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Engraving overleaf: Laurentius Heister, Institutiones chirurgicae, Amsterdam 1750. Illustration provided by the Institute for History of Medicine and Foreign Languages.
Extracorporeal Oxygenation Techniques in Adult Critical Airway Obstruction: A Review

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Abstract: Extracorporeal life support has been increasingly utilized in different clinical settings to manage either critical respiratory or heart failure. Complex airway surgery with significant or even total perioperative airway obstruction represents an indication for this technique to prevent/overcome a critical period of severe hypoxaemia, hypoventilation, and/or apnea. This review summarizes the current published scientific evidence on the utility of extracorporeal respiratory support in airway obstruction associated with hypoxaemia, describes the available methods, their clinical indications, and possible limitations. Extracorporeal membrane oxygenation using veno-arterial or veno-venous mode is most commonly employed in such scenarios caused by endoluminal, external, or combined obstruction of the trachea and main bronchi.

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Introduction
A complex airway surgery includes a spectrum of interventional bronchological, otorhinolaryngological, and thoracic surgical procedures for various malignant and benign lesions of the upper airway and tracheobronchial tree. The requirements for the unobstructed surgical field and sufficient oxygenation with ventilation represent a challenging task for both surgeon and anaesthetist (Abdelmalak and Doyle, 2020). A majority of these procedures can be successfully and safely performed using a variety of ventilation techniques including intermittent apnea, jet ventilation (Pearson and McGuire, 2017) or high flow nasal oxygenation (HFNO). However, in cases with significant or even total airway obstruction, these methods are not sufficient in providing adequate respiratory support with the risk of developing severe and life-threatening hypoxemia and hypercapnia. Furthermore, following reconstructive surgery on the tracheobronchial tree, the suture line or the anastomosis site may be at high risk of dehiscence or disruption due to the endotracheal tube presence or pressures exerted by mechanical ventilation (Auchincloss and Wright, 2016). In such circumstances, extracorporeal life support devices including cardio-pulmonary bypass, extracorporeal membrane oxygenation, and pumpless lung assist devices should be utilized.

Methodology
A comprehensive electronic and manual search of databases PubMed, Web of Science, and Scopus was performed for a period from January 1980 till April 2020 using the following terms: “extracorporeal oxygenation”, “airway surgery”, “airway obstruction”, “tracheal surgery”. The following types of articles were retrieved: randomized controlled trials, prospective and retrospective cohort studies, case reports, and reviews.

Cardio-pulmonary bypass
Historically, the first documented use of conventional cardio-pulmonary bypass (CPB) for airway surgery was published by Woods et al. in 1961 and this technique was further used by many centers worldwide (Tyagi et al., 2006). The main advantage of CPB is intraoperative haemodynamic stability and a possibility of “recycling” surgical field suctioned blood returning it back to the circuit, thus significantly limiting the total intraoperative blood loss (Pillai and Suri, 2008). On the other hand, the necessity for high level of anticoagulation with heparin during the procedure, and serious CPB-related sequelae such as induction of postoperative coagulopathy, development of systemic inflammatory response, acute renal failure or acute lung injury has resulted in the development of less invasive methods (Pillai and Suri, 2008).

Extracorporeal membrane oxygenation
Extracorporeal membrane oxygenation (ECMO) is a modified cardio-pulmonary bypass machine providing extracorporeal decarboxylation and oxygenation. It can be
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configured in a veno-venous (VV) used for the management of critical respiratory failure, or in a veno-arterial (VA) setting providing both respiratory and cardiac support (Fierro et al., 2018). VV ECMO (Figure 1) can be instituted peripherally using the two cannulas (femoro-jugular, femoro-femoral) or with the double-lumen cannula inserted under the ultrasound and echocardiographic guidance via the internal jugular vein. VA ECMO can be cannulated peripherally by a transcutaneous Seldinger technique into the femoral vessels or centrally using a surgical approach via median sternotomy into the right atrium and ascending aorta (Fierro et al., 2018). Most ECMO cannulations are carried out after induction of general anaesthesia, however in cases of anticipated high likelihood of losing the airway during a complicated endotracheal intubation, awake cannulation can be safely performed (Gardes and Straker, 2012). When used for this indication, patients are usually weaned off and disconnected from ECMO at the end of surgical procedure or within the immediate postoperative period, when definite airway access is secured and native lung function has proven adequate (Lang et al., 2015). However, in cases of unresolved pulmonary pathology with ongoing respiratory insufficiency or a significant haemodynamic instability, ECMO has to be continued and subsequently weaned off gradually in the postoperative period as the underlying condition improves.

Figure 1 – A. Photograph showing VV ECMO (veno-venous extracorporeal membrane oxygenation) (Cardiohelp, Getinge, Sweden) in a patient with severe influenza pneumonitis; B. A detail of the drainage cannula in the right femoral vein; C. A detail of the return cannula in the left internal jugular vein (patient’s written consent for publication of these images obtained in 2018).

Extracorporeal Oxygenation in Airway Obstruction
The current scientific evidence on ECMO use in high-risk airway surgery is based only on case reports and small case series. Initially, most of the reports came from the paediatric population. ECMO has been successfully used in children undergoing invasive procedures for foreign body related critical airway obstruction (Park et al., 2014), congenital tracheal stenosis (Kunisaki et al., 2008) and malignant or benign endotracheal masses (Smith et al., 2009). Children, and especially infants are extremely prone to serious airway obstruction due to the already narrowed subglottic diameter of the trachea. Thus, according to Hagen-Pouiselle’s law, even a small airway narrowing of any cause can lead to critical airway obstruction and respiratory arrest (Harless et al., 2014). In adults, the first use of ECMO in a patient undergoing tracheal resection was published by Onozawa et al. (1999). Since then, more adult ECMO experience has been increasingly reported. ECMO has been reliably used in a variety of high-risk airway surgical and endoscopic procedures in patients presenting with airway and thoracic tumours (Lang et al., 2015), trauma (Yuan et al., 2008), intrinsic obstruction (Jeon et al., 2006) or extrinsic compression syndromes (De Piero et al., 2018).

The important advantage of ECMO compared to CPB is the minimal spread of tumour cells in oncological airway surgery. The suction of blood from the surgical field into a CPB reservoir creates a risk for disseminating cancer cells into remote organs. Such a case was published by Hasegawa et al. (2002), who reported the pulmonary dissemination of thyroid cancer cells after a CPB-assisted thyroidectomy. As ECMO consists of a closed circuit without a reservoir, no blood from the surgical field is reinfused into the patient and therefore such risk is negligible. Alternatively, the suctioned blood from the field should be processed by a cell saver machine, and reinfused to the patient after filter through a leukocyte depletion filter that’s known to filter out cancer cells because of their negative electrical charge (Gwak et al., 2005).

**Veno-venous ECMO**

By providing an adequate gas exchange, VV ECMO can enable extensive airway surgical interventions with superior visualisation and access to the surgical field. As a sole respiratory support, VV ECMO is fully sufficient in most cases having the advantage of less invasive cannulation and reduced risk of mechanical and bleeding complications compared to VA ECMO with arterial cannulation (Guglin et al., 2019). Venous drainage cannulas of 21-27 Fr in femoral position, return cannulas of 17-23 Fr in femoral or jugular position or 27-31 Fr jugular double-lumen cannulas are commonly used in adult patients to provide blood flow of 50–100 ml/kg. Inspired oxygen fraction (FiO₂) and sweep gas flow in the ECMO oxygenator are tailored to achieve proper gas exchange with physiologic values of PaO₂ and PaCO₂ in the arterial blood gas analysis (Sen et al., 2016). The most common complication of VV ECMO is post-cannulation venous thrombosis that can be present in up to 62% of cases of prolonged ECMO (Fisser et al., 2019). However, due to the short duration
of ECMO in airway surgical patients, such complication is likely to be minimal and has not been reported so far.

**Veno-arterial ECMO**

VA ECMO should be used in cases with supposed or already ongoing haemodynamic instability including impending cardiac arrest (Willms et al., 2012). There have been several studies published, reporting the feasibility of VA ECMO use in patients undergoing complex oncological airway or goitre surgery (Shao et al., 2009). It may be technically difficult to cannulate ECMO in the emergency settings including respiratory or cardiac arrest situations. In such circumstances, femoro-femoral cannulation in the sitting or semirecumbent position is the method of choice and successful cases of such approach have been reported (Kim et al., 2015). Arterial cannulas of 15-21 Fr and drainage venous cannulas of 21-27 Fr should provide sufficient ECMO blood flow of 50–70 ml/kg/min, fully substituting the patient’s own cardiac output. Extensive monitoring during VA ECMO generally includes invasive arterial blood pressure, evaluation of oxygen delivery adequacy by measuring arterial lactate and mixed central venous oxygen hemoglobin saturation, and possibly tissue oximetry (Guglin et al., 2019). Limb ischaemia is a frequent complication of peripheral VA ECMO; however, its incidence has been markedly reduced by the routine use of the distal limb reperfusion with 6-8 Fr antegrade catheter inserted into the superficial femoral artery (Guglin et al., 2019).

**Pumpless lung assist**

The third option for extracorporeal respiratory support during complex airway surgery represents the use of pumpless extracorporeal lung-assist devices (lung membrane). These are based on extracorporeal CO₂ removal with only limited oxygenation capacity using low transmembrane gradient oxygenators with blood flow driven by the patient’s own cardiac output (Walles, 2007). Typical cannulation sites include femoral artery and vein, however, cases with central cannulation via thoracotomy used for the treatment of pulmonary hypertension have been reported. The main disadvantages of this method compared to ECMO are the limited gas exchange capacity, dependence on adequate cardiac output, and inability of hemodynamic support if needed (Walles, 2007). However, potential benefits of the membrane lung include a reduction in cellular trauma and minimal inflammatory activation compared to ECMO and CPB (Iglesias et al., 2008). There is only anecdotal experience with this method.

**Anticoagulation and fluid management in ECMO**

A need for full anticoagulation represents a major drawback of using CPB for airway surgery. ECMO with heparin-coated cannulae and circuit tubings requires a much lower level of heparinization, usually aiming for activated clotting time of 160–180 s, thus offering the advantage of less bleeding complications. For
instance, in a recent study, no bleeding complications and no more than the usual intraoperative blood loss were observed in 10 patients undergoing complex tracheobronchial surgery on VA ECMO with only minimal anticoagulation of 3,000–5,000 units of heparin (Lang et al., 2015). Furthermore, in another study, no anticoagulation at all was used in a patient on VV ECMO undergoing tracheal surgery without any thrombotic events (Antonacci et al., 2018). Usually, at the end of the airway procedure, protamine is administered for heparin anticoagulation effect reversal and prevention of postoperative bleeding. Afterwards, ECMO cannulas can be removed with manual compression and skin suture. In cases of complicated arterial cannulation, a vascular surgical revision and repair may be required. ECMO requires a smaller amount of circuit priming compared to CPB, thus the risk of dilutional coagulopathy and volume overload associated complications including pulmonary oedema may be reduced (Wiebe et al., 2006).

Immunosuppressive effects of ECMO
The theoretical disadvantage of extracorporeal methods lies in the temporary immunosuppressive effect caused by the increases in immunoregulatory factors including interleukin-10, tumor necrosis factor, and transforming growth factor-β. This may cause changes in the function of the immune system and cancer surveillance, possibly leading to the spread and growth of cancer cells (Sablotzki et al., 1997). This is supported by the study, in which a trend towards the progression of cancer was observed in patients undergoing cardiac surgery with the use of CPB compared to off-pump surgery (Pinto et al., 2013). On the other hand, ECMO is a mini-invasive extracorporeal method comparable to miniaturized CPB systems, which have been shown to have a diminished immune reaction (Vohra et al., 2009). Therefore, the effect of ECMO on potential cancer growth is likely to be insignificant and has not been documented in the literature so far.

Complications of ECMO
Generally, there are many mechanical, haemorrhagic, thromboembolic and infectious complications associated with the use of ECMO described in critically ill patients requiring lung or heart support. All of these seriously affect the patient’s outcome, increasing both morbidity and mortality (Marasco et al., 2008). Mechanical problems, represented by oxygenator or pump failure, circuit thrombosis, cannulae-associated, and limb ischaemic complications require multidisciplinary approach including intensive care physicians, vascular surgeons, cardiologists, and perfusionists. Nevertheless, patients undergoing airway surgery with short ECMO runs are likely to have a much lower incidence of such serious complications compared to the critically ill, and to our knowledge, they have not been reported yet.

One case series reported successful use of extracorporeal lung membrane in combination with intermittent apnoeic ventilation in 15 patients undergoing
complex airway surgery without any complications and with minimal effect on coagulation and inflammatory response (Sanchez-Lorente et al., 2012).

**Extracorporeal oxygenation in tracheal reconstructions**

A potential advantage of extracorporeal techniques in reconstructive procedures on trachea (Figure 2) is the allowance of stable surgical field without risks of hypoxemic adverse events. Most case reports are related to new-born or toddler reconstructions due to congenital malformations of the trachea. Three larger case series report the use of ECMO in 32 adult patients undergoing open tracheal resections or extended reconstructions of the tracheobronchial tree (Chang et al., 2014; Kim et al., 2015; Lang et al., 2015). While two former studies employed the VA ECMO circuit in all cases, Kim et al. (2015) have used mostly VV ECMO. Only one patient died within 30 days after surgery due to multiorgan system failure, all remaining patients were successfully discharged. Another retrospective study reported a cohort of 18 patients with the central airway obstruction syndrome caused by malignant conditions (Hong et al., 2013). These patients had a VV ECMO support as a bridge before the procedure of interventional bronchology aiming to restore patency of the airways. Five of these patients (27.8%) died at 60 days.

**Extracorporeal oxygenation in the external airway compression**

An anterior mediastinal mass (AMM) (Figure 3), for example, lymphomas, retrosternal goitre or thymoma may result in a life-threatening external airway compression in the intraoperative period. VV ECMO has been reported in several cases of the significant external lower trachea or main bronchi obstruction, as planned femoral vessel cannulation at the start of the procedure (Goh et al., 1999) or as a rescue technique in sudden ventilation and oxygenation difficulties during a lymphatic node excision (Netri et al., 2016). This technique may also be used awake,
as a respiratory support tool, in patients undergoing chemotherapy for extensive intrathoracic lymphatic masses causing significant breathing problems (Worku et al., 2015). It should be noted that such approach may not be necessary at all in low and moderate risk AMM cases, however it should be entertained in the high risk AMM situations causing severe symptoms and >50% tracheal compression. Even in severe cases conservative approach with awake intubation, and gradual deepening of the anesthetic preserving spontaneous ventilation has proved successful in the past (Abdelmalak et al., 2010). Clinicians must keep in mind that having CPB, or ECMO equipment on stand-by may not always ensure a favorable outcome (Slinger and Karsli, 2007). Despite the immediate availability of the ECMO team, it may take approximately 5–10 minutes to achieve adequate oxygenation after complete airway obstruction (Tempe et al., 2001). This delay might pose a risk of hypoxic brain injury. Therefore, a well thought out anesthetic plan that allows for maintaining the airway and ventilation throughout lessens the chances of the need for such an invasive intervention.
Extracorporeal oxygenation in and whole lung lavage

Many case reports described the use of ECMO in whole lung lavage procedure for pulmonary alveolar proteinosis (PAP) (Sivitanidis et al., 1999). However, that option should be considered carefully in light of the known potential complications of ECMO, and also the fact that this procedure can be performed effectively even in the severe cases of PAP in a sequential manner in centers with extensive experience in such procedures (Abdelmalak et al., 2015).

Awake extracorporeal oxygenation

Extracorporeal oxygenation has been used in one case of awake video-assisted thoracic surgery, in the patient with advanced chronic obstructive pulmonary disease, emphysema, and hypoxaemia at rest (Drosos et al., 2020). Although awake tracheal reconstructions have also been reported in the literature, the use of the extracorporeal oxygenation support while awake has been limited only to the “suture sparing” strategy of tracheal reconstruction during the immediate postoperative period (Schieren et al., 2017).

Conclusion

In conclusion, extracorporeal respiratory support represents the method of choice in patients undergoing complex high-risk airway procedures with the risk of profound hypoxaemia and hypercapnia due to significant intraluminal, external or mixed airway obstruction. Given the risk to benefit ratio, ECMO, either in VV or VA mode is the most viable method providing adequate oxygenation and ventilation support with the acceptable risk of complications. However, it has to be mentioned, that the techniques of extracorporeal oxygenation must be accompanied by other methods of restoring the airways such as tracheal or bronchial dilation and stenting, the mass removal or debulking, or surgical reconstruction of the airways.

References


Extracorporeal Oxygenation in Airway Obstruction


**Extracorporeal Oxygenation in Airway Obstruction**


Remarks on the Hormonal Background of the Male Equivalent of Polycystic Ovary Syndrome

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Key words: Polycystic ovarian syndrome – Premature androgenic alopecia – Androgens – SHBG – Insulin resistance

Abstract: The hypothesis that the most common female endocrine disease, the polycystic ovarian syndrome (PCOS), has a male equivalent, has recently become more widely accepted. The male form of PCOS is marked by alterations in the secretion of gonadotropins, increased insulin resistance, and changes of the levels of several steroid hormones, with clinical manifestations including premature androgenic alopecia (AGA). Because these symptoms are not always found in men with genetic predispositions, knowledge of the male equivalent of PCOS needs to be supplemented by measurements of adrenal 11-oxygenated C19 steroids, particularly 11-keto-, and 11β-hydroxy-derivatives of testosterone and dihydrotestosterone, by focusing on the newly-realized role of skin as an endocrine organ, and by confirming any age-related factors in glucose metabolism disorders in such predisposed men.

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Introduction
Polycystic ovaries are inherited as an autosomal dominant trait (Carey et al., 1993; Govind et al., 1999), so from the genetic point-of-view it seems likely that in families with women suffering from this genetic predisposition for PCOS (polycystic ovarian syndrome), certain symptoms of PCOS may also appear in males. Starting with early hypotheses that male symptoms may include hypertrichosis, insulin resistance, disorders in the secretion of gonadotropins, and premature androgenic alopecia (AGA), more recent results have confirmed the existence of the male equivalent of PCOS, with premature AGA being the most conspicuous symptom (Dušková and Stárka, 2006). Experts on PCOS generally agree (Aversa et al., 2020) that the male equivalent of PCOS is a disease that brings not just dermal and metabolic symptoms and the increasing the risk of “diseases of civilization”, but in affected males it could also influence fertility.

PCOS
Polycystic ovarian syndrome is one of the most common endocrine diseases in women, affecting about 6–15% of the female population in developed countries. Azziz et al. (2004) stated that 82% of female hyperandrogenemia is a result of PCOS. The syndrome has been long recognized, though under various names such as sclerotic ovarian degeneration (1845), a cystic form of chronic oophoritis (1895), and from 1935 as Stein-Leventhal syndrome (Stein and Leventhal, 1935) after the discoverers of the hormonal nature of the disease. In the 1970s, the term polycystic ovarian syndrome became more widely used, and 1980 saw the inclusion of glucose metabolism disorders with emphasis on increased insulin resistance to the syndrome spectrum (Burghen et al., 1980). One key marker is a lowered follicle-stimulating hormone (FSH) level compared to luteinizing hormone (LH), so a defect in the hypothalamic-pituitary axis may be hypothesized, but the pathogenesis of PCOS is not yet entirely clear.

Even a precise definition of this disease was long lacking. According to the Rotterdam criteria of the International Gynecological Association, updated in 2012 (Fauser et al., 2012), affected women must fulfil two of three criteria; namely disorders in the menstrual cycle with anovulation, hyperandrogenemia (whether clinically or according to laboratory hormonal results), and polycystic ovaries demonstrated by imaging methods (Dumesic et al., 2015). When considering the male equivalent of PCOS, this definition is clearly inappropriate.

AGA
Androgenic alopecia is the loss of hair due to shortening the anagen phase of hair cycle, and can be diffuse or general but usually proceeds with hair loss at the so-called “widow’s peaks” and at the vertex. Such hair loss affects up to 80% of men and 50% of women over the course of their life. Premature androgenic alopecia (AGA) is defined as alopecia that occurs before 30 years of age, or according to
some authors before 35 years of age, and reaching at least stage III on the Hamilton-Norwood scale. It seems that dihydrotestosterone likely plays the most significant role of hormonal factors affecting hair loss. Changes in the hair growth cycle during the onset of AGA have been described in detail, but the actual causes of AGA are still largely unknown (Kaliyadan et al., 2013). It is clear, however, that the onset of AGA is multifactorial, with genetic background playing a key role (Martinez-Jacobo et al., 2018).

**Associations of AGA and PCOS in men**

Based on the autosomal genetic transmission of PCOS, and symptoms of PCOS in the men of families where women have been often stricken with PCOS, researchers have proposed the existence of a male equivalent of PCOS. Suggested signs have included excessive body hair loss (Cooper et al., 1968), changes in gonadotropin and testosterone levels (Givens and Andersen, 1975), premature balding (Ferriman and Purdie, 1979; Lunde et al., 1989; Govind et al., 1999), and insulin resistance (Legro, 2000). At first, most studies dealt with just hypotheses, causalities, or data on men from families with an incidence of PCOS in females. According to a recent meta-analysis (Di Guardo et al., 2020), the first study on the hormonal status of premature AGA was from 2005 (Stárka et al., 2005). Some studies of our group were published even earlier (Stárka et al., 2000, 2004, 2005; Dušková et al., 2004). Currently, however, the male equivalent of PCOS has been demonstrated by multiple studies and has generally been accepted (Cannarella et al., 2017, 2018, 2020; Di Guardo et al., 2019, 2020).

It is also important to note that the attribute “premature” regarding AGA as being before 30 or 35 years of age was chosen more-or-less randomly. Not all men with premature AGA can be considered to be carriers of a male equivalent of PCOS (Dušková et al., 2004; Cannarella et al., 2020). And there is no reason not to search for such an equivalent in men with a later onset of hair loss. In light of the higher prevalence of (non-premature) AGA in men than the prevalence of PCOS in women would indicate, it is likely that only some men with AGA indeed suffer from a male equivalent of PCOS. Especially in these men, hormonal changes may give us clues (Dušková et al., 2004).

Additional signs of a male equivalent of PCOS include clinical epidermal markers attributed to hyperandrogenemia like premature AGA, such as acne and increased body-hair, as a result of hormonal changes. Such changes include increased adrenal secretion activity (dehydroepiandrosterone sulfate – DHEAS, 17α-hydroxyprogesterone), low FSH or increased LH/FSH ratio, and increased free testosterone and anti-mullerian hormone. Metabolic abnormalities include insulin resistance, hyperglycemia, hyperinsulinemia, a tendency to be overweight, and lower levels of sex hormone binding globulin (SHBG). At more advanced ages, metabolic disorders often lead to diabetes mellitus type 2, cardiovascular disease, and more frequent prostate disease (Cannarella et al., 2020).
While in PCOS women slight hyperandrogenemia is present (indeed by definition) with increased levels of circulating total testosterone (Dumesic et al., 2015), in men with AGA levels of total testosterone have been found to be within reference ranges (Dušková et al., 2004), slightly increased (Sanke et al., 2016) or even slightly lowered (Canarella et al., 2020). And even though levels of total testosterone in men with AGA may differ significantly from controls, there are still within reference ranges. Lowered SHBG likely plays a role, so that even in men with lower total testosterone the free fraction is still normal. Lowered SHBG levels were already documented in the first hormonal studies on the male equivalent of PCOS (Stárka et al., 2000) and confirmed in many later studies (Arias-Santiago et al., 2011; Sanke et al., 2016; Cannarella et al., 2017, 2018, 2020). Levels of free testosterone, however, do not by themselves explain the clinically observed hyperandrogenization, so other androgens must be considered, including the already-mentioned adrenal androgens, but also other less-studied markers such as adrenal 11-oxygenated C19-steroids. In fact, these have been found at higher concentrations in women with PCOS (O’Reilly et al., 2017). The question remains, if it could be a specific marker for PCOS. A large study focused only on the relationship between body hair distribution and serum androgens concentrations revealed that 11-oxygenated androgens were not positively associated with greater body hair (Skiba et al., 2020). The principal limitation of this study is only self-reported information of body hair distribution. Personal perception of body hair intensity could influence the results.

Both women with PCOS and men with premature androgenic alopecia have shown increased DHEAS levels (Dušková et al., 2004; Stárka et al., 2004, 2005; Dumesic et al., 2015; Sanke et al., 2016). Significantly higher DHEAS levels were found in 75% of women fulfilling criteria for PCOS, while both 17α-hydroxy-progesterone and DHEAS were higher in about half (21 of 46) of men with premature AGA (Cannarella et al., 2020). These results reflect the higher activity of the zona reticularis in the adrenal cortex, and a slightly increased activity of adrenal steroidogenesis in PCOS patients is an accepted explanation. From this point-of-view, it would be useful to measure a wide spectrum of adrenal androgens in men with AGA or with suspected male-equivalent PCOS, as has been done in women (O’Reilly et al., 2017). It has recently been shown (Storbeck et al., 2013; Turcu et al., 2018) that important roles in adrenal androgen effects are played by the above-mentioned 11-oxygenated C19 steroids. Of these, 11-keto-testosterone, 11-keto-dihydrotestosterone, and 11β-hydroxy-testosterone are similar to testosterone itself in concentrations and biological activities. How these 11-oxygenated androgens affect hair loss has not yet been studied. It may be speculated, however, that higher adrenal androgen secretion, as yet only observed only for DHEAS, 17α-hydroxy-progesterone, and androstenedione, leads also to higher levels of these highly-effective androgens, and that these adrenal products likely play a role in the yet poorly-studied function of skin as an endocrine organ (Zouboulis, 2009; Slominski et al., 2015).
As for metabolic disorders, premature AGA has been associated with insulin resistance (Matilainen et al., 2000; Vrbíková et al., 2002; Stárka et al., 2005; González-Gonzáles et al., 2009; Arias-Santiago et al., 2011; Sanke et al., 2016; Cannarella et al., 2017; Di Guardo et al., 2020). This has been proposed as a marker for the male equivalent of PCOS (Matilainen et al., 2000), but in light of its wide population distribution range it is likely not a very useful diagnostic marker. In addition, not all authors have found worsening insulin sensitivity. This is also the reason that some authors have concluded that premature balding under the age of 30 years in brothers of women with PCOS should not be considered a symptom of male-equivalent PCOS (Lenarcik et al., 2011). These ambiguous findings may indeed be due to the higher incidence of AGA compared to the incidence of female PCOS in the population. The prevalence of premature AGA compared to PCOS is not yet clearly known, and we do not yet know what precise role is played by a higher sensitivity to androgens in women than in men, or how many cases of non-premature AGA can be categorized as equivalent. Lowered insulin resistance has in fact been found in some men with premature AGA that had characteristic hormonal changes (Dušková et al., 2004; Cannarella et al., 2020).

Questions regarding metabolic changes and disorders of insulin resistance are even ongoing in women with PCOS, with results complicated by the higher incidence of obesity in these women, introducing problems with interpretation. Only some studies have found insulin sensitivity disorders in slender women with PCOS, and most authors have not confirmed these results (Šimková et al., 2020). This adds even greater complexity to the search for a precise definition of a male equivalent of PCOS. Moreover, while in women with PCOS it is known how alterations of various factors in glucose metabolism change with age, this is not yet the case in males with premature AGA or the male equivalent of PCOS.

Conclusion

The existence of a male equivalent of PCOS has been supported by a number of recent findings. These include hormonal changes, premature androgenic alopecia, and a tendency for insulin resistance. However, a precise definition and diagnostic criteria still need to be delimited, and this will be just as difficult in men as it has been in women.

References


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Male Equivalent of PCOS
Comparison of the Chemiluminescence Immunoassay LIAISON®
with the Radioimmunoassay for Aldosterone and Renin Measurement

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Abstract: Determination of renin plasma levels is useful in the diagnosis of hypertension and in the therapeutic follow-up of hypertensive patients. Plasmatic concentration of renin decreases in patients with hypertension due to a primary hyperaldosteronism, contrary to renovascular hypertension where concentrations of renin and aldosterone are both elevated. Blood samples (serum, EDTA plasma) were analysed using two different chemiluminiscent methods CLIA LIAISON® and radioimmunoassay for aldosterone (IMMUNOTECH Beckman Coulter) and renin (Cisbio Bioassay) measurements were compared. We used both methods to ascertain the correlation between serum vs. EDTA plasma levels of aldosterone (RIA, CLIA) and renin (IRMA, CLIA) and to compare aldosterone to renin ratios for CLIA and for radioimmunoassay: serum aldosterone to plasma renin and plasma aldosterone to plasma renin. We compared serum aldosterone CLIA vs. RIA (r_P=0.933, P<0.001) and plasma renin determined using CLIA vs. IRMA (r_P=0.965, P=0.062). Furthermore, we used both methods to establish the correlation between the serum vs. plasma levels of aldosterone: RIA (r_P=0.980, P<0.001); CLIA (r_P=0.994, P=0.353) and serum vs. plasma levels of renin: IRMA (r_P=0.948,

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Comparison of Aldosterone and Renin Immunoassays

P<0.001); CLIA (r_p=0.921, P=0.011). Aldosterone (serum, plasma) to plasmatic renin ratios for CLIA (r_p=0.999, P=0.286) and for radioimmunoassay (r_p=0.992, P=0.025). Our data demonstrate that renin and aldosterone concentrations obtained using CLIA correlate with renin and aldosterone concentrations using radioimmunoassay methods. Correlation coefficients of pair results ranged from 0.921 to 0.994. Aldosterone (serum, EDTA plasma) to plasmatic renin ratios are comparable and any of them can be used with no significant differences found.

Introduction
Primary hyperaldosteronism (PH) is nowadays the most frequent form of secondary endocrine-mediated hypertension. Primary hyperaldosteronism (PH) diagnostics based on the determination of aldosterone levels, plasma renin activity and their ratio has become obsolete. Instead of the demanding determination of plasma renin activity, direct renin measurement is now performed (Wedatilake et al., 2011; Jensen et al., 2014; Douillard et al., 2016). Renin also plays a role in the release of aldosterone, a hormone that also controls the body sodium and water balance. Measurement of serum aldosterone in conjunction with plasma renin and their ratio are used clinically to differentiate between primary and secondary hyperaldosteronism (Trenkel et al., 2002; Reincke et al., 2003; Barigou et al., 2015).

At our laboratory, we used to perform determination of serum levels of aldosterone and plasma renin by radioimmunoassay. Our tests on two analytical systems were mainly focused on comparing the results of serum aldosterone (RIA vs. CLIA) and plasma renin (IRMA vs. CLIA). Subsequently, we tested how much the choice of material (serum, plasma) affects the results of the analysis of aldosterone and renin (CLIA vs. RIA or IRMA). The aldosterone and renin concentrations dependency on sample material (serum and EDTA plasma) was evaluated using both analytical systems. Our task was to compare the CLIA LIAISON® technology with a radioimmunoassay method in samples of serum and EDTA plasma. Parallel sample testing was conducted using both analytical methods, using the LIAISON® automated immunoanalyzer (CLIA) and the STRATEC SR 300 automated immunoanalyzer (RIA, IRMA). For both analytical systems (CLIA, RIA or IRMA) we compared the ratios: serum aldosterone to plasmatic renin and plasmatic aldosterone to plasmatic renin.

Plasma renin activity (angiotensin I) was not determined in tested samples.

Material and Methods
Material
From single blood draw we prepared two sets of identical serum samples and EDTA plasma samples for parallel determination of aldosterone (CLIA and RIA) and two sets of identical EDTA plasma samples for renin determination (CLIA and IRMA). The samples were stored at –20 °C until the measurements, but not longer than one month. Fresh samples analysed by routine method were chosen to cover low to high values along the calibration curve. Icteric and lipemic samples as well as samples...
with results out of calibration range for any of analytes (renin, aldosterone) were excluded. Samples tested were obtained from men and women aged 20–60 years. Samples come from both inpatient and outpatient hospital populations after appropriate diet. All were taken in sitting position after at least 30 minutes long resting from fasting patients in morning hours. Samples were transported in 5–15 °C and centrifuged in room temperature within one hour at 3,000 rpm for 10 min. Samples were stored at –20 °C for maximum of 1-month prior analysis.

**Methods**

Aldosterone and renin were determined by two methods, in parallel CLIA and radioimmunoassay. Aldosterone and renin were determined according to the instructions for use given by producers.

**RIA procedure of aldosterone**

Aldosterone was determined using the ALDOSTERONE RIA kit (Beckman Coulter, France). The kit is intended for direct quantitative determination of aldosterone in serum and EDTA plasma for *in vitro* diagnostics. Aldosterone determination is a competitive radioimmunoassay (RIA, a radionuclide marked $^{125}$I-aldosterone). A total of 50 µl plasma or serum and 500 µl of the tracer ($^{125}$I-aldosterone) are incubated for 3 hours at 18–25 °C with a solid phase anti-aldosterone monoclonal antibody. At the end of the incubation the unbound material is removed, the concentration of aldosterone is calculated by extrapolation of a spline curve with six calibrators.

Performance characteristics of aldosterone RIA assays: limit of quantification (LOQ) 6.0 ng/l, intra-assay: CV (coefficient of variation) ≤ 9.5%, inter-assay: CV ≤ 10.4%, measurement range 6.0–2,000 ng/l.

**CLIA procedure of aldosterone**

Chemiluminescence tests provided by the LIAISON®Aldosterone assay (DiaSorin, USA). The kit is intended for quantitative determination of aldosterone in human serum and EDTA plasma for the purposes of *in vitro* diagnostics. The method for the quantitative determination of the aldosterone assay is a competitive assay that uses sheep monoclonal antibody to capture the aldosterone molecule. The principle components of the test consist of magnetic particles (solid phase) coated with anti-sheep antibody that binds sheep anti-aldosterone monoclonal antibody. An aldosterone labelled conjugate containing an isoluminol derivative competes with aldosterone from the calibrators, controls and patient samples. During the first incubation (55 minutes), sample is incubated with a specific anti-aldosterone monoclonal antibody. Following this incubation, the conjugate is added and competes with aldosterone for an additional amount of time. After the second incubation the unbound material is removed with a wash cycle. The starter reagents are then added and a flash chemiluminescent reaction is initiated. The light signal is measured by a photomultiplier as relative light units (RLU) and is inversely proportional to
the concentration of aldosterone present in the calibrators, controls and patient samples. The final values of aldosterone are calculated with a two-point working curve adjusted against a stored master curve. The analyser automatically calculates renin concentrations for the unknown samples expressed as ng/l and grades the results.

Performance characteristics of aldosterone CLIA assays: limit of quantification (LOQ) 19.1 ng/l, intra-assay: CV = 1.8–4.2%, inter-assay: CV = 5.6–10.5%, measurement range 9.7–1,000 ng/l.

IRMA procedure of renin
Renin was measured using the RENIN III GENERATION kit (Cisbio Bioassay, France). The kit is intended for quantitative determination of direct renin in EDTA plasma for in vitro diagnostics. The principle is based on non-competitive immunoradiometric assay (IRMA, two anti-renin monoclonal mouse antibodies (MAb); 1st MAb is fixed to the vial wall (specific for renin, prorenin); 2nd MAb is marked with radionuclide $^{125}$I (specific for renin). A total of 300 µl plasma and 100 µl of the tracer (2nd MAb-$^{125}$I) are incubated for 3 hours at 18–25 °C with a solid phase monoclonal specific antibody for renin (1st MAb). After incubation, the unbound material is removed with a wash cycle, the concentration of renin is calculated by extrapolation of a spline curve with six calibrators.

Performance characteristics of renin IRMA assays: limit of quantification (LOQ) 1.0 ng/l, intra-assay: CV ≤ 3.6%, inter-assay: CV ≤ 5.0%, measurement range 2.5–320.0 ng/l.

CLIA procedure of renin
The LIAISON® Direct Renin kit (DiaSorin, Italy) is intended for quantitative determination of renin concentration in human EDTA plasma samples for the purposes of in vitro diagnostics. The method for the quantitative determination of renin is a sandwich CLIA. A specific mouse monoclonal antibody is coated on the magnetic particles (solid phase), that recognizes both renin and prorenin; another mouse monoclonal antibody (specific for renin) is linked to an isoluminol derivative (isoluminol-antibody conjugate). During the incubation (38 minutes), renin present in calibrators or controls as well as renin and prorenin present in samples bind to the solid phase monoclonal antibody, and subsequently the antibody conjugate reacts with renin already bound to the solid phase. A sandwich is formed only in the presence of renin molecules that bridge both antibodies. After incubation; the unbound material is removed with a wash cycle. Subsequently, the starter reagents are added and a flash chemiluminescence reaction is thus induced. The light signal, and hence the amount of isoluminol-antibody conjugate, is measured by a photomultiplier as relative light units (RLU) and is directly proportional to renin concentration present in calibrators, patient samples or controls. The final values of plasma renin concentration are calculated with a two-point working curve.
adjusted against a stored master curve. The analyser automatically calculates renin concentrations for the unknown samples expressed as pg/ml and grades the results.

Performance characteristics of renin CLIA assays: limit of quantification (LOQ) 0.3 ng/l, intra-assay: CV ≤ 2.38%, inter-assay: CV ≤ 7.31%, measurement range 2.5–316.5 ng/l.

Scheme of experiments of CLIA vs. RIA or IRMA assays
1) CLIA vs. IRMA of plasma renin levels
2) CLIA vs. RIA of serum aldosterone levels
3) CLIA vs. IRMA of renin levels between serum and plasma
4) CLIA vs. RIA of aldosterone levels between serum and plasma
5) Aldosterone (serum, plasma) to renin (plasma) ratios for CLIA and for RIA

Statistical analysis
The statistical analysis was conducted using MedCalc version 4.31.010. Continuous variables are expressed as means and 95% confidence interval (CI) for the mean. For continuous variables, Bland-Altman plots, Passing-Bablok regression analysis and Pearson’s correlation coefficient (r_p) were used to assess differences of data measured by CLIA and radioimmunoassay (RIA, IRMA). Preselected level of significance was P<0.05.

Results
Comparison of EDTA plasma renin levels between CLIA and IRMA
For the evaluation of plasmatic renin levels differences between CLIA and IRMA 79 samples were measured. Statistical characteristics of renin concentrations using CLIA and IRMA are reported in Table 1. Pearson’s correlation coefficient is: r_p=0.965 (slope = 0.964, intercept = –0.654, P=0.062) demonstrates sufficient compliance between CLIA and IRMA.

Comparison of serum aldosterone levels between CLIA and RIA
For the evaluation of serum aldosterone level differences between CLIA and RIA 90 samples were measured. Table 1 contains statistical parameters. Serum aldosterone levels using CLIA are lower in comparison with serum aldosterone levels using RIA. Pearson’s correlation coefficient is: r_p=0.933 (slope = 0.607, intercept = 23.81, P<0.001). Differences in average values, medians and standard deviations are reported in Table 1.

Comparison of aldosterone levels obtained by RIA and CLIA for serum and EDTA plasma
We used RIA to compare aldosterone concentrations (serum vs. plasma, n=45) and CLIA to compare aldosterone concentrations (serum vs. plasma, n=43). Basic statistical characteristics is shown in Table 2 (RIA, CLIA). The results of the Bland-
Table 1 – Statistical characteristics of measuring serum aldosterone by CLIA vs. RIA and of measuring plasma renin by CLIA vs. IRMA

<table>
<thead>
<tr>
<th>Statistical parameters</th>
<th>Aldosterone serum levels</th>
<th>Renin plasma levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CLIA</td>
<td>RIA</td>
</tr>
<tr>
<td>Sample size</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>Range (ng/l)</td>
<td>24.0–536.0</td>
<td>26.4–840.2</td>
</tr>
<tr>
<td>Arithmetic mean (ng/l)</td>
<td>153.41</td>
<td>195.31</td>
</tr>
<tr>
<td>95% CI for the mean</td>
<td>132.32–174.50</td>
<td>161.07–229.56</td>
</tr>
<tr>
<td>Median (ng/l)</td>
<td>124.50</td>
<td>159.70</td>
</tr>
<tr>
<td>Pearson’s correlation coefficient (r_p)</td>
<td>0.933 (0.899–0.955)</td>
<td>0.965 (0.945–0.977)</td>
</tr>
<tr>
<td>Slope</td>
<td>0.607 (0.610–0.750)</td>
<td>0.964 (0.898–1.049)</td>
</tr>
<tr>
<td>Intercept</td>
<td>23.81 (13.83–31.32)</td>
<td>–0.654 (–1.687–[–0.007])</td>
</tr>
<tr>
<td>Paired samples t-test (T)</td>
<td>5.066</td>
<td>1.892</td>
</tr>
<tr>
<td>Degrees of freedom (DF)</td>
<td>89</td>
<td>78</td>
</tr>
<tr>
<td>P(T≤t) two-tail</td>
<td>&lt;0.001</td>
<td>0.062</td>
</tr>
<tr>
<td>t (t critical two-tail)</td>
<td>1.986</td>
<td>1.989</td>
</tr>
</tbody>
</table>

CI – confidence interval

Table 2 – Statistical characteristics of measuring aldosterone levels by RIA and by CLIA between serum and EDTA plasma

<table>
<thead>
<tr>
<th>Statistical parameters</th>
<th>Aldosterone levels by RIA</th>
<th>Aldosterone levels by CLIA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>serum EDTA plasma</td>
<td>serum EDTA plasma</td>
</tr>
<tr>
<td>Sample size</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>Range (ng/l)</td>
<td>6.6–359.3</td>
<td>8.1–391.6</td>
</tr>
<tr>
<td>Arithmetic mean (ng/l)</td>
<td>111.96</td>
<td>134.01</td>
</tr>
<tr>
<td>95% CI for the mean</td>
<td>83.23–140.69</td>
<td>102.17–165.87</td>
</tr>
<tr>
<td>Median (ng/l)</td>
<td>96.90</td>
<td>128.0</td>
</tr>
<tr>
<td>Pearson’s correlation coefficient (r_p)</td>
<td>0.980 (0.963–0.989)</td>
<td>0.994 (0.988–0.997)</td>
</tr>
<tr>
<td>Slope</td>
<td>1.137 (1.059–1.264)</td>
<td>0.978 (0.938–1.016)</td>
</tr>
<tr>
<td>Intercept</td>
<td>5.35 (0.406–10.927)</td>
<td>0.727 (–1.613–4.585)</td>
</tr>
<tr>
<td>Paired samples t-test (T)</td>
<td>–6.501</td>
<td>0.002</td>
</tr>
<tr>
<td>Degrees of freedom (DF)</td>
<td>44</td>
<td>42</td>
</tr>
<tr>
<td>P(T≤t) two-tail</td>
<td>&lt;0.001</td>
<td>0.353</td>
</tr>
<tr>
<td>t (t critical two-tail)</td>
<td>2.015</td>
<td>2.017</td>
</tr>
</tbody>
</table>

CI – confidence interval

Comparison of Aldosterone and Renin Immunoassays
Figure 1 – The Bland-Altman plots and the Passing-Bablog regression line. A) The Bland-Altman plots for data of aldosterone between average levels of serum and plasma vs. ratio aldosterone levels of serum to plasma using RIA. With the representation of the limits of agreement (dot-and-dash line), from −1.96 SD to +1.96 SD (SD – standard deviation). B) The Passing-Bablog regression line of aldosterone concentrations between serum and plasma using RIA (Pearson’s r=0.980, P-value < 0.001, y = 1.137x + 5.35).
Figure 2 – The Bland-Altman plots and the Passing-Bablok regression line. A) The Bland-Altman plots for data of aldosterone between average levels of serum and plasma vs. ratio aldosterone levels of serum to plasma using CLIA. With the representation of the limits of agreement (dot-and-dash line), from –1.96 SD to +1.96 SD (SD – standard deviation). B) The Passing-Bablok regression line of aldosterone concentrations between serum and plasma using CLIA (Pearson’s r=0.994, P-value < 0.353, y = 0.978x + 0.727).

Comparison of Aldosterone and Renin Immunoassays
Altman analysis are shown on Figure 1A (RIA); Figure 2A (CLIA) and Passing-Bablog analysis on Figure 1B (RIA); Figure 2B (CLIA) respectively.

Average plasma aldosterone values determined by RIA are higher (19.7%) in comparison with serum values. Pearson’s regression analysis demonstrates correlation coefficient, $r_P = 0.980$ (slope = 1.137, intercept = 5.35, $P<0.001$).

Average plasma aldosterone values determined using CLIA were not different in comparison with serum values. Pearson’s regression analysis demonstrates an acceptable correlation coefficient, $r_P = 0.994$. The slope value of 0.978 and the intercept value 0.727 are also acceptable.

Comparison of renin levels with IRMA and CLIA between serum and EDTA plasma

To compare renin levels, we used CLIA technology (serum vs. plasma, $n=38$) and IRMA technology (serum vs. plasma, $n=44$). Table 3 contains basic statistical characteristics for IRMA and CLIA. The results of the Bland-Altman analysis are shown of Figure 3A (IRMA) and Figure 4A (CLIA) and Passing-Bablog analysis on Figure 3B (IRMA) and Figure 4B (CLIA).

Plasma renin levels determined using IRMA are lower by 34.4% on average in comparison to serum levels. The value of Pearson’s correlation coefficient is: $r_P = 0.948$, as well as the slope value of 0.625 are acceptable, however, the intercept value of 0.126 is not sufficient.

Plasma renin levels determined using CLIA are higher by 46.3% on average in comparison to serum levels. The value of Pearson’s correlation coefficient is: $r_P = 0.921$ (slope = 1.597, intercept = 0.332, $P=0.011$).

Comparison of aldosterone to renin ratios

We compared aldosterone to renin ratios (ng/dl) for CLIA and for RIA or IRMA: serum aldosterone to EDTA plasma renin and EDTA plasma aldosterone to EDTA plasma renin. Statistical characteristics of these ratios of aldosterone and renin are shown in Table 4 for CLIA and for RIA. For CLIA ratio values for aldosterone (serum or EDTA plasma) to EDTA plasma renin Pearson’s correlation coefficient is: $r_P = 0.999$, slope 0.959 and intercept 0.014 is acceptable. For radioimmunoassay ratio values for aldosterone (serum or EDTA plasma) to EDTA renin ratios Pearson’s correlation coefficient is: $r_P = 0.992$, slope 1.133 and intercept 0.059 is also acceptable.

Discussion

Contemporary laboratory diagnostics of primary hyperaldosteronism (PH) provides for the determination of aldosterone and renin analytical procedures that are easy to perform and more time affordable than nowadays used radioimmunoassay procedures. These are mainly automated non-isotopic determinations of aldosterone and renin, from which we tested CLIA. It seems that for the diagnostics of PH are preferred non-isotopic direct determinations of both aldosterone and renin and their
### Table 3 – Statistical characteristics of measuring renin levels by CLIA and IRMA between serum and EDTA plasma

<table>
<thead>
<tr>
<th>Statistical parameters</th>
<th>Renin levels by IRMA</th>
<th>Renin levels by CLIA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>serum</td>
<td>EDTA plasma</td>
</tr>
<tr>
<td>Sample size</td>
<td>44</td>
<td>44</td>
</tr>
<tr>
<td>Range (ng/l)</td>
<td>1.10–148.5</td>
<td>0.60–127.2</td>
</tr>
<tr>
<td>Arithmetic mean (ng/l)</td>
<td>27.89</td>
<td>18.29</td>
</tr>
<tr>
<td>95% CI for the mean</td>
<td>17.83–37.95</td>
<td>10.78–25.80</td>
</tr>
<tr>
<td>Median (ng/l)</td>
<td>14.60</td>
<td>9.50</td>
</tr>
<tr>
<td>Pearson’s correlation coefficient (r&lt;sub&gt;p&lt;/sub&gt;)</td>
<td>0.948 (0.906–0.872)</td>
<td>0.921 (0.853–0.959)</td>
</tr>
<tr>
<td>Slope</td>
<td>0.625 (0.532–0.811)</td>
<td>1.597 (1.214–2.123)</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.126 (–1.882–1.531)</td>
<td>0.332 (–1.877–2.523)</td>
</tr>
<tr>
<td>Paired samples t-test (T)</td>
<td>5.127</td>
<td>–2.693</td>
</tr>
<tr>
<td>Degrees of freedom (DF)</td>
<td>43</td>
<td>37</td>
</tr>
<tr>
<td>P(T≤</td>
<td>t)</td>
<td>two-tail</td>
</tr>
<tr>
<td>t (t critical two-tail)</td>
<td>2.018</td>
<td>2.025</td>
</tr>
</tbody>
</table>

CI – confidence interval

### Table 4 – Statistical characteristics of aldosterone (serum, EDTA plasma) to of renin (EDTA plasma) ratios for radioimmunoassay and for CLIA

<table>
<thead>
<tr>
<th>Statistical parameters</th>
<th>Aldosterone to renin ratios for radioimmunoassay</th>
<th>Aldosterone to renin ratios for CLIA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ALDO&lt;sub&gt;S&lt;/sub&gt;/REN&lt;sub&gt;P&lt;/sub&gt;</td>
<td>ALDO&lt;sub&gt;P&lt;/sub&gt;/REN&lt;sub&gt;P&lt;/sub&gt;</td>
</tr>
<tr>
<td>Sample size</td>
<td>38</td>
<td>38</td>
</tr>
<tr>
<td>Range (ng/dl)</td>
<td>0.07–17.0</td>
<td>0.09–23.0</td>
</tr>
<tr>
<td>Arithmetic mean (ng/dl)</td>
<td>2.138</td>
<td>2.618</td>
</tr>
<tr>
<td>95% CI for the mean</td>
<td>0.856–3.421</td>
<td>0.964–4.272</td>
</tr>
<tr>
<td>Median (ng/dl)</td>
<td>0.73</td>
<td>0.85</td>
</tr>
<tr>
<td>Pearson’s correlation coefficient (r&lt;sub&gt;p&lt;/sub&gt;)</td>
<td>0.992 (0.985–0.997)</td>
<td>0.999 (0.998–1.000)</td>
</tr>
<tr>
<td>Slope</td>
<td>1.113 (1.049–1.298)</td>
<td>0.959 (0.924–1.007)</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.059 (0.003–0.100)</td>
<td>0.014 (–0.001–0.038)</td>
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<tr>
<td>Paired samples t-test (T)</td>
<td>2.34</td>
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<td>Degrees of freedom (DF)</td>
<td>37</td>
<td>26</td>
</tr>
<tr>
<td>P(T≤</td>
<td>t)</td>
<td>two-tail</td>
</tr>
<tr>
<td>t (t critical two-tail)</td>
<td>2.026</td>
<td>2.056</td>
</tr>
</tbody>
</table>

ALDO<sub>S</sub> – aldosterone of serum; ALDO<sub>P</sub> – aldosterone of EDTA plasma; REN<sub>P</sub> – renin of EDTA plasma; CI – confidence interval

Comparison of Aldosterone and Renin Immunoassays
Figure 3 – The Bland-Altman plots and the Passing-Bablog regression line. A) The Bland-Altman plots for data of EDTA plasma renin between average levels of serum and plasma vs. ratio renin levels of serum to plasma using IRMA. With the representation of the limits of agreement (dot-and-dash line), from –1.96 SD to +1.96 SD (SD – standard deviation). B) The Passing-Bablog regression line of renin levels between serum and plasma using IRMA (Pearson’s r=0.948, P-value < 0.001, y = 0.625x + 0.126).

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Figure 4 – The Bland-Altman plots and the Passing-Bablog regression line. A) The Bland-Altman plots for data of EDTA plasma renine between average levels of serum and plasma vs. ratio plasma renine levels of serum to plasma using CLIA. With the representation of the limits of agreement (dot-and-dash line), from $-1.96 \, SD$ to $+1.96 \, SD$ (SD – standard deviation). B) The Passing-Bablog regression line of plasma renin between serum and plasma using CLIA (Pearson’s $r=0.921$, P-value = 0.011, $y = 1.597x + 0.332$).

Comparison of Aldosterone and Renin Immunoassays
ratio value as well (Dorrian et al., 2010; Horváth et al., 2012; Burrello et al., 2015; Glinicki et al., 2015).

PH laboratory diagnostics is based over last 45 years also on the ratio of aldosterone and renin or renin activity respectively (Ferrari et al., 2004). Its usage helps to find out patients with PH and distinguish between those who bear the disease and those who do not. Test of renin plasmatic activity is continuously replaced by concentration measurement only (Reincke et al., 2003; Ferrari et al., 2004). Optimization of the ratio for screening purposes is being studied (Jensen et al., 2014; Ma et al., 2018; Russmann et al., 2019). Till now none widely used optimized ratio calculation is known. It is probably caused by different analytical procedures used (RIA, CLIA, ELISA), by the choice of biological material (serum, plasma), by the usage of renin concentration or renin activity and by different units. Even more there is no international consensus on aldosterone/renin ratio cut-off and no guidelines for its interpretation. Discussion published in Clinical Chemistry shows non-comprehensive opinions on ratio usage in screening of PH (Raizman et al., 2015; Vecchiola et al., 2019).

Most studies on PH diagnostics are focused more on optimizing ratio of aldosterone to renin or renin activity calculation, but they do not address correlation between serum and plasmatic levels. In the study by Belaidi et al. (2015), who measured aldosterone in serum using RIA assay (coat-a-count, Siemens, Marburg, Germany) and CLIA using a LIAISON automated analyser (Diason, Saluggia, Italy) RIA and CLIA aldosterone serum concentration were linearly correlated with a slope of 0.988 and an intercept of 70.4 pmol/l. The variations of aldosterone serum concentration obtained with two assays during postural tests were very consistent. Contrary to it in our comparison of serum aldosterone levels between RIA (Beckman) and CLIA (DiaSorin) slope was 0.607 and intercept 23.81 pmol/l.

Glinicki et al. (2015) studied the comparison of aldosterone levels between the serum and EDTA plasma. Aldosterone was measured by RIA (ZenTech, RIAZENco, Belgium). Measured concentrations of aldosterone in plasma (EDTA2K) and serum samples showed high correlation ($r_P=0.979$). The differences between pairs of plasma and serum samples ranged from 37% to 144% (median 75%) (Belaidi et al., 2015). In our study concentrations of aldosterone in serum and EDTA plasma were measured by RIA (Beckman) with correlation 0.980, while differences between pairs of plasma and serum sample were lower 5% to 85% (median 30.6%). Good regression dependency determination of aldosterone in serum between CLIA and RIA was shown. We found the significant effect of sample material (serum vs. EDTA plasma), except of aldosterone determination using CLIA ($r_P=0.994$, slope = 0.978, intercept = 0.727).

Statistical evaluation shows that the choice of material for the determination of renin influences both IRMA and CLIA methods. Renin concentration measurements show significant variations between measurement serum and plasma however both systems have a similar range of the calibration curve (IRMA to 320 pg/ml, CLIA to
316.5 pg/ml) and in both assay systems two mouse monoclonal antibodies are used. The first monoclonal antibody is fixed to the solid phase anti-renin and prorenin (IRMA wall tubes, CLIA, magnetic particles). The second monoclonal antibody specific to renin is labelled with a corresponding detection substance (IRMA, $^{125}$I, CLIA, isoluminol). The assays are referenced to the World Health Organization International Reference Preparation, NIBSC code 68/356. Our values of primary concentration of renin show considerable variability between serum and plasma, which does not yet support determining renin in serum.

The discrepancies among renin assay results could be caused by different specificity of antisera or antibodies may vary between assays. Most commonly it is due to heterophile antibodies or due to endogenous circulating antibodies, of cross-reacting steroids or other interfering substances (Lonati et al., 2014).

Contemporary laboratory diagnostics of PH provides for the determination of aldosterone and renin analytical procedures that are easy to perform and time affordable than radioimmunoassay procedures. These are mainly the automated non-isotopic determination of aldosterone and renin, from which we tested CLIA (Dorrian et al., 2010; Jensen et al., 2014; Burrello et al., 2015).

The main advantage of CLIA is analysis quickness (renin 38 minutes, aldosterone 55 minutes) and shortening of the turnaround time. It is also the main reason for daily availability of determinations, for RIA methods the real frequency is usually just once or twice a week.

**Conclusion**

Current requirements on biochemistry laboratories include increasing workflow and productivity with rapid responses from the laboratory to clients. In contrast to radioimmunoassay methods, the automated non-isotopic technology improves analytical comfort and the possibility of sample processing completion on the day of receipt by the laboratory. Our study has shown that these requirements are met by a fully automated immunoassay LIAISON XL, which has enabled us fluently transfer the determination of aldosterone and renin from RIA/IRMA to CLIA method. We found the significant effect of sample material (serum vs. plasma) with exception of aldosterone determination using CLIA. We can conclude that our results show good concordance also in plasmatic but not in serum renin (IRMA vs. CLIA). Automated aldosterone and renin chemiluminescent assays are a reliable alternative to the radio-immunometric method. Aldosterone to renin ratios (ng/dl) for CLIA and for RIA or IRMA (both serum aldosterone to EDI plasma renin and EDTA plasma aldosterone to EDTA plasma renin) are comparable and any of them can be used. The main advantage of CLIA methods are good standardization, automatization and simple sample processing.

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Salmonella Paratyphi Infection: Use of Nanopore Sequencing as a Vivid Alternative for the Identification of Invading Bacteria

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Key words: Enteric fever – Salmonella – Paratyphoid fever – Nanopore sequencing – Pathogen identification

Abstract: In our study we present an overview of the use of Oxford Nanopore Technologies (ONT) sequencing technology on the background of Enteric fever. Unlike traditional methods (e.g., qPCR, serological tests), the nanopore sequencing technology enables virtually real-time data generation and highly accurate pathogen identification and characterization. Blood cultures were obtained from a 48-year-old female patient suffering from a high fever, headache and diarrhea. Nevertheless, both the initial serological tests and stool culture appeared to be negative. Therefore, the bacterial isolate from blood culture was used for nanopore sequencing (ONT). This technique in combination with subsequent bioinformatic analyses allowed for

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prompt identification of the disease-causative agent as *Salmonella enterica* subsp. *enterica* serovar Paratyphi A. The National Reference Laboratory for *Salmonella* (NIPH) independently reported this isolate also as serovar Paratyphi A on the basis of results of biochemical and agglutination tests. Therefore, our results are in concordance with certified standards. Furthermore, the data enabled us to assess some basic questions concerning the comparative genomics, i.e., to describe whether the isolated strain differs from the formerly published ones or not. Quite surprisingly, these results indicate that we have detected a novel and so far, unknown variety of this bacteria.

**Introduction**

Rapid and unambiguous identification of the pathogenic organism represents a crucial step for the subsequent treatment of patients. However, in many cases this might be complicated, for example by atypical clinical symptoms of a patient. Furthermore, atypical behavior of a pathogen itself may hamper the usual routines as well as the choice of relevant identification techniques. Despite the number of available, well established and certified methodologies and procedures designed for the determination of infectious disease causative agents (Váradi et al., 2017), an atypical behavior of a pathogen could lead to erroneous outcomes.

Within this study we want to focus on Enteric fever, which is a human disease caused by the pathogenic bacteria *Salmonella enterica*. Its clinical identification is usually based on some of the following approaches, or rather, a combination of those approaches: microbiological cultivation methods (Wain et al., 1998); serological tests, e.g., the Widal test (Olopoenia, 2000); PCR based tests (Song et al., 1993; Tennant et al., 2015) and of course the symptomatic manifestation of the disease itself (Matono et al., 2017). The disadvantage of these, let’s say, standard approaches represent the fact that the diagnostics is based upon many variables including for example idiosyncratic human reactions, ambiguous clinical manifestation of closely related causative agents (Maskey et al., 2006), as well as the experience and erudition of the responsible physician.

On the other hand, the application of the Third Generation Sequencing (Schadt et al., 2010), Oxford Nanopore Technologies (ONT) sequencing (Magi et al., 2018), offers unique performance, which already plays its role in current, but more importantly, in tomorrow’s medical and biological research (Fuselli et al., 2018; Ton et al., 2018) and maybe even in the future daily routines.

The main advantage of the ONT nanopore sequencing is represented by several facts, the sequencing device (i.e., the MinION) is delivered within so called “starter pack” (ONT) basically for free, furthermore, the MinION is a portable USB powered device, which allows its deployment even in field conditions, e.g., literally even in a jungle (Watsa et al., 2019). Nevertheless, its major benefits are the real-time generation of the sequencing data and the read length representing the key parameter for subsequent bioinformatic analyses (Norris et al., 2016).
Within this study we point out that this rapidly evolving techniques have a great potential and maybe, even if not in their current form, represent the future of diagnostic and analytical practice.

**Case report**

A 48-year-old female was admitted to the Department of Infectious Diseases with a diarrhea following a 10-day travel in India (Delhi and the northern part of India). She had been vaccinated against typhoid fever and cholera three years ago. Initial stool sample culture was negative as well as parasite detection in the stool. The diarrhea was resolved by symptomatic therapy within 14 days. After five days, the patient developed a severe headache, fever with spikes up to 39 °C, chills and myalgias. Even after the following four days these symptoms persisted and the patient visited an emergency department. She had no chronic disease and her medical history included a tonsillectomy in childhood due to recurrent episodes of tonsillitis and meningitis of unknown etiology at the age of ten.

The clinical examination upon admittance was unremarkable other than a temperature of 37.6 °C. Initial laboratory investigations revealed raised liver enzymes and a C-reactive protein (CRP) level of 16 mg/l (normal value < 5 mg/l). Full blood count, serum electrolytes, and creatinine were within normal limits. Chest and abdominal ultrasound showed no significant abnormalities except mild splenomegaly. Malaria blood microscopical and immunochromatographic tests, Widal test, Dengue and Zika serology tests were all negative.

Empirical treatment with doxycycline 100 mg twice daily was initiated after microbiological sampling. However, three blood cultures taken on admission yielded *Salmonella enterica* and therefore the therapy was switched to ceftriaxone 2 g once daily intravenously. Urine and stool cultures were negative for bacterial pathogens. Fever spikes persisted over the next two days on therapy. Therefore, dosing of ceftriaxone was increased to 2 g twice daily intravenously, and metronidazole 500 mg three times daily was added. A repeat abdominal ultrasound test and CT (computed tomography) radiograph revealed no abscess formation.

All symptoms of the patient subsequently resolved and from the 9th day of hospitalisation she remained afebrile. The therapy was switched to co-ampicillin and metronidazole and on the 13th day of hospitalisation the patient was discharged. CRP and liver function tests normalized after 14 days and completion of a 2-week course of antibiotic treatment. After her final check-up in the clinic, she was clinically well and had made a full recovery.

**Material and Methods**

The initial blood culture isolate of the patient was grown in hemocultivation bottles using the Bact/Alert 3D platform (bioMérieux, Durham, North Carolina, USA) and it was preliminarily identified as *Salmonella* sp. using mass spectrometer IVD MALDI Biotyper/System/Microflex™ LT/SH System (Bruker Daltonic GmbH,
Bremen, Germany). The identification scores of this technique measured in duplicate were 2.09 and 2.25, respectively. Nevertheless, the strain was sent to the National Reference Laboratory for Salmonella of the National Institute of Public Health (NIPH) in Prague for further analysis.

In order to proceed with the ONT nanopore sequencing, the DNA was isolated and purified from the blood culture using High Pure PCR Template Preparation Kit (Roche). Obtained DNA was quantified on the Qubit fluorometer (Invitrogen) and used for sequencing library preparation (SQK-LSK109, ONT). The sequencing itself was performed on the ONT GridIONx5 platform using the R9.4.1 chemistry (Flow Cell). The so called “base-calling”, i.e., the conversion from raw electric-current signal measured by the device to biologically meaningful bases, was performed in real time using the ONT GridION sequencing software version 19.12.2.

Acquired reads were subject to the read classification pipeline, i.e., each single read was compared to a database in order to estimate its taxonomic origin (based upon sequence similarity criteria). The database was built using the NCBI (National Center for Biotechnology Information) archives from Archaea, Bacteria and Viral species with project status “complete genome” in June 2019 and contains over 9,000 unique bacterial genomes/isolates. We used the freely available software Centrifuge for this purpose (Kim et al., 2016), whose outputs were further analyzed and re-interpreted using custom R scripts (R Core Team, 2019), which take advantage of the R packages taxize (Chamberlain and Szöcs, 2013) and data-table (Dowle and Srinivasan, 2019). The classification results were further analysed using the Krona interactive tool (Ondov et al., 2011).

Since this methodology is based upon analysis of “erroneous” data (Kolmogorov et al., 2019) and thus might be easily doubted, despite the fact that we try to account for this type of error, we use another mostly independent methodology to confirm our results. We thus assembled the sequence data into a draft genomic assembly, which technically results in a consensus sequence, which is at least by an order of magnitude more accurate. This data sequence has been compared to custom BLAST (Zhang et al., 2000) database. This database was built from 1,498 genomic sequences that were downloaded from the NCBI “nuccore” database on 2.2.2020. Included were only those sequences that fulfilled all the following conditions: 1) contained the term “Salmonella enterica” within the organism name; 2) contained the term “complete genome” within their description (title); and 3) their length was between 3.5 to 10 million base pairs.

Furthermore, in order to obtain some insights about the isolated strain, i.e., some insights about its topological properties or stability (e.g., deletions, duplications, insertions...), we compared our isolate to the reference strain Salmonella enterica subsp. enterica serovar Paratyphi A str. ATCC 11511 (GenBank accession number NZ_CP019185.1). We mapped all the reads against the reference using the Minimap2 ver. 2.12 (Li, 2018). Some necessary operations, e.g., file format conversions, were conducted with SAMtools ver. 1.9 (Li et al., 2009). Structural
variants (SV) themselves were estimated using the Sniffles ver. 1.0.11 variant-calling tool (Sedlazeck et al., 2018). Inferred SV were visually inspected, i.e., we inspected aligned reads to the reference in the pre-defined presumably SV regions using the CLC Genomics workbench tool ver. 11.0.1 (Qiagen). Genes, or sequences with meaningful open reading frames present within predicted SV regions in the reference strain were further annotated against the NCBI “nr” (protein) database using the Blast2GO tool (Götz et al., 2008), only genes that were not annotated as “hypothetical protein” or “putative ...” were taken into account.

**Results and Discussion**

In total we acquired 127,161 reads that passed the quality threshold criteria estimated by the base-calling software, i.e., primarily the mean per base quality of a read (Phred quality score > 7). The mean read length equalled 1,527 base pairs (bp) while the longest one achieved 43,235 bp.

**Classification**

The reads were attempted to be classified with the Centrifuge and acquired classification results were further re-analyzed using three different stringency thresholds. Unlike, e.g., BLAST (Altschul et al., 1990), the Centrifuge seeks only so called “exact match” against the database records and extends it as far as possible within given read (due to computational feasibility of such a demanding task). In result, Centrifuge reports for each read inferred classification to given taxonomic group and length of the match (in base pairs) upon which the estimate was based. Logically, the longer the match, the higher the credibility of the result. Therefore, we re-evaluated the classification results with three thresholds concerning the match length: 23 bp which is the default setting, 50 bp and 75 bp; the latter values were arbitrarily chosen based upon our experience.

We successfully classified 88.93% of reads, which represents quite satisfactory results given the presumable bacterial origin of the sample (DNA). To achieve a 100% rate of classified reads is illusory given the technological, both bioinformatic and the sequencing technology, limitations. While the ONT technology possesses many factual benefits, most importantly the read length and real-time sequencing, still it has some disadvantages compared to the so called “Next Generation Sequencing” (Illumina technology), especially higher error rates (Kolmogorov et al., 2019). This is also another reason why to evaluate the data more strictly than Illumina data.

Therefore, when we applied more stringent criteria as described above, we classified 71.71% and 60.06% of reads, respectively. It is obvious that the numbers logically decrease, nevertheless, these numbers make more sense when we look at individual species (Table 1), i.e., reads classified to the rank of species or higher rank, e.g., subspecies or strain, that were converted to the desired taxonomic rank species, or ignored (e.g., reads classified only to taxonomic rank genus or reads without any classification).

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Table 1 – Summary of classification results at the taxonomic rank

<table>
<thead>
<tr>
<th>taxID</th>
<th>Species name</th>
<th>Stringency 1</th>
<th>Stringency 2</th>
<th>Stringency 3</th>
<th>Drop (%)</th>
<th>Superkingdom</th>
</tr>
</thead>
<tbody>
<tr>
<td>28901</td>
<td><em>Salmonella enterica</em></td>
<td>103764</td>
<td>88053</td>
<td>74974</td>
<td>27.75</td>
<td>Bacteria</td>
</tr>
<tr>
<td>54736</td>
<td><em>Salmonella bongori</em></td>
<td>507</td>
<td>145</td>
<td>58</td>
<td>88.56</td>
<td>Bacteria</td>
</tr>
<tr>
<td>562</td>
<td><em>Escherichia coli</em></td>
<td>324</td>
<td>180</td>
<td>87</td>
<td>73.15</td>
<td>Bacteria</td>
</tr>
<tr>
<td>573</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>74</td>
<td>40</td>
<td>26</td>
<td>64.86</td>
<td>Bacteria</td>
</tr>
<tr>
<td>545</td>
<td><em>Citrobacter koseri</em></td>
<td>55</td>
<td>13</td>
<td>3</td>
<td>94.55</td>
<td>Bacteria</td>
</tr>
<tr>
<td>546</td>
<td><em>Citrobacter freundii</em></td>
<td>48</td>
<td>12</td>
<td>4</td>
<td>91.67</td>
<td>Bacteria</td>
</tr>
<tr>
<td>208962</td>
<td><em>Escherichia albertii</em></td>
<td>47</td>
<td>13</td>
<td>2</td>
<td>95.74</td>
<td>Bacteria</td>
</tr>
<tr>
<td>550</td>
<td><em>Enterobacter cloacae</em></td>
<td>41</td>
<td>10</td>
<td>1</td>
<td>97.56</td>
<td>Bacteria</td>
</tr>
<tr>
<td>67825</td>
<td><em>Citrobacter rodentium</em></td>
<td>33</td>
<td>8</td>
<td>1</td>
<td>96.97</td>
<td>Bacteria</td>
</tr>
<tr>
<td>35703</td>
<td><em>Citrobacter amalonaticus</em></td>
<td>32</td>
<td>7</td>
<td>1</td>
<td>96.88</td>
<td>Bacteria</td>
</tr>
<tr>
<td>548</td>
<td><em>Klebsiella aerogenes</em></td>
<td>32</td>
<td>7</td>
<td>3</td>
<td>90.62</td>
<td>Bacteria</td>
</tr>
<tr>
<td>622</td>
<td><em>Shigella dysenteriae</em></td>
<td>27</td>
<td>9</td>
<td>5</td>
<td>81.48</td>
<td>Bacteria</td>
</tr>
<tr>
<td>61647</td>
<td><em>Pluralibacter gergoviae</em></td>
<td>27</td>
<td>4</td>
<td>1</td>
<td>96.30</td>
<td>Bacteria</td>
</tr>
</tbody>
</table>

Per each row we provide: the species taxID; its scientific name; the number of classified reads according to three different Stringency thresholds (details provided in text); the drop of successfully classified reads between the Stringency1 and Stringency3 in percent according to the given species; and the superkingdom of a given species. Only 13 most abundant “putative” species within the sample are presented.

A high rate of dropout of classified reads, in a response to more stringent thresholds, belonging to individual species represents an important hint, suggesting that the signal pointing to such species (at the lowest threshold value) represents “noise” in the analysis, i.e., false positives, rather than true positive evidence. So, while our bioinformatic pipeline clearly pointed out all the positives as well as the negatives of the Centrifuge tool, which indeed represents the core of this pipeline and was not primarily designed to deal with the ONT data, we came to the conclusion that we have identified *Salmonella enterica* as the prevalent DNA donor within our analysis.

Furthermore, based on the Centrifuge classification results, which encompass roughly 4%, i.e., 3,109 individual reads considering the highest threshold value (i.e., exact match of 75 bp), of the classified reads, we were able to classify the bacterial serovar to *Salmonella Paratyphi A* (Figure 1). The reason why only such a sub-portion of reads was able to point directly to this serovar (subspecies) lies in the fact that most of the bacterial species share a set of common genes (Bochkareva et al., 2018), while only some of the genes or even noncoding sequences (features) are able to distinguish between the individual serovar (or even strain).

The data were sufficient for reasonable genome assembly, but they were insufficient for chromosome-level assembly. Therefore, we chose the two longest contigs, 328,615 bp and 233,901 bp, and compared them to the database of all *Salmonella enterica* species (described in Material and Methods). Both contigs...
show highest similarity to *Salmonella Paratyphi* A, which is in concordance with the previous results. However, this methodology has not enough power to distinguish between individual strains, which is in agreement with the fact that this tool was not actually meant as a classification tool, even despite it is often being used in this manner.

Both, these results are in agreement with the NIPH certified laboratory results. The serovar *Salmonella Paratyphi* A was confirmed by biochemical tests and agglutination tests. According to the exact strain identification we used the results provided by the Centrifuge, which classified roughly 0.3% of reads, i.e., 260, to *Salmonella Paratyphi* A str. ATCC 11511. Despite the fact these numbers seem to be low, they represent a reasonable result. Nevertheless, the exact confirmation of such a deep classification could only be attained by a combination of both ONT and Illumina sequencing, and a set of bioinformatic analyses (i.e., phylogenomics and comparative genomics).

**Exploratory genomic analysis**

Beyond the classification of the disease causative pathogen, we were also able to provide an exploratory analysis of the strain characteristics, i.e., to test whether it possesses some significant structural variation (SV) compared to the reference strain of *Salmonella Paratyphi* A.

Quite surprisingly, we have identified two major deletion events, one encompasses region 1,848 bp long and comprises 5 well annotated genes (protein sequences) while the other one encompasses a region roughly 43,746 bp long which in the reference strain harbour 40 well annotated genes (according to criteria described in Material and Methods).
Despite the fact that bacterial strains are, with respect to genomic evolution and gene content, probably much more plastic (Bochkareva et al., 2018) than for example vertebrates; compare loss of 45 most likely functional genes with the massive impact on the phenotype and even viability caused by simple variation in the copy number of certain blocks of genes, i.e., chromosomal aneuploidy (Hassold et al., 1996).

Still, it is questionable whether such significant loss of part of a likely ancestral genome might be neglected as an example of the bacterial plasticity and variability, or whether it would rather deserve careful description and status of evolutionary independent and novel entity (Simpson, 1951). Nevertheless, these and other questions fall beyond the scope of this study and would require further research before they could be seriously answered.

**Conclusion**

Oxford Nanopore Technologies sequencing provides a new and easy-to-use tool for both rapid and unambiguous identification of causative agents of infectious diseases. In this study, we successfully demonstrate application of this new technology for the purposes of identification of the invasive pathogenic organism. The initial bacteria culture was cultivated from the patient blood sample. Subsequently, we sequenced the DNA content of the culture using the Nanopore (ONT) sequencing platform. Then, we analysed the data by two methodologically independent bioinformatic pipelines, which allowed us to conclude that the disease causative agent was *Salmonella* Paratyphi A. These results were in a perfect agreement with the classification obtained for this isolate from the Czech National Reference Laboratory for *Salmonella* in Prague.

In comparison to the mass spectrometry identification technique (mentioned in Material and Methods), which might be considered a standard method nowadays, nanopore sequencing is utterly different. Mass spectrometry identification based on proteins analysis possesses few drawbacks in its limits, e.g., the impossibility to detect synonymous mutations, deletions etc., which might be essential for precise identification. Nanopore sequencing overcomes these limits by working directly with bare DNA of causative agents.

In addition, this technique allows not only for the identification of the pathogen, but it also allows the exploration of its genomic architecture. We demonstrate this point of view as well as its importance.

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Rare Cause of Left Upper Abdominal Pain

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Key words: Jejunal diverticulitis – Diverticular disease – Acute abdomen – Duodenal diverticulum

Abstract: Inflamed diverticular disease of the small bowel is an uncommon cause of acute abdominal pain. Despite its low prevalence rate (0.3–2%), it is associated with a high mortality rate between 20–25% (Fisher and Fortin, 1977; Ferreira-Aparicio et al., 2012). This is due to complications including perforation, bleeding, and obstruction. This case report presents the diagnosis and management of Mr. X, a 70-year-old male with jejunal diverticulitis and a duodenal diverticulum. Mr. X has a background of type 2 diabetes mellitus and sigmoid diverticulosis, he presented with a three-day history of left upper quadrant pain radiating to the left iliac fossa. He was haemodynamically stable despite his elevated inflammatory markers (C-reactive protein 161 mg/l and neutrophils 13.3×10⁹/l) and computerised tomography (CT) of the abdomen and pelvis showing jejunal diverticulitis and a duodenal diverticulum. Mr. X was successfully treated with intravenous antibiotics and analgesia and a follow up CT scan showed that the jejunal diverticulitis had resolved. Previous operative management of the discussed pathology has been reported, the current report is novel as the diagnosis was made early and the case managed conservatively.

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Introduction
Small bowel diverticular disease excluding, Meckel’s diverticulitis, is an uncommon cause of acute abdominal pain. It occurs in approximately 0.3–1.3% of post-mortem cases and 0.5–1.9% of contrast media study cases (Fisher and Fortin, 1977). Whereas, more common small bowel emergencies are bowel obstruction, small bowel inflammation (especially terminal ileum) and/or perforation in patients with Crohn’s disease (Vallicelli et al., 2011). In these cases, if the affected small bowel is unsalvageable then resection is the definitive surgical approach. Moreover, multiple small bowel diverticuli have been linked with various clinical conditions including Fabry’s disease, Marfan’s and Ehlers Danlos syndrome (Cunliffe and Anderson, 1967; Friedman et al., 1984; McLean et al., 1985; Shapira et al., 1992; Yağmur et al., 2004; Aksoy et al., 2005).

The jejunum is the least common site of small bowel diverticula with an incidence of less than 0.5% in upper GI (gastrointestinal) radiographs and 0.3–1.3% autopsy incidences (Chow et al., 1997). Duodenal diverticula are approximately five times more common than jejunoileal diverticula. Small bowel diverticula are often symptomatic, with approximately 10% of patients presenting with acute clinical symptoms (Cunningham et al., 2006).

The prevalence of jejunal diverticular disease increases with age, mostly occurring in older males between the age of 60–80 years old (Akhrass et al., 1997). Complicated small bowel diverticular disease has a mortality rate as high as 24% with serious complications including perforation, bleeding, abscess formation and obstruction (Ferreira-Aparicio et al., 2012). Therefore, it is important for clinicians to consider small bowel diverticula in patients who present with symptoms such as abdominal pain, nausea and fever. There is little research on the presentation and management of patients with small bowel diverticulitis. Nonetheless, previous case reports have shown that both medical and surgical intervention may be required to treat the complications (Kouraklis et al., 2001).

This report presents a case of a male patient with jejunal diverticulitis and a duodenal diverticulum, with discussion of literature on this rare condition.

Case report
A 70-year-old male with a history of sigmoid diverticulosis and type 2 diabetes mellitus presented to the emergency department with a three-day history of progressive, constant left upper quadrant pain. The pain was dull in nature and radiated to the left iliac fossa. Pain severity at its worst was 8/10, worse with movement and relieved by lying flat. It was associated with reduced appetite and fever but no nausea. Physical examination showed marked left lumbar and iliac fossa tenderness with no peritonism. There was a suspected mass in the left lumbar region.

The patient’s white cell count was 14.8×10^9/l (4.3–11.2×10^9/l), C-reactive protein 161 mg/l (0–5 mg/l), neutrophils 13.26×10^9/l (2.1–7.4×10^9/l), lymphocytes
Figure 1 – Computed tomography scan of the abdomen and pelvis for the patient. Jejunal diverticulitis is shown in white arrows.

Figure 2 – Another slice of the same computed tomography abdomen and pelvis scan as in Figure 1 for the patient. In white arrows, the duodenal diverticulum is shown.

Figure 3 – Computed tomography abdomen and pelvis scan is being shown post treatment of the jejunal diverticulitis shown in the white arrow. The black arrow shows the duodenal diverticulum.
0.65×10^9/l (1.0–3.6×10^9/l), lactate < 2 mmol/l and all other laboratory data was unremarkable. An abdominal and pelvis computed tomography (CT) scan with contrast showed large inflamed jejunal diverticulum with surrounding inflammatory mass and no obvious features of bowel ischaemia (Figure 1). Diverticular disease of the duodenum and the left colon was also noted (Figure 2).

The patient was treated with intravenous co-amoxiclav and metronidazole and remained nil by mouth. He was discharged home on day 5 with oral co-amoxiclav for an additional seven days and followed-up with repeat outpatient CT in six weeks’ time.

Repeat imaging showed that the inflammation in the left flank had resolved without complication (Figure 3) and the patient was discharged from the general surgery clinic.

**Discussion**

We present a case of a 70-year-old man with a history of sigmoid diverticular disease who presented with acute left sided abdominal pain and was found to have jejunal diverticulitis.

Compared to large bowel diverticular disease, small bowel diverticular disease is uncommon. The duodenum is more common than the jejunal-ileal sites for small bowel diverticulosis except for Meckel’s diverticulum (Hobson and Roberts, 2004). One theory of the pathophysiology of small bowel diverticulosis is that there are abnormalities of intestinal peristalsis and high intraluminal pressure, so the diverticulum exists at the site where mesenteric vessels enter the muscular layer of the small intestine (Hobson and Roberts, 2004). There is little information on the incidence of duodenal, jejunal and ileal diverticular simultaneously. Krishnamurthy et al. (1983) showed in their study of 10 patients that 40% (n=4) had simultaneous duodenal, jejunal and ileal diverticulae; however, this was a small sampled study, so it is difficult to make generalisations from this study.

Diverticula can also be classified as either congenital or acquired. Except for Meckel’s diverticulum, small bowel diverticula tend to be acquired rather than congenital (Albert et al., 2009). Diverticula can also be classed as either true or pseudodiverticula. A true diverticulum such as in Meckel’s diverticulum (on the antimesenteric border of ileum) involves all layers of the bowel whereas pseudodiverticula consists of the uncus of the mucosa and the submucosa (Albert et al., 2009). Jejunal diverticulum tend to be pseudodiverticula (Chow et al., 1997; Mantas et al., 2011).

On radiological findings, inflamed diverticula tend to appear as non-specific changes suggestive of inflammation. This may include an inflammatory mass, bowel wall thickening of an involved segment, surrounding fat stranding and fluid collections (Coulier et al., 2007; Kirbas et al., 2007).

Patients can present with complicated diverticulitis including abscess formation, perforation and ischaemia. These complications generally require surgical
intervention. The case we present was diagnosed early without complication and therefore antibiotic treatment alone was effective. Previous reports describe failed initial medical management where patients went on to suffer complications including diverticular bleeding and perforation (Kumar, 2017; Prough et al., 2019). In Prough et al. (2019) case report, the patient was suspected of having acute perforated jejunal diverticulitis, but this was disproved at operation. Nonetheless, the affected segment was resected.

Conclusion
It is important to consider a differential diagnosis of small bowel diverticulitis in patients with acute abdominal pain. Early radiological investigation is important and surgical intervention should be considered if medical management is insufficient or complications occur. Future studies could investigate the number of cases of small bowel diverticulitis which are managed medically vs surgical management, however this may be difficult as this is not a common condition.

References


Soft Tissue Perivascular Epithelioid Cell Tumour: An Unusual Finding

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Abstract: Perivascular epithelioid cell tumour (PEComa) is a rare mesenchymal tumour made up of clear perivascular cells with epithelioid characteristics, which co-expresses muscle and melanocytic markers with a component of spindle cells, like sarcoma and variety of other tissues. This time, we present the case of a young patient with a tumour in the dorsal region of progressive growth, compatible with PEComa of soft tissue after histopathological and immunohistochemical analysis.

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Introduction
Perivascular epithelioid cell tumour (PEComa) is a rare mesenchymal tumour made up of clear perivascular cells with epithelioid characteristics, which co-express muscle and melanocytic markers with a component of spindle cells, like sarcoma and variety of other tissues (Chen et al., 2016). It occurs more frequently in women in a 5:1 ratio compared to men, and with an average age of 45 years, benign behaviour prevails (Bao et al., 2019). These types of tumours have similar morphological and immunophenotypic characteristics, including epithelioid angiomyolipoma, lymphangioleiomyomatosis, and common clear cells (sugar tumour) (Lim et al., 2007; Bao et al., 2019), although malignant variants have also been described, mainly of gastrointestinal, gynecological, retroperitoneal, and uterine origin (Llamas-Velasco et al., 2016). Their presence in soft tissues is exceptional (Harris et al., 2004). Despite current advances in the biomedical sciences, no solid protocol has been established for the diagnosis or treatment of this specific disease. A hypothesis was recently proposed in which it is stated that perivascular epithelioid cells originate from pluripotent cells of the neural crest due to the expression of S-100, CD56, and CD-99 (Lim et al., 2007), which can give rise to cells smooth muscle and melanocytes, which may explain the histological pattern of this tumour. The objective of this article is to report the experience with a patient who presented a spontaneously appearing tumour in the dorsal region, who received standard definitive surgery, and subsequently found the unusual histopathological finding PEComa.

Case report
A 27-year-old female patient who consulted for a 3-month evolution disease manifesting with a tissue mass in the dorsal region of a progressive growth, without
pain, without bleeding, without weight loss. She reported that a year ago a similar lesion was resected in the same location, but the histopathological examination was inconclusive. There was not relevant pathological history. On physical examination, a raised nodular lesion at the level of skin was evident, it had firm consistency, 2 cm × 3 cm, was immobile, not painful, violet in colour at the location described (Figure 1). Based on the above, there was the diagnostic impression of a tumour with uncertain behaviour at the soft tissue level, so it was proposed to take the patient to surgery for excision of the lesion with flap.

This process was carried out without complications and extracted tissue was sent to pathology (Figures 2 and 3). During the postoperative period, she was a patient with good evolution, asymptomatic, with a healed surgical hound, without pain or bleeding. A pathology report described fibrohistiocytic lesion of uncertain behaviour with epithelioid areas and atypia, so immunohistochemistry procedures were performed, finding reactivity in Melan-A, MITF-1 and calponin, which suggested a definitive diagnosis of PEComa.

**Discussion**

PEComa was first described in the 1950s, wrongly reported as a renal angiomyolipoma (Morgan et al., 1951). In 2002, the World Health Organization recognized it for the first time as a heterogeneous mesenchymal tumour (Petersen, 2013), and subsequently, few isolated cases have been reported worldwide to date. It is considered the base of its origin in cell lines related to muscle and adipose
tissue, having different patterns of intensity and sensitivity for the co-expression of melanocytic cells and muscle cell markers, this being one of its main characteristics (Folpe and Kwiatkowski, 2010).

These types of tumours are of extraordinary presentation, representing an incidence of less than 1% of all benign tumours (Fenz et al., 2017), and their most frequent location are the adrenal glands (Kwazneski Ii et al., 2016), although they can be established from the retroperitoneum, bone, thyroid, lung, kidney, pancreas, liver, skin, uterus, gastrointestinal tract, etc. (D’Andrea et al., 2016; Ferrari et al., 2016; Fenz et al., 2017; Leal-Medrano et al., 2017; Touloumis et al., 2019; Zhao et al., 2019). These tumours have a nonspecific clinical presentation, being able to simulate any type of neoplasm, however, they usually debut with a painful sensation of mass, with typical characteristics of the affected organ (Cuevas et al., 2015), such as intestinal obstruction and bleeding in tumours of the gastrointestinal tract or bleeding vaginal in the case of uterine tumours.

Despite being considered mainly benign, those PEComas that are larger than 5 cm, infiltrative growth pattern, high nuclear grade, cells with necrosis and atypical mitosis, are associated with malignancy (Folpe and Kwiatkowski, 2010; Schaefer and Fletcher, 2018). That is why the current diagnostic gold standard is through histopathology and immunohistochemistry, thus allowing to accurately examine the phenotypic characteristics of each particular case. For immunohistochemistry, CD-68 markers, S-100 protein, Calponin, cytokeratin AE1/AE3, epithelial membrane antigen (EMA), Human Melanoma Black-45 (HMB-45), Vimentin, anti-muscle smooth antibodies (SMA) and CD-117 are measured (Folpe et al., 2005; Petersen, 2013; Schaefer and Fletcher, 2018; Liu et al., 2019), which are taken by means of biopsy. Among the most common differential diagnoses are melanoma, sarcoma, epithelial tumours, and oxyphilic carcinomas (Petersen, 2013; Fenz et al., 2017), because melanosomes can be observed by ultrastructure, and a large amount of glycogen and cytoplasmic (Bao et al., 2019). The expression of this type of marker supports the hypothesis that PEComa is neoplasm originating from stem cells, which suffer defects acquired during differentiation.

So far, no specific treatment has been established, so it is resolved by surgical intervention, taking into account free margins of the tumour (Cuevas et al., 2015). It has a very good prognosis in the short and long term, since it is generally benign, but recurrence may occur if the resection margins of the lesion are not taken into account (Harris et al., 2004; Folpe et al., 2005).

In this case, fortunately the patient presented a benign neoplasm in a location that did not compromise any vital organ; the recommendations described as the resection of margins were taken into account when performing the operation, as well as the protocol to obtain the final diagnosis by means of immunohistochemistry, finding the expression of common markers registered in the literature.
Conclusion
In this case, PEComa can be seen, a neoplasm of strange presentation, of uncertain behaviour. The tumor was studied with histopathological and immunohistochemical methods, to define the tumour phenotype and its association with malignancy. Reviewed literary data have shown, the need to take into account the resection margins that should be identified and avoided.

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