcomes, as has already been shown successfully in the past three decades in the very important and globally applied field of mass spectrometry based newborn screening for inborn errors of [146–148], multiplexing is an advantage in situations in which a multitude of analytes must be monitored for a certain diagnostic purpose, e.g., if drug exposure monitoring is advised. In TDM, the broad availability of IVD-CE-certified LC–MS/MS assays covering more than 150 analytes will become increasingly automated and will improve the availability of drug monitoring. As a consequence, drug application will become safer and better adapted to the individual needs of the patients, fostering the aim of personalized diagnosis and therapy. This change in paradigms will not only take place in neuropsychiatry but also – and maybe more importantly from a clinical point of view – in anticoagulant therapy, antibiotic/anti-infective stewardship or geriatric medicine. In clinical toxicology, LC–MS/MS has the potential to replace initial screening procedures currently utilizing immunoassays, which show performance limited to the given cross reactivity of the utilized diagnostic antibodies. A prerequisite for such change is, of course, the 24/7 availability of LC–MS/MS in a completely automated manner including a measurement readout, which is as easy to understand as an immunological test result with its associated decision limits. Quite often, an analytical need is situated in the gray zone between TDM and toxicology. The ongoing "opioid crisis" in pain management [149] does show impressively that the overall IVD industry is not in the position to meet upcoming diagnostic needs in a timely and qualitatively appealing manner. Here, mass spectrometry combined with liquid chromatography has been shown to be a valuable alternative regardless of the technology (LC–MS/MS, LC–HR-MS) used [150,151].

In endocrinology, LC–MS/MS will become the analytical method of choice. In steroid hormone monitoring, diagnostic panels will benefit not only from the selectivity, specificity and sensitivity of LC–MS/MS but also from its unmet multiplexing capabilities, replacing several individual immunoassays with parallel quantification of a single sample. As already stated above, metanephrine quantification by LC–MS/MS will become a standard of care in pheochromocytoma diagnosis, and a future can be imagined in which immunological thyroid hormone measurements, including the free forms, are transitioned from immunoassays to mass spectrometry.

The marked progress made in the past few years in understanding the analytical needs and limitations of quantitative proteomics – especially in SRM/MRM-based approaches – allows us to speculate that another leap forward will prime LC–MS/MS technologies to allow for the safe and unequivocal analysis of proteins and peptides [36], e.g., apo-lipoproteins [38], thyroglobulin, insulin/C-peptide, PTHrP, PTH, or angiotensin I, as markers of renin activity [152]. Candidate reference methods will play a pivotal role in this respect since key components, e.g., SRM transitions or stationary phase/mobile phase combinations, might be transferable from reference to routine methods.

Other applications that are currently still more research-oriented

and employ other mass spectrometry-based ionization strategies such as “paper spray” [153], “iKnife” [154,155], VOC (volatile organic compound) analysis from breath [156], or DART (“direct analysis in real time”) in combination with different mass spectrometry detectors will be of importance in both pathology and clinical chemistry. If analyte separation from the matrix is waived or minimized (flow injection analysis, solid phase extraction cartridge filtration, desorption from a solid matrix, etc.) and the detector setting allows the complete analysis of an ionizable matrix (e.g., in high-resolution mass spectrometry as realized in ToF or Orbitrap instruments), the multitude of detectable signals will require pattern recognition technologies to isolate meaningful information. This principle has been very successfully applied in microbiology, where MALDI-TOF-based analysis platforms allow swift and secure identification of pathogens [2]. Such analytical achievement, which led to a significant change in the diagnostic pathway and became broadly applied in a very short time, is missing in all other “omics” approaches. It can be easily envisioned that it is only a matter of time that a similar breakthrough will bring the one of the game-changing “omics” applications forward – most likely in preventive medicine or in disease-staging approaches where surrogate parameter measurements might add valuable information to grading by clinical scores – e.g., in cardiovascular disease management [157,158].

8. Conclusion: making clinical mass spectrometry fit for the future

The application of mass spectrometry in laboratory medicine has developed very well in the last decade. Due to technological development initiatives triggered mainly by needs in biomedical research and the pharmaceutical industry, LC–MS/MS instrumentation matured such that most small-molecule concentrations in the lower picomolar range can be successfully assessed. Instruments are, however, still designed to be utilized in research settings with specialized personnel available. Since another major trend in laboratory medicine is the industrialization of diagnostic services, mass spectrometry operation lags behind in this respect. This poses a chance for mass spectrometry to be readily accepted, for example, when novel medical or recreational drugs entering the market need monitoring or if industrialized high-throughput analysis platforms do not meet the analytical goals to aid in meaningful medical diagnoses. In this context, the competence to design, validate and operate LDTs will still represent a major advantage of highly specialized central laboratories in the decades to come. With this statement, the thread for mass spectrometry in laboratory medicine is obvious. To become a part of routine clinical chemistry operations, the industry must strive for the automation and “push-button-design” of their instruments. The past decade saw some attempts to move in this direction, but only recently were the first closed LC–MS/MS system platforms marketed. The coming years will certainly see major changes, and in ten to twenty years, the scientific community will look back to a path of development very similar to the path of ligand-binding assay automation [159]. Until then, the proper use and application of mass spectrometry and the art of method development and validation need to be thought of by lab personnel with distinctively different educational backgrounds. It must not be questioned, that albeit MS/MS instruments tend to become increasingly complex, the safe and robust use in the specific environment of the clinical laboratory is a *conditio sine qua non* for its successful application in patient care. Handing over LC–MS/MS technology from industrial support or from research personnel to routine laboratory personnel and/or to coworkers with an educational focus in natural sciences or medicine not exposed to instrumental analytical chemistry requires permanent educational support and well-communicated responsibility sharing. Fortunately, all major national and international scientific organizations in the laboratory medicine, instrument and IVD industry and even some organizers of congress series as MSACL have taken on this challenge and provide the interested audience with seminars, workshops, webinars, etc. to foster knowledge transfer. The future of mass spectrometry in laboratory medicine is certainly in our hands. It is the obligation of the scientific and professional community to persuade the stakeholders in clinical medicine, IVD industry, public health authorities, and care providers that this technology is a major cornerstone of contemporary and future modern, safe patient-centered diagnosis.

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