Advances in Health and Disease

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Volume 15

Lowell T. Duncan
Editor

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ADVANCES IN HEALTH AND DISEASE

VOLUME 15

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VOLUME 15

LOWELL T. DUNCAN
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PREFACE

Advances in Health and Disease. Volume 15 explores the current knowledge relating extracellular calreticulin to human pathologies, focusing on cancer, rheumatoid arthritis, systemic lupus erythematosus, wound repair and the retroviral infection with human T-cell lymphotropic virus type 1.

Following this, the authors provide an overview of the tumor markers and scoring systems as well as its utilization for effective diagnosis of female adnexal masses. Appropriate discrimination between benign and malignant mass results in the correct choice between conservative and surgical management.

This collection also presents the way in which two B-glucans play a vital role in innate immunity modulation, and are therefore predicted to be candidates for alternative therapies against inflammatory diseases.

Subsequently, the authors present indications, techniques, complications and treatment regarding the use of polyacrylamide for facial wasting rehabilitation. Medical literature shows how facial wasting is recognized as a stigma of the infection for the patient-self, and as such facial features restoring is crucial to the social life of HIV positive patients.

In conclusion, the major issues regard reduced nostril aperture and nasal fossae volume, and degree of sinus pneumatization are presented. Endoscopic sinus surgery and its main use in pediatric patients is described, as well as the advantages of this less invasive surgery.

Chapter 1 - Calreticulin (CRT), initially described as an endoplasmic reticulum (ER)-resident chaperone with high affinity for Ca^{2+}, also localizes in the nucleus, cytoplasm, plasma membrane and the extracellular medium, suggesting multifunctional roles in different systems and cell types. Cell surface (ecto-CRT) and extracellular CRT have been found driving diverse processes and, therefore, CRT is now associated to several diseases. Here, the authors explore the current knowledge relating extracellular CRT to human pathologies, focusing on cancer, rheumatoid arthritis, systemic lupus erythematosus, wound repair and the retroviral infection with human T-cell lymphotropic virus type 1 (HTLV-1). In cancer, CRT shows immunomodulatory functions by exposing to the cell surface of dying cells, acting as a critical danger pattern for the recognition and engulfment of tumor cells. Cell surface CRT is relevant for the immunological clearance of tumors induced by some chemotherapeutic and physical agents, a process called "immunogenic cell death" (ICD), characterized by rapid translocation of CRT to the surface and/or its release to the extracellular space. Among novel antitumor treatments, a combination of ICD inducers together with immune checkpoint inhibitors have shown effective synergistic effects against resistant tumors. In autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis, the increased CRT serum levels and the presence of anti-CRT autoantibodies are common and may be involved in the disease progression. It has been found that cell surface CRT mediates cell signaling associated to main risk factors in patients with rheumatoid arthritis, suggesting key extracellular functions contributing to autoimmune pathogenesis. *In vitro* studies and animal models show that wound treatment with exogenous CRT increase epithelization rate via migration of keratinocytes and formation of new epidermis by fibroblasts, which are required for successful wound repair. Secretion of CRT has also been found in HTLV-1-infected lymphocytes from patients with spastic paraparesis. ER CRT interacts with two viral proteins, p12I and Tax, which affect viral replication and survival. The CRT-Tax interaction may promote CRT secretion and potentially block the extracellular deleterious effects of the viral infection. CRT association to disease not only serves as a biomarker of disease progression, but also extracellular CRT emerges as a potential

therapeutic target and drug. The variety of processes involving extracellular CRT opens new treatment possibilities in multifactorial diseases, such as cancer and autoimmune disorders.

Chapter 2 - Adnexal masses, including ovarian cancer, have increased in incidence during the last decade. Extensive introduction of screening programs and progress in diagnostic imaging methods have led to a progressive increase in adnexal masses detection, which can have gynecologic or nongynecologic etiologies, ranging from functional cysts to ovarian cancer, and intestinal masses. Appropriate discrimination between benign and malignant mass results in the correct choice between conservative and surgical management. Pelvic examination has low sensitivity for detecting an adnexal mass; therefore detailed workup required employing different indicators. Currently, development and application of cancer biomarkers have significantly increased opportunities for improving cancer diagnosis and treatment. Early detection of malignancy can be achieved using serum levels of tumor markers such as Ca 125 combined with Ca 19-9, Ca 15-3, HE 4, and CEA. Nowadays, a multimodal approach including the evaluation of serum tumor biomarkers combined with imaging techniques seems to be the best strategy for adnexal tumor detection. Malignancy risk estimation of ovarian cysts could be also achieved by employment of new multivariate logistic regression models and scoring systems that have been introduced into clinical practice to improve the diagnostics. The most useful algorithms utilizing tumor markers are Risk of Malignancy Index (RMI), Risk of Ovarian Malignancy Algorithm (ROMA), OVA-1, and Triple screen. In this chapter, the authors provide an overview of the tumor markers and scoring systems as well as its utilization for effective diagnosis of female adnexal masses.

Chapter 3 - Immunomodulating β-glucans show potent and unique properties as biological response modifiers (BRM) within the immune system. Interest in the medicinal properties of β-glucans has increased due to their immunomodulating activities and prevention of pathological effects for many diseases. Recently, these glucans have been made available as alternative medicine for the inhibition of inflammation and alleviation of various disorders, such as inflammatory bowel disease (IBD). In this chapter

the authors describe two β-glucans of mushroom origin, lentinan (LTN) from *Lentinula edodes* and *Pleurotus citrinopileatus* glucan (PCPS), which inhibit inflammatory responses and prevent related diseases such as IBD. Various immunocompetent cells and active molecules have recently been identified within the innate immune system, and the concept of pathogen-associated molecular patterns (PAMPs) pattern recognition receptors (PRRs) was developed. Toll-like receptors (TLRs) are already recognized as typical receptors within the scope of innate immunity. Moreover, regarding the specific receptor for β-glucan, Dectin-1 has been discovered as a PRR playing an important role. Dectin-1 and several TLRs are synergistically involved within glucan's effect on immunocompetent cell modulation, including for monocytes, macrophages and dendritic cells. The authors show here that two β-glucans play a vital role in innate immunity modulation, and are therefore predicted to be candidates for alternative therapies against inflammatory diseases.

Chapter 4 - The highly active antiretroviral therapy (HAART) against HIV infection has been showed as an efficient treatment, and in recent years guaranteed a longer life for infected patients, however this therapy is not without side effects; patients receiving HAART may develop a typical syndrome, the so-called HAART-related lipodystrophy. This syndrome is both characterized by metabolic alterations and abnormal distribution of fat in the body, such as loss of subcutaneous fat of the face (also known as facial wasting) and extremities, and relative increase of the fat of the trunk. Medical literature shows how facial wasting is recognized as a stigma of the infection for the patient-self, due to this reason facial features restoring is a keypoint in social life for HIV positive patients. In this clinical situations polyacrylamide hydrogel based fillers can be suitable to restore facial features. The authors present indications, techniques, complications and their treatment regarding the use of polyacrylamide for facial wasting rehabilitation.

Chapter 5 - Vesicoureteral reflux (VUR) is a common pathology encountered in pediatric urology. It may affect from 1 to 3% of children. Potential complications of VUR are upper urinary tract infections and renal scarring, which can lead to loss of renal function and hypertension. During

the last three decades, endoscopic treatment of VUR has evolved to become the first line of intervention in several countries, especially for low grade VUR. This growing interest in subureteral injection has prompted additional research on different types of bulking agents. The ideal bulking agent is described as biocompatible, nontoxic, non-antigenic, non-migratory, causing minimal inflammation and keeping its shape and volume. The perfect agent which meets all of these criteria has yet to be found. Nowadays, the most popular bulking agent is dextranomer hyaluronic acid (Dx/HA, Deflux®), which is the only agent approved by the Food and Drug Administration for treatment of VUR. Polyacrylamide hydrogel (PAHG, Bulkamid®) is currently approved for the treatment of stress urinary incontinence through urethral injections. Its safety in the urinary tract has been shown in different clinical trials. Recent publications have evaluated the efficacy and safety of PAHG in the endoscopic treatment of VUR in pediatric patients. In this chapter, the authors propose a review of the use of PAHG endoscopic injection as an alternative bulking agent for the treatment of children with VUR.

Chapter 6 - Introduction of pediatric endoscopic sinus surgery (PESS) is relatively recent. Owing to the excellent outcomes of functional endoscopic sinus surgery observed in the adult population, in 1990s the development of suitable pediatric instruments and optics was encouraged. Moreover, improvements in technology and surgical techniques enabled pediatric otolaryngologists to progressively expand the surgical indications of endoscopic nasal approaches. Nowadays, PESS is routinely performed in children affected by sinonasal inflammation pathologies, anatomic malformations, benign expansible lesions, and lacrimal stenosis. Furthermore, this surgical management is favoring PESS over open approaches for CSF leak repair and other skull base disorders, including some neoplastic masses. However, specific considerations in PESS must be addressed. The major issues regard reduced nostril aperture and nasal fossae volume, and degree of sinus pneumatization. Herein, the authors describe this surgical technique and its main use in pediatric patients. Finally, the advantages of this less invasive surgery are described compared to traditional surgical treatments.

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Chapter 1

EXTRACELLULAR FUNCTIONS OF CALRETICULIN IN DISEASE

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ABSTRACT

Calreticulin (CRT), initially described as an endoplasmic reticulum (ER)-resident chaperone with high affinity for Ca^{2+}, also localizes in the nucleus, cytoplasm, plasma membrane and the extracellular medium, suggesting multifunctional roles in different systems and cell types. Cell surface (ecto-CRT) and extracellular CRT have been found driving diverse processes and, therefore, CRT is now associated to several diseases. Here, we explore the current knowledge relating extracellular CRT to human

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pathologies, focusing on cancer, rheumatoid arthritis, systemic lupus erythematosus, wound repair and the retroviral infection with human T-cell lymphotropic virus type 1 (HTLV-1). In cancer, CRT shows immunomodulatory functions by exposing to the cell surface of dying cells, acting as a critical danger pattern for the recognition and engulfment of tumor cells. Cell surface CRT is relevant for the immunological clearance of tumors induced by some chemotherapeutic and physical agents, a process called "immunogenic cell death" (ICD), characterized by rapid translocation of CRT to the surface and/or its release to the extracellular space. Among novel antitumor treatments, a combination of ICD inducers together with immune checkpoint inhibitors have shown effective synergistic effects against resistant tumors. In autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis, the increased CRT serum levels and the presence of anti-CRT autoantibodies are common and may be involved in the disease progression. It has been found that cell surface CRT mediates cell signaling associated to main risk factors in patients with rheumatoid arthritis, suggesting key extracellular functions contributing to autoimmune pathogenesis. *In vitro* studies and animal models show that wound treatment with exogenous CRT increase epithelization rate via migration of keratinocytes and formation of new epidermis by fibroblasts, which are required for successful wound repair. Secretion of CRT has also been found in HTLV-1-infected lymphocytes from patients with spastic paraparesis. ER CRT interacts with two viral proteins, p12I and Tax, which affect viral replication and survival. The CRT-Tax interaction may promote CRT secretion and potentially block the extracellular deleterious effects of the viral infection. CRT association to disease not only serves as a biomarker of disease progression, but also extracellular CRT emerges as a potential therapeutic target and drug. The variety of processes involving extracellular CRT opens new treatment possibilities in multifactorial diseases, such as cancer and autoimmune disorders.

Keywords: calreticulin, CRT, immunogenic cell death, cancer therapy, immune checkpoint inhibitor, systemic lupus erythematosus, rheumatoid arthritis, wound healing, HTLV-1, viral proteins

1. INTRODUCTION

CRT is a multifunctional protein discovered by Ostwald and MacLennan (1974), initially described as an endoplasmic reticulum (ER)

lectin-like chaperone. Inside the ER, CRT regulates several processes, such as Ca^{2+} homeostasis and quality control during protein folding, which result essential for embryogenesis, cellular stress response and expression/trafficking of proteins (Michalak et al., 1999, 2009; Gold et al., 2010; Van Duyn Graham et al., 2010; Wang et al., 2012; Raghavan et al., 2013; Lu et al., 2015; Zimmermann et al., 2013, 2015; Blees et al., 2017; Arshad and Cresswell, 2018; Owusu et al., 2018; Venkateswaram et al., 2018). As a consequence of its fundamental ER functions, CRT contains two ER targeting signals; an N-terminal hydrophobic sequence for insertion into the ER and an ER retention sequence (KDEL) at its C-terminal domain (Michalak et al., 1999, 2009). Despite the ER targeting signals and the initial reports of CRT as an ER-resident chaperone, several reports within the last two decades have shown CRT associated also to other cellular locations, such as the cytosol, the nucleus, secretory granules and the cell surface playing essential biological functions (Michalak et al., 1999; Holaska et al., 2001, 2002; Afshar et al., 2005; Wang et al., 2012). In this chapter, we will discuss the current extracellular CRT functions associated to immune (dys)regulation in cancer, rheumatoid arthritis, systemic lupus erythematosus, the infection with the human lymphotrophic virus type 1 (HTLV-1) and wound healing taking into consideration the recent advances and possible therapies involving extracellular CRT.

1.1. General Characteristics of Calreticulin (CRT)

CRT is intrinsically disordered in nature, yet three distinct domains associated to different CRT functions are recognized (Lu et al., 2015; Wijesakere et al., 2016; Varricchio et al., 2017; Venkateswaran et al., 2018; Schcolnik-Cabrera et al., 2019):

1) The N-terminal domain (residues 18-197), which has chaperone function. Residues 1 to 17 contain the NH_3+ signal peptide targeting to the ER lumen. This domain interacts with a variety of proteins, such as α-integrin, the DNA-binding site of steroid receptors, the

MHC class I complex, the protein disulfide isomerases (PDI) such as ERp57, trombospondin and even RNA. The N-terminal domain is a Zn^{2+}-binding conserved sequence essential for the ability of CRT to bind unfolded proteins and glycoprotein substrates.

2) The P-domain (residues 198-308), rich in proline, is also responsible of CRT's lectin-like chaperone function. The P-domain is a high-affinity, low-capacity Ca^{2+}-binding site that can interact with PDI and perforin (a component of cytotoxic T cell granules), ERp57, C1q with Ca^{2+}, and glucosylated oligosaccha-rides.
3) The C-terminal domain (residues 309-417), a highly acidic region with low affinity and high capacity for Ca^{2+}, acts as a calcium buffer and also binds other chaperones in the ER. CRT binding to Ca^{2+} regulates its interaction with PDI, ERp57 and other ER chaperones. The protein terminates with the KDEL (Lys-Asp-Glu-Leu) ER retention sequence.

1.2. Mechanisms of CRT Mobilization

On its journey to the extracellular medium, CRT must first retrotranslocate to the cytoplasm. CRT retrotranslocation from the ER lumen to the cytoplasm usually requires a peptidase that removes the ER-retention KDEL sequence (Afshar et al., 2005). The proteolytic cleavage of KDEL sequence is triggered by ER Ca^{2+} depletion and accounts also for the nuclear, cell surface and extracellular localization of CRT (Schcolnik-Cabrera et al., 2019). CRT phosphorylation by a tyrosine kinase is required for the cleavage of its KDEL retention signal (Schcolnik-Cabrera et al., 2019). It was found that the phosphorylation of CRT by Bruton's tyrosine kinase (BTK) activates its process of mobilization to the macrophage cell surface and secretion, participating in the efficient uptake of apoptotic cancer cells (Byrne et al., 2013; Feng et al., 2015; Schcolnik-Cabrera et al., 2019). Alternatively, CRT retrotranslocation may result as the consequence of CRT interaction with other proteins masking CRT ER-retention signal (Asfar et al., 2005).

CRT co-translocation requires activation of the ER stress response, the formation of reactive oxygen species (ROS) and the reduction of Ca^{2+} levels in the ER. In some cell types, CRT co-translocation together with ER-resident and cytoplasmic proteins is also involved in apoptosis, mitochondrial damage, induction of ATP release and caspase activation (Gold et al., 2010; Zitvogel et al., 2010; Garg et al., 2014; Wiersma et al., 2015). Translocation of CRT to the cell surface may additionally involve the co-translocation with other ER-resident proteins, such as disulfide isomerase ERp57 (Panaretakis et al., 2008; Raghavan et al., 2013). In response to some chemotherapeutic agents as well as radiotherapy and photodynamic therapy (PDT) the CRT exposure pathway is activated, resulting in anterograde transport of CRT bound to ERp57 via Golgi apparatus and SNARE-dependent exocytosis mediated by several events, such as pre-apoptotic ER stress; phosphorylation of the eukaryotic translation initiation factor eIF2α by PERK (protein kinase RNA-like ER kinase); inhibition of PP1, an eIF2α-specific phosphatase; proteolysis of BAP31 mediated by caspase-8; and activation of pro-apoptotic proteins, such as Bax and Bak (Panaretakis et al., 2008, 2009; Zitvogel et al., 2010; Garg et al., 2014; Fucikova et al., 2018; Schcolnik-Cabrera et al., 2019).

Arginylation of cytosolic CRT (R-CRT) has also been associated with CRT mobilization to the cell membrane (Lopez-Sambrooks et al., 2012). R-CRT is recruited to stress granules under conditions that promote a decrease in cytoplasmic Ca^{2+} levels. Therefore, R-CRT is currently emerging as a novel protein participating in the regulation of stress granule scaffolding and also playing a role in the effect of pharmacological treatments of cancer (Carpio et al., 2013; Decca et al., 2007; Tarr et al., 2010a; Comba et al., 2019).

2. INTRACELLULAR AND EXTRACELLULAR FUNCTIONS OF CRT

2.1. Cytoplasmic CRT

Cytoplasmic CRT, retrotranslocated from the ER, must have a different structure from that of the isolated ER protein, because it is more resistant to digestion with trypsin either in the presence or absence of Ca^{2+} (Asfar et al., 2005). In the cytoplasm, CRT can also act as a Ca^{2+} buffer (Tarr et al., 2010a). Contrary to ER-resident CRT, cytoplasmic CRT is citrullinated, a post-translational modification characteristic of nuclear proteins. Citrullinated CRT in the cytoplasm therefore suggests that CRT could also be transported from the nucleus to the cytoplasm (Wiersma et al., 2015).

Cytosolic Ca^{2+} regulates the amount of the phospholipid phosphatidylserine (PS) in the inner and outer surface of the plasma membrane (Tarr et al., 2010a). In the outer plasma membrane, PS is an "eat-me" signal in apoptotic cells. Cytoplasmic CRT increases upon apoptosis and promotes the generation of intracellular nitric oxide (NO). Nitrosative stress inhibits the aminophospholipid translocase, an enzyme that maintains PS at the cytosolic face of the cell membrane; therefore, CRT-induced NO stimulates PS translocation to the exoplasmic face. CRT also binds PS in the cytoplasmic side and translocates together with PS, suggesting a caspase-independent, Ca^{2+}-mediated mechanism of CRT externalization (Tarr et al., 2010b).

Cell adhesion and protein translation are among the cytoplasmic functions of CRT, which are dependent on Ca^{2+} concentration (Wang et al., 2012). Cytoplasmic CRT binds to the highly conserved KXGFFKR motif in the cytoplasmic tails of α-integrins, producing the stabilization between integrin-ligand binding and the activation of focal adhesion kinase, essential for integrin-mediated adhesion (Gold et al., 2010; Lu et al., 2015). CRT control of cell adhesiveness involves also the nuclear regulation of fibronectin, vinculin and N-cadherin expression, along with matrix deposition and activation of c-SRC (Papp et al., 2007, 2008; Lu et al., 2015).

2.2. Cell Surface CRT

Cell surface CRT is an ecto-protein (also called ecto-CRT) that has important roles in antigen-processing events, phagocytosis of apoptotic cells, immunogenicity of dying tumor cells, cell migration by disassembly of focal adhesion and wound healing rate (Jiang et al., 2014). CRT does not contain a transmembrane domain or a glycosylphosphatidylinositol (GPI) attachment to the cell surface. Therefore, CRT is anchored to the plasmatic membrane on lipid rafts via adaptor proteins, such as CD59, CD69, CD91 and thrombospondin 1 (TSP1) (Kroemer et al., 2013; Garg et al., 2014; Varricchio et al., 2017). TSP1 binds to CRT at its N-terminus, enhancing the interaction with CD91 and activating signaling cascades via PI3K (phosphoinositide-3-kinase) and ERK. Activation of CRT-TSP1-CD91-mediated signaling promotes cytoskeleton changes affecting focal adhesions, which regulate cell shape and cytoskeletal structures conferring cell motility (Goicochea et al., 2013).

Increasing levels of CRT in the cell surface can derive in its secondary release into the extracellular environment, where it can act in a paracrine form in other cells (Osman et al., 2017).

2.3. Extracellular Soluble CRT

The initial report of CRT in the extracellular matrix describes its presence in odontoblast and pre-dental matrix associated to a mineralization function (Somogyi et al., 2003). Since then, several studies of extracellular CRT have been reported in association to a variety of functions. Both intracellular and extracellular CRT participates in T cell activation, peptide loading with tumor antigens and the phagocytosis of tumor cells (Obeid et al., 2007a; Wemeau et al., 2010; Wang et al., 2012; Feng et al., 2015). Exogenous CRT has been shown to improve wound healing by enhancing of epithelial migration and granulation tissue formation (Nanney et al., 2008; Greives et al., 2012; Gold et al., 2010; Wiersma et al., 2015; Eggleton et al., 2016; Owusu et al., 2018). It has also been reported that exogenously added

CRT rescues CRT-deficient cell adhesion, migration, phagocytosis, immunoregulation and also enhances the clearance of apoptotic debris (Nanney et al., 2008; Obeid et al., 2007a; Gold et al., 2010). Exogenously administered CRT consequently has significant therapeutic potential, including impaired diabetic wound healing and cancer therapy (Eggleton et al., 2016).

Several studies report the use of recombinant CRT (rCRT) on mice CT26 colon cancer cells associated to immunogenic cell death (ICD) inducers (Obeid et al., 2007a, 2007b, 2007c, Obeid 2008; Panaretakis 2008, 2009: Kroemer et al., 2013). ICD is a special cell death pathway mediated by immune cells to eliminate cancer cells. Pretreatment with ICD inducers, such as mitomycin C, anthracyclines, γ-radiation and/or UVC light originate efficient immunogenic antitumor responses in the presence of rCRT, increasing *in vivo* and *in vitro* phagocytosis by dendritic cells (DCs). Studies using mouse tumors originated from injected CT26 cells showed that rCRT requires direct injection into the tumor area to generate an effective ICD (Obeid 2007b).

In addition, dying cells efficiently release CRT to the extracellular medium (Osaman et al., 2017). As a consequence of CRT extracellular release during cellular death, several diseases have been associated to unusual levels of extracellular soluble CRT. In some inflammatory diseases, such as rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis, increased plasmatic levels of CRT have been found (Gold et al., 2010; Wemeau et al., 2010; Ni et al., 2013; Wiersma et al., 2015; Wang et at., 2017a). Cancer patients also show high concentration of CRT in plasma samples, such as in patients with acute myeloid leukemia. Similarly, our group has shown increased CRT levels in patients with HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), a neurodegenerative central axonopathy associated to HTLV-1 infection, where CRT may co-secrete with a viral protein involved in the disease progression (Medina et al., 2014).

The presence of autoantibodies against CRT in plasma, along with post-translationally modified CRT, could be explained by the increase of intracellular CRT levels that translocate to the cell surface and the

extracellular environment (Eggleton et al., 1999, 2000; Wiersma et al., 2015; Clarke et al., 2017). When a protein that normally resides intracellularly is secreted or exposed to the cell surface, it commonly induces the production of autoantibodies (Wiersma et al., 2015). The generation of anti-CRT antibodies have been detected in several autoimmune diseases, such as rheumatoid arthritis, juvenile idiopathic arthritis, systemic lupus erythematosus, autoimmune hepatitis, myasthenia gravis, primary biliary cirrhosis, systemic sclerosis, and also in some cancers such as colorectal carcinoma, refractory celiac disease, pancreatic cancer, melanoma and hepatoma (Wiersma et al., 2015).

3. IMMUNOMODULATORY ROLES CRT

The immunomodulatory functions of CRT depend on its cellular localization and cell type. As an ER chaperone, CRT participates in the processing and cell surface expression of the major histocompatibility complex class I (MHC-I) for antigenic presentation to $CD8^+$ T lymphocytes (Gao et al., 2002; Del Cid et al., 2010; Wearsh et al., 2011). However, one of the principal roles of CRT in the adaptive immune system is associated to ICD (Obeid et al., 2007b; Raghavan et al., 2013; Fucikova et al., 2016a).

3.1. Immunogenic Cell Death

The immunogenic cell death (ICD) is a complex process with variants and has different causative agents, one of the most interesting is its induction by chemotherapeutic drugs such as doxorubicin, mitoxantrone, oxaliplatin, bortezomib (Casares et al., 2005; Obeid et al., 2007b; Tesniere et al., 2010; Chang et al., 2012), as well as by physical signals such as ionizing irradiation and UV-C light (Panaretakis et al., 2009; Adkins et al., 2014; Golden et al., 2014). ICD is also induced by pathogens (viruses, bacteria) and by necroptosis (Kepp et al., 2014; Galluzi et al., 2017). The ICD is a highly studied process at the present, to a large extent using murine models, where

it has been demonstrated that is not only associated with phenomena such as apoptosis or necrosis, and it has been necessary to apply new definition models, considering its characteristic immunogenicity (Casares et al., 2005; Kepp et al. 2014; Galluzi et al., 2017). A new way of defining ICD, at least in murine models, consists in vaccination assays, in which the proposed specific inducer agent is used to induce *in vitro* cell death in tumor cells, these treated cells are inoculated into immunocompetent syngenic mice (such as a vaccine); after a time on another flank of the same mouse the same untreated tumor cells are inoculated as a challenge and the proportion of tumor-free mice is monitored. The chemotherapeutic agents that allow the lower development of the tumor, in this murine model, will be the best inducers of ICD (Kepp et al. 2014).

ICD activates the immune response against the tumor in cancer patients and, therefore, is important in considering anticancer therapy strategies (Gardai et al., 2005; Caput et al., 2007; Obeid et al., 2008; Zitvogel et al., 2010; Wemeau et al., 2010; Chen et al., 2017; Schcolnik-Cabrera et al., 2019; Rapoport and Anderson, 2019).

3.2. CRT and Immunogenic Cell Death

Treatments that promote CRT mobilization to the cell surface stimulate ICD through ecto-CRT, which serves as an “eat-me” signal, activating the innate and adaptive immune responses. ER-retained CRT is not immunogenic, however, upon transport onto the cell surface and/or the extracellular milieu, CRT acquires immunogenic characteristics and is considered a danger molecule or “alarmin” (Nanini et al., 2018). Mediators released from cancer cells, known as DAMPs (danger-associated molecular patterns), include CRT, heat shock proteins, ATP, HMGB1 and IFN-I (Garg et al., 2014; Pandolfi et al. 2016). DAMP signals act as antigens, activate anti-tumorigenic $CD4^+CD8^+$ T cell immunity and lead to the recruitment of immature DCs (Casares et al., 2005; Fucikova et al., 2016a; Galluzzi and Kroemer, 2017; Wang et al., 2018a). ATP and HMGB1 promote the response of the immune system using specific pattern recognition receptors

(PRR) (Garg et al., 2014; Pandolfi et al. 2016). Also annexin A1 (ANXA1) and CXC-chemokine ligand 10 (CXCL10) are secreted (Casares et al., 2005; Fucikova et al., 2016a; Galluzzi and Kroemer, 2017). CRT translocation to the cell surface of dying cells depends on cellular ER-stress response and is one of the relevant events in ICD (Obeid et al., 2007b, 2007d; Panaretakis et al., 2009).

Dying cells also produce cell debris that can be engulfed by DCs, which have been recruited into the tumor area fueling the adaptive immune response through antigenic presentation to T lymphocytes (Obeid et al., 2007b, 2007d). The magnitude of the ICD response will depend on the type of tumor and the neo-antigens that the tumor possesses. Accordingly, tumor cells may present antigenic characteristics different from normal cells and generate ICD (Galluzi et al., 2017; Fucikova et al., 2018).

In order to understand the immunological properties of the ICD, it is necessary to bear in mind the immunogenicity (antigenicity) of tumors through the tumor antigen presentation and the adjuvanticity associated with the expression of DAMPs mediators (Galluzi et al., 2017; Fucikova et al., 2018). Therefore, the importance of the ICD process in the immune system lies in anticancer therapeutic application because it allows to activate the immune response of the cancer patient.

3.3. CRT and Apoptotic Cells

Apoptotic cells secrete “find-me” signals that attract phagocytes, such as sphingosine-1-phosphate (S1P), lysophosphatidylcholine (LPC), ATP and CX3CL motif chemokine ligand 1 (CX3CL1) (Lauber et al., 2003; Gude et al., 2008; Truman et al., 2008; Peter et al., 2008; Elliot et al., 2009). Apoptosis also induce the expression of “eat-me” signals on the outer surface of the cell membrane, such as PS (Tarr et al., 2010a). The recruited phagocytes possess a wide variety of PS receptors that can either directly bind PS or recognize bridging proteins to allow efficient cellular engulfment (Kumar et al., 2017). Among the co-receptors that serve as bridge between

PS of apoptotic cells and PS receptors of phagocytes we can find MGF-8, protein S, Gas6 and CRT (Park and Kim, 2017).

In dying cells induced by ER stress, cell surface CRT-PS complex is an "eat-me" signal for phagocytes that increases the recognition of dying cells and allows antigen presentation in macrophages and DCs (Michalak et al., 2009; Gardai et al., 2005; Jiang et al., 2014; Park and Kim, 2017; Osman et al., 2017; Venkateswaram et al., 2018; Schcolnik-Cabrera et al., 2019). CRT-PS interaction is recognized by the CD91 receptor of phagocytes (Obeid et al., 2007d; Garg et al., 2012; Park and Kim, 2017). CRT similarly binds the first complement component C1q as an interactive bridge to PS (Païdassi et al., 2011; Park and Kim, 2017). The CRT-C1q-PS complex is required for recognition of apoptotic cancer cells by phagocytes and the subsequent clearance of apoptotic cells.

CRT-mediated phagocytosis is inhibited by integrin-associated protein (IAP or CD47). Healthy cells present "do not eat-me" signals characteristic of normal cellular function that are based essentially on the interaction of cellular CD47 with signal regulatory protein alpha (SIRPα) of phagocytes (Chao et al., 2010; Raghavan et al., 2013; Fucikova et al., 2018). Therefore, tumor cell surface CRT corresponding to an "eat-me" signal for DCs is counterbalanced by CD47, an anti-phagocytic signal. Because CD47 and CRT are both induced in murine cancer cells, their balance will determine the final cellular fate (Jaiswal et al., 2009; Eric-Nikolic et al., 2012).

4. Functional Roles of Extracellular CRT in Disease

Extracellular CRT has been found associated to diverse pathologies. Table 1 summarizes some of the main reported diseases associated to CRT. We will focus on the current extracellular roles of CRT in cancer, autoimmune diseases and the HTLV-1 retroviral infection. In several pathologies, cellular stress and death are involved in CRT release or exposure with the concomitant production of anti-CRT autoantibodies. In

cancer, CRT is associated to ICD, as well as the innate and the adaptive immunity, which could be useful for possible therapeutic applications. Autoimmune rheumatic disorders, such as systemic lupus erythematosus and rheumatoid arthritis are characterized by the production of several autoantibodies against cellular self-antigens. Parasites and viruses are known to take advantage of extracellular CRT functions to promote infectivity and evade immune responses (Gold et al., 2010). We will discuss some findings of extracellular CRT in the retroviral infection with HTLV-1.

Table 1. Pathologies associated to calreticulin

Metabolic disorders: obesity and diabetes	Neurological alterations: multiple sclerosis, amyotrophic lateral sclerosis
Cancers: oral, esophagus, breast, lung, pancreas, gastric, colon, bladder, prostate, vagina, ovarian, endometrium, liver, neuroblastoma	Hematological diseases: myeloproliferative diseases, myelofibrosis and essential thrombocytopenia
Renal disorders: diabetic nephropathy, renal fibrosis, allograft rejection	Chagas and other parasites diseases that cause disease in humans
Autoimmune diseases: rheumatoid arthritis, systemic lupus erythematosus	Chronic inflammatory bowel diseases. Crohn's disease and ulcerative colitis
Stress induced fibrocitic disorders: lung, cardiac fibrosis, atherosclerosis and vascular injuries	

Lu et al., 2015; Charonis et al., 2017; Nanini et al., 2018; Owusu et al., 2018.

4.1. CRT Function in Cancer

CRT and Ca^{2+} signals contribute to the progression of cancer by promoting cell proliferation (cancer adhesion/invasion), migration (metastasis) and survival (Zamanian et al. 2013; Venkateswaren et al. 2018). CRT participates in cell proliferation and survival by directly binding the transcription factor STAT3, producing the activation of PI3K/AkT signaling pathway and ultimately inhibiting apoptosis. On the other hand, extracellular

CRT promotes metastasis by binding cell surface CD91 and TSP1. The CRT-CD91-TSP1 complex reduces E-cadherin levels and promotes Ca^{2+} release from the ER, which are two major factors responsible of cell migration. During metastasis, free CRT upregulates VEGF (vascular endothelial growth factor), which increases angiogenesis, as well as reduces the activity of non-liganded estrogen receptor alpha (ERα) and E-cadherin (Zamanian et al., 2016).

Several reports show a positive correlation between CRT overexpression and invasiveness of tumors, which is associated to an unfavorable prognosis (Sun et al., 2017). In eleven different cancerous types, a positive correlation between CRT expression (either as RNA and/or protein levels) and tumorigenesis has been found (Zamanian et al., 2013; 2016). Furthermore, CRT and/or anti-CRT antibodies may be useful biomarkers to improve the clinical efficacy of therapeutic treatments. CRT-associated biomarkers used to predict therapy-associated immune responses have been suggested in colon, gastrointestinal, pancreatic, bladder urothelial and breast cancers, as well as in oral squamous cell carcinoma (Sun et al., 2017).

4.2. Anti-Cancer Therapies

Extracellular CRT functions in cancer prognosis and immunological response to therapy are complex and depend on the cell type, the clinical stage of cancer progression and the balance between CRT exposure and CD47 levels in the tumor environment (Venkateswaram et al., 2018; Fucikova et al., 2018). Tumors where CRT does not translocate to the surface are resistant to chemotherapy, which could be associated with defects in the ER stress response (Galluzzi et al., 2017; Sun et al., 2017; Fucikova et al., 2018). CRT is heterogeneously expressed in different cancer cells, suggesting that in some patients, tumor cells submitted to chemotherapy can, independent of stress, release danger signals as a consequence of the malignant transformation (Fucikova et al., 2018). *In vitro* studies with tumoral cells have shown three different strategies that may

restore ICD properties: (1) administration of ER stressors, such as thapsigargin, tunicamycin, pyridoxine and reticulon-1; (b) co-administration of eIF2α phosphatase inhibitors, such as tautomycin, calyculin A and salubrinal; and (c) administration of exogenous rCRT (Sun et al., 2017; Fucikova et al., 2018). Applications of these strategies, however, have neither been reported in mice nor in clinical studies. Instead, therapies consist on treatments with combined chemotherapeutic agents, CRT vaccination (as DNA vaccines encoding CRT) or pro-apoptotic peptides and the so-called checkpoint inhibitors (described in next section).

ICD inducers may increase ROS production or RE stress triggering CRT exposure (Gold et al., 2010; Zitvogel et al., 2010; Wiersma et al., 2015). Therefore, inducers have been classified in two types, type I (ROS generation) and type II ER (stress induction) (Garg et al., 2015; Wang et al., 2018a). Type I inducers produce cancer cell death by acting on non-ER associated target as DNA/chromatin or DNA replication and repair proteins, cytosolic proteins, mitochondria, cellular membranes and surface transmembrane proteins/channels. By contrast, Type II inducers selectively target the ER to induce cell death and produce more DAMPs through ROS-induced ER stress release, being more effective at inducing ICD. In both cases, the type of induced cell death includes: apoptosis, autophagic cell death and necrosis (Wang et al., 2018a). The currently used ICD inducers approved by the U.S.A Food and Drug Administration (FDA) in diverse cancer trials are summarized in Table 2 (Wang et al., 2018a).

Therapy-driven CRT in cancer cells correlates with better prognosis in cohorts of acute myeloid leukemia and non-small cell lung cancer (Fucikova et al., 2016b; Schciolnik-Cabrera et al., 2019). High CRT expression was also associated with an increase in the number of mature and myeloid DCs, as well as with more infiltration in the tumor microenvironment by effector memory $CD4^+$ and $CD8^+$ T cells. CRT is also present in the granules of cytotoxic T lymphocytes (CTL), where it interacts with perforin and assists CTL-induced lysis.

Monitoring the levels of extracellular and surface CRT in cancer tissue samples is useful for the clinical management of therapies (Fucikova et al., 2018). CRT sample analysis includes quantification of exogenous soluble

CRT by ELISA, flow cytometry of non-permeabilized cells and immunohistochemistry of paraffin-embedded tissue using specific antibodies (Wu et al., 2013; Fucikova et al., 2018). Patients can be classified into those with high CRT levels, which have good prognosis and response to therapy; and those with low CRT levels, probably non-responders to therapy and usually considered high-risk patients. The former group is subjected directly to treatment, whereas the latter requires additional analyses to find appropriate immunological restoration strategies prior to treatment.

Table 2. Clinically used ICD inducers

Chemotherapeutics	Associated DAMPs	Types of cancer in clinical applications
Cylophosphamide	CRT at the surface Secretion of HMGB1 and ATP	Lymphomas, brain cancer, leukemia and some solid tumors
Oxaliplatin	CRT at the surface Secretion of HMGB1 and ATP	Colorectal cancer
Mitoxantrone and Antracyclins (Doxirubicin, Idarubicin)	Cell-surface CRT and ERp57 HMGB1 and ATP secretion	Leukemia, Hodgkin's lymphoma, breast, colon, lung cancer
Bleomycin and Bortezomib	Cell-surface CRT and ERp57, Cell-surface HSP90	Testicular cancer, ovarian cancer, Multiple melanoma, mantle cell lymphoma

Wang et al., 2018a.

More recent biotechnological developments include antibodies that can be delivered at the tumor site, such as biospecific antibodies engineered against the ED-A domain of fibronectin (highly expressed in malignant tissues) fused to CRT (Ziffels et al., 2019). Intravenous administration of the CRT-anti-ED-A fusion protein to mice-bearing colon carcinoma resulted in important tumor retardation, which led to a complete remission of the tumor when administered together with programed cell death 1 (PD1)-blocking

antibodies (a checkpoint blocker). Additional increase of $CD8^+$ T cell, DC and macrophage infiltration into the tumoral tissue was found as a result of the antibody therapy, suggesting a possible use in future clinical tests (Ziffels et al., 2019).

Increase of arginylated CRT accumulation in the cytoplasm, stress granules and plasma membrane of glioma cell lines has been found. CRT arginylation, which provides a longer half-life and increased stability to CRT, is catalyzed by the enzyme arginyltransferase-1 (Lopez-Sambrooks, 2012; Comba et al., 2019; Schcolnik et al., 2019). Therefore, modulation of arginyltransferase-1 could serve as a target for improving chemotherapy outcome and cancer prognosis (Lopez-Sammbrook et al., 2012; Comba et al., 2019). Other reports using oxaliplatin in murine glioma cells reported activity reduction of the transcription factor STAT3 and the DNA repair enzyme *O*-6-methylguanine-DNA methyltransferase, together with an increase of cell surface CRT and reduction of the pro-tumorigenic macrophage phenotype. Arginylation of CRT has also been found relevant in the apoptotic response of bortezomib-treated glioma cells, where arginylated CRT results a better "eat-me" signal than the unmodified CRT (Schcolnik-Cabrera et al., 2019).

Rapoport and Anderson (2019) described mechanisms that could restrict the efficacy of ICD such as low mutational load in tumor cells, reduced levels of tumor-infiltrating macrophages, overexpression of CD47 on tumor cells, expression of MHC class I molecules by tumor cells, and release of immunosupressor factors.

4.3. Cancer, CRT and Immune Checkpoint Blockade

Among the new antitumor treatment approaches and with the purpose of achieving synergistic effects, a combination of ICD inducers with immune checkpoint inhibitor (ICI) agents has been explored (Iwai et al., 2017; Wei et al., 2018; Yan et al., 2018; Wang et al., 2018b). Conventional cancer treatments, such as cytotoxic chemotherapy, radiation therapy and targeted therapy, have immunomodulatory effects in addition to direct cell-

killing activity. The clinical relevance of conventional therapy in combination with ICIs have recently been explored, aiming to achieve synergistic effects with improved and durable clinical response. Currently, checkpoint inhibitors are being used in combination with ICD inducers.

Lymphocytes are regulated by co-stimulatory and co-inhibitory molecules of the immune response, some of which can be pharmacologically regulated (Chen et al., 2013; Finetti and Baldari, 2018). Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) is a regulatory protein that is expressed in activated T-lymphocytes and inhibits cell activation. PD-1 is another inhibitory receptor, which similar to CTLA-4, participates in immune response termination (Esensten et al., 2016; Wei et al., 2018). CTLA-4 and PD-1 are co-inhibitors, with their corresponding ligands PD-L1 and B7-1 (CD80), B7-2 (CD86) (Esensten et al., 2016; Wei et al., 2018; Wang et al., 2018a).

Cancer immunotherapy based on blocking/inhibiting these checkpoints has shown an enormous development in recent years (Iwai et al., 2017; Wei et al., 2018; Yan et al., 2018). Several monoclonal antibodies authorized as drugs are among checkpoint blockers, currently including anti-CTLA-4, anti-PD-1 and anti-PD-L1 monoclonal antibodies (Topalian et al., 2015; Wei et al., 2018). ICIs inhibit the interaction between ligands and inhibitory receptors by blocking the activation of inhibitory pathways, allowing the antitumor action of T-lymphocytes.

Numerous pre-clinical studies based on murine and cellular models of ICD induction have demonstrated the significant increase in the antitumor response associated to higher expression of CRT and mediators on the cell surface. The basic experimental design of most studies uses a specific ICD-inducing agent in *in vitro* cells and *in vivo* murine models to demonstrate the expression of DMAPs and the translocation of CRT to the cell membrane (Leshem et al., 2019). Studies in a murine mesothelioma model of ICD induction with immunotoxin anti-mesothelin, showed the *in vitro* secretion of ATP and the expression of CRT on the surface of murine mesothelioma AE17M cells (Leshem et al., 2019). Furthermore, the same authors found an important regression of the tumor after injecting the immunotoxin together with anti-CTLA-4 monoclonal antibodies. The immunogenicity of tumor

cells and the translocation of CRT to the plasma membrane were also demonstrated in epithelial ovarian cancer treated with cisplatin and anti-PD-L1 antibodies (Grabosh et al., 2019), as well as in non-small cell lung (Liu et al., 2019), and the tumoral cell lines CT26WT, MC38, CT26 and MB49 (Sakakibara et al., 2017; Hossain et al., 2018).

Nevertheless, studies using human experimental models are not very abundant. An interesting murine breast cancer cell line model that expresses human epidermal growth factor 2 receptor (hHER2) develops ICD with an antibody-conjugate derivative of anthracycline and trastuzumab, which produced a significant antitumor response together with anti-PD-1 antibody treatment *in vivo* (D'Amico et al., 2019). In a different case using a human cellular model, inhibitor agents of bromodomain regulatory proteins show membrane translocation of CRT and Erp57, the downregulation of PD-1 and the secretion of ATP and HMGB1 in malignant pleural mesothelioma (MPM) cells (Riganti et al., 2018).

Cancer therapy has taken advantage of the progress made in different areas of science and has combined distinct treatments, both traditional and novel (Yan et al., 2018). The use of immune checkpoint inhibitors (ICI) has incorporated a new and very important treatment approach. The combination of ICD-induced therapy, such as chemotherapy and radiotherapy, along with ICI contribute to an efficient antitumor response and results, in general, very promising for applications in human pathology (Figure 1).

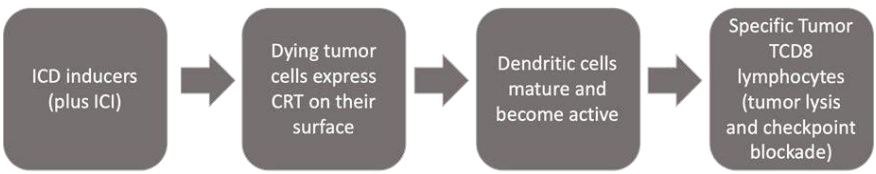

Figure 1. Action of ICD inducers in combination with ICI. The ICD inducers allow the expression of various DAMPs, including the expression of CRT and the secretion of HMBG1and ATP. These mediators activate DCs that can engulf tumor cells and present antigens to T lymphocytes and eventually lyse the tumor. The immunotherapeutic action of ICIs optimizes the tumor lysis.

This synergistic approach has been studied in some cancer types, with the most promising clinical results in non-small cell cancer in randomized

trials using PD-1/PD-L1 inhibitor (humanized antibody) together with chemotherapy (Zhou et al., 2018; Xia et al., 2019) This combined therapy improved the progression-free survival, the objective response rate and the overall survival. Also some success has been observed in clinical trial of patients with chronic myeloproliferative neoplasms, and acute myeloid leukemia (both with a dysregulation of the immune system) using immune checkpoint blocking antibodies in combination with hypomethylating agents (Holmström and Hasselbalch, (2019).

4.4. Autoimmune Disorders

Autoimmune disorders are characterized by the production of varied autoantibodies against self-antigens. CRT is a recognized autoantigen leading to the production of anti-CRT autoantibodies in diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (Eggleton, 2003). The reason of CRT immunogenicity is not completely understood. Moreover, extracellular and cell surface CRT also participate as ligands to different effectors of rheumatic diseases. Given the multifunctional roles of extracellular CRT, diseases such as SLE and RA are key to understand the extracellular mechanisms and potential therapeutic opportunities of CRT in autoimmunity.

4.4.1. Systemic Lupus Erythematosus

SLE is an autoimmune disease characterized by several nonspecific symptoms affecting various parts of the body that widely range among patients, from cutaneous rash to a mortal multi-organ disease (Eggleton et al., 1997). SLE patients produce autoantibodies against several self-proteins and nucleic acids, though the production of autoantibodies alone is considered insufficient to explain SLE symptomatology (Eggleton et al., 2003). The mechanism by which the body produces autoantibodies in SLE is not clear. SLE is also characterized by increased levels of apoptosis and an impaired ability of the immune system to clear apoptotic debris (Huggins et al., 1999; Donnelly et al., 2006). It has been hypothesized that the

defective clearance of apoptotic material in SLE contributes to the loss of immunological tolerance by providing an extracellular source of cytoplasmic and nuclear antigens. Protein and nucleic acid complexes available to antigen-presenting cells in the extracellular milieu may stimulate an autoimmune response and the production of autoantibodies (Staikou et al., 2003; Eggleton, 2003).

CRT association to SLE can be traced back 50 years when Clark et al. (1969) first reported the Ro/SSA autoantigen. Ro/SSA, an extractable nuclear antigen target of autoantibodies found in various autoimmune diseases (Yoshimi et al., 2012), is a soluble ribonucleoprotein complex comprising at least three proteins of 48-, 52- and 60-kDa. The 60-kDa protein was thought to be CRT (Collins et al., 1989; McCauliffe et al., 1990), but instead was found by other groups to correspond to a different protein (Deutscher et al., 1988; Ben-Chetrit et al. 1989). The disparity of the results was attributed to the interaction of CRT with the Ro/SSA complex and their co-purification during early attempts of Ro/SSA characterization (Rokeach et al., 1991). CRT was later found to interact with the Ro52 and Ro60 components of the Ro/SSA complex (Kinoshita et al., 1998; Staikou et al., 2003) and was frequently found associated with apoptotic blebs containing cell debris (Huggins et al., 1999).

CRT association with apoptotic material may be a consequence of its interaction with the complement protein C1q during macropinocytosis of apoptotic blebs (Ogden et al., 2001). C1q promotes the clearance of apoptotic cells by binding to CRT and its co-receptor CD91 at the cell surface of phagocytic cells. However, in apoptotic SLE neutrophils, C1q binding to CRT is impaired, even though SLE patients show increased apoptotic cells (Kishore et al., 1997a; Donnelly et al., 2006). Consequently, C1q/CRT/CD91-mediated clearance of SLE apoptotic neutrophils is defective and may account for the increased quantity of apoptotic cells observed in SLE patients. It is interesting that C1q functional loss and impaired C1q-mediated clearance of apoptotic debris generates autoimmunity and potentially conduces to SLE, as observed in most C1q-deficient humans and mice developing a SLE-like autoimmune disease (Trinder et al., 1997; Racila et al., 2003).

Soluble extracellular CRT has also been reported to bind and sequester available C1q, interfering with complement activation and impairing the uptake of apoptotic cells (Kishore et al., 1997a; Kovacs et al., 1998; Donnelly et al., 2006). Serum CRT levels are elevated in SLE patients compared to healthy individuals or patients affected by other autoimmune diseases (Hong et al., 2010; Duo et al. 2014). Elevated extracellular CRT in SLE may therefore promote deficient clearance of apoptotic blebs (Kishore et al., 1997b; Racila and Sontheimer et al., 1999; Byrne et al., 2013), contributing to the disease progression, activity and organ damage observed in SLE patients with higher levels of serum CRT (Wang et al., 2017a). Alternatively, interfering with cell surface CD91/CRT binding to C1q in phagocytic cells may also conduce to SLE (Eggleton, 2003). For example, anti-CRT autoantibodies potentially disrupt the CRT bridging function during clearance of apoptotic material. It has been documented by several independent groups that many SLE patients produce anti-CRT antibodies (Boehm et al., 1994; Kishore et al., 1997a; van den Berg et al., 1998; Racila and Sontheimer et al., 1999; Eggleton et al., 2000; Sánchez et al., 2000; Scofield et al., 2000).

The mechanism by which B and T cell tolerance to CRT fails is, however, unknown. It is proposed that the CRT chaperone function exposes cryptic sequences in otherwise non-antigenic proteins present in extracellular ribonucleoprotein complexes, a mechanism termed “epitope spreading” (Eggleton et al., 1997; 2000; Eggleton, 2003). It is possible that the CRT association to the Ro/SSA complex in apoptotic ER blebs induces immune intolerance toward CRT as well (Kinoshita et al., 1998). Consequently, the CRT-Ro/SSA complex potentially elicits an early immune response in SLE, supported by observations that the major antigenic CRT epitopes were mapped to the N and P domains of the protein and, in particular, on fragments that bind to C1q for complement inhibition (Eggleton et al., 2000; Huang et al., 2013). In addition, the production of autoantibodies against CRT may also relate to its molecular mimicry with protein homologous from several parasites, such as *Schistosoma mansoni*, *Onchocerca volvulus*, *Opisthorchis viverrini*, *Plasmodium falciparum* and *Trypanosoma cruzi* (Eggleton et al., 1997; Ramírez-Toloza and Ferreira,

2017; Chaibangyang et al., 2018). Parasite CRT homologs share extensive sequence identity with human CRT, which provides them a sophisticated mechanism for immunological escape (Schroeder et al., 2009). Because of sequence similarity, antibodies produced against immunogenic parasite CRT homologs may induce autoimmunity by binding human CRT (Ribeiro et al., 2009).

Besides interfering with the clearance of C1q-opsonized apoptotic cells, recombinant extracellular CRT—active after self-oligomerization— has been shown to stimulate proliferation and secretion of cytokines by macrophages and B cells (Huang et al., 2013). Soluble extracellular CRT potentially transduces MAPK phosphorylation, IκBα degradation and NF-κB phosphorylation via scavenger receptor A in murine macrophages (Duo et al., 2014). NF-κB activation further induces TNF-α and IL-6 expression (Duo et al., 2014). TNF-α secretion and immunoglobulin class switching was also stimulated by oligomerized CRT in B cells via binding to TLR-4/CD14 (Hong et al., 2010). It is interesting that Bruton's tyrosine kinase, a downstream target of TLRs (Horwood et al., 2006), has been found to directly phosphorylate CRT and promote CRT translocation at the cell surface of myeloid cells during C1q-mediated clearance of apoptotic blebs (Byrne et al., 2013).

Therefore, via different mechanisms, extracellular CRT promotes opposite responses; (1) the clearance of apoptotic cells by stimulating CRT association with CD91 at the cell surface of phagocytic cells, and; (2) inhibition of C1q-mediated macropinocytosis and generation of a pro-inflammatory response. A fine regulation of extracellular CRT functions may be impaired in SLE, generating an immunological disequilibrium at inflammation sites that translates into deficient apoptotic clearance and loss of immunological tolerance in SLE.

4.4.2. Rheumatoid Arthritis

RA is a chronic systemic autoimmune disorder, primarily resulting in inflammation, pain and stiffness of joints. The disease most commonly affects hands and wrists, progressively worsening with aging (Goronzy and Weyand, 2005). The specific causes of RA remain unclear. Several

autoantibodies have been found in RA patients that may shed light on the disease etiology, particularly anti-CRT antibodies (Goëb et al. 2009; Huang et al., 2013; Corsiero et al., 2018). Serum autoantibodies against CRT and other ER chaperones, such as HSP60 and BiP, suggest a possible role of CRT in the disease that has been recognized for over two decades (Routsias et al., 1993; Tishler and Shoenfeld, 1996). CRT is therefore considered a target of the RA autoimmune response. However, as described above for SLE, similar autoimmune responses to CRT have been found associated to other pathologies as well, such as mixed connective tissue disease and Sjögren's syndrome (Routsias et al., 1993; Eggleton et al, 2000). Therefore, the auto-calreticulin autoantibodies found in RA patients may not respond to a specific disease mechanism and may instead correspond to a generic overactive response observed in autoimmune diseases.

Similar to SLE, not only have anti-CRT antibodies been found associated with RA. Increased levels of serum CRT —usually higher than SLE patients (Hong et al., 2010; Ni et al., 2013; Hayashi et al., 2015; Hashaad et al., 2017)— and impaired apoptosis (Tarr et al., 2010b; Jiao et al., 2018) are frequently found in RA patients as well. Besides binding C1q and inhibiting effective clearance of apoptotic cells, extracellular CRT is capable of binding directly to the Fas ligand (FasL), an extracellular inductor of apoptosis (Duus et al., 2007). CRT was shown to inhibit FasL-mediated apoptosis in Jurkat T cells (Tarr et al., 2010b). CRT-FasL interaction is blocked by anti-CRT antibodies and therefore increased autoantibodies in the synovial fluid may neutralize CRT inhibition of apoptosis in RA. In addition, CRT produced an increased expression of anti-apoptotic proteins Bcl-XL and Mcl-1 in RA fibroblast-like synoviocytes *in vitro* (Jiao et al., 2018). Apoptosis inhibition in the synovial lining is a hallmark of RA and therefore the induction of apoptosis of synovial macrophages, fibroblasts and lymphocytes have been considered as a possible therapeutic strategy.

There is a strong association of RA patients with the major histocompatibility complex (MHC) HLA-DR cell surface receptor bearing a particular small amino acid sequence. HLA-DRB1 alleles codify for the HLA-DRβ chain of the MHC class II surface protein involved in antigen presentation. A small sequence in position 70–74 of the HLA-DR4 protein

is shared in over 90% of RA patients (Gregersen et al, 1987). This five-amino acid sequence, or shared epitope (SE) with the consensus motif 70Q/R-K/R-X-X-A74, is associated with elevated risk, increased severity and poor prognosis of RA (Gorman et al., 2004; Ling et al., 2007a; 2007b; Mewar et al., 2008). The SE hypothesis establishes this small MHC sequence as the leading cause for RA, although the mechanisms by which the SE promotes and increases the severity of RA remain unclear. One intriguing possibility is that, as observed previously with MHC class I, the SE-containing HLA-DR4 acts as a membrane-bound ligand to other receptors, transducing a signal that may trigger and worsen RA symptoms. The group led by Dr. Holoshitz has done remarkable efforts toward this idea over the past decade by identifying CRT as a specific receptor of the SE (Ling et al., 2007a: Holoshitz et al., 2013)

According to the SE-CRT ligand hypothesis, binding of SE-bearing HLA-DR4 to cell surface CRT transduces a response that activates NO signaling in fibroblasts, mediating ROS production in opposite cells (Ling et al., 2006; Ling et al., 2007b). The NO-mediated oxidative stress significantly impacts the disease progression and correlates with increased NO and its proinflamatory effects in RA (Stichtenoth and Frolich, 1998), as well as increased oxidative DNA damage and cell death found in the RA synovium (Maurice et al., 1997). CRT expressed in RA synovial tissue was shown to stimulate angiogenesis via NO production by increasing the activity of endothelial nitric oxide synthase (eNOS) (Ding et al., 2014). A recent study also found CRT-mediated increase of eNOS activity involved in PI3K/Akt/eNOS/p38 signaling, leading to ICAM-1 (intercellular adhesion molecule 1) and VCAM-1 (vascular cell adhesion molecule 1) upregulation (Liu et al., 2019). High ICAM-1 and VCAM-1 levels correlate to CRT levels from serum and synovial fluid of RA patients, respectively, leading to an increase in chemotaxis and transendothelial infiltration of inflammatory cells (Liu et al., 2019).

In addition, SE-mediated signaling via CRT in DCs was found to polarize CD4+ T cell differentiation toward Th17 cells *in vitro* and *in vivo* (de Almeida et al., 2010; 2011). SE-CRT signaling in CD11c+CD8+ DCs inhibits the activity of indoleamine 2,3-dioxygenase (IDO), which induces

immune tolerance by promoting immunosuppressing Foxp3+ T regulatory cell differentiation, whereas CRT ligand stimulates IL-6 (and IL-23 in the presence of LPS) production in CD11c+CD8– cells (de Almeida et al., 2010). IL-6 and IL-23 lead to the differentiation of Th17 cells at the expense of Treg differentiation. Th17 cells have been found increased in RA joints, along with its product IL-17, a proarthritogenic and autoimmunogenic cytokine (Ziolkowska et al., 2000). Accordingly, the SE-CRT-mediated unbalance or immune dysregulation toward Th17 cells and production of IL-17 has been shown to be proarthritogenic and to enhance disease severity in arthritis models (Nakae et al., 2003).

A 15-aa region of CRT (278–292) has been found associated to the MHC by analyzing HLA-DR/peptide immunoprecipitated complexes in the early studies of Max et al. (1994) and Verreck et al. (1995). Given the, at the time, recent findings of CRT as a possible autoantigen (Routsias et al., 1993) and the canonical role of MHC class II in antigen presentation, it was reasonable to hypothesize that the associated CRT peptides corresponded to antigenic groove peptides (de Almeida et al., 2011). Ling et al. (2007a), however, first showed the possibility that CRT peptides could represent fragments of CRT binding to the SE. Native, cell-bound SE-positive HLA-DR as well as soluble HLA-DR tetramers containing the SE sequence trigger NO-mediated oxidative signaling via binding to cell surface CRT (Ling et al., 2006; 2007b). In addition, the SE expressed in other heterologous proteins, as well as short synthetic peptides also produced the observed CRT-mediated signaling effects of the native SE-containing HLA-DR. CRT binding to the SE was demonstrated by SE-bearing peptide affinity chromatography, surface plasmon resonance, cell binding assays and time-resolved FRET, showing CRT binding with a biologically significant affinity that results in the NO pathway activation (Ling et al., 2007a).

CRT-mediated effects are blocked by anti-CRT antibodies, CRT-directed siRNA and antisense oligonucleotides, as well as anti-CD91 antibodies, suggesting that the transmembrane CD91 protein acts as CRT co-receptor and signal transducer, as previously observed (Basu et al., 2001). CRT-deficient murine embryonic fibroblast (MEF) as well as cells from CD91-negative mice fail to generate SE-mediated signaling. Addition of

soluble CRT restores SE-triggered signal in crt–/– cells, but not in CD91-deficient cells, indicating that soluble extracellular CRT is able to translocate to the cell membrane, interact with CD91 and mediate the SE transduction signal (Ling et al., 2007a).

The CRT binding site to the SE has been identified to be part of the P-domain around the residues 217–223, based on in silico analyses, domain-deletion mutants and site-directed mutagenesis (Ling et al., 2010). CRT E217A and E223A mutants showed lower binding to SE ligands and, along with a D220A mutant, showed diminished SE-activated ROS production in MEF cells. Therefore, CRT binding to the SE and the consequent SE-triggered signal depends on Glu217, Glu233 and Asp220 CRT residues (Ling et al., 2010). The binding of the SE to cell surface CRT and its physiological effects were also observed *in vivo* using 15-mer synthetic linear and cyclic SE-mimetic peptides injected in mice (Naveh et al., 2012; Fu et al., 2013; Holoshitz et al., 2013). Particularly, the conformationally and biochemically more stable cyclic peptides carrying the SE sequence not only bind to cell surface CRT at low-nM concentrations, activating ROS signaling in mouse osteoclast precursor cell line RAW 264.7, but also enhance osteoclast differentiation from murine primary bone marrow cells and from human PBMCs. In addition, the effect of cyclic SE-mimetic peptides facilitate Th17 polarization, reduce disease onset and increase incidence and bone damage in collagen-induced arthritis mouse model (Naveh et al., 2012; Fu et al., 2013; Holoshitz et al., 2013). The immune dysregulation produced by potent peptidomimetic SE ligands and their arthritogenic effects by facilitating osteoclast-dependent bone damage highlight the importance of the SE in inflammatory arthritis etiology.

The CRT-mediated SE hypothesis may also explain the role of environmental factors in RA. Although strong genetic factors are associated to RA, studies in monozygotic twins have shown a disease concordance rate of only 12-15% (Silman et al., 1993). This difference is attributable to environmental factors that are thought to contribute to around 40% of the disease risk (de Almeida et al., 2011). It is therefore believed that RA onset and progression involves a combination of genetics and environmental factors. One such a risk factor is cigarette smoke. Smoking greatly increases

disease susceptibility, particularly in genetically predisposed people (Karlson et al., 2010). Smoking has been found to increase pulmonary peptidylarginine deiminase expression and activity, which increases protein citrullination (deimination of arginine to citrulline). Citrullinated proteins are characteristic of the RA synovial fluid, tissue and fibroblast-like synoviocytes (Makrygiannakis et al., 2008). Intriguingly, citrullinated CRT, particularly in residues critical for its interaction with the SE, has been found to increase CRT binding to the SE and enhance SE-mediated signaling (Ling et al., 2013). All three arginine residues in the CRT binding site to the SE were found citrullinated by mass spectrometry in RA patients. CRT mutants showed that citrullination at position Arg205 may increase CRT-SE binding affinity and transduce SE-mediated signaling to a 10,000-fold higher potency compared to non-citrullinated CRT (Ling et al., 2013). Overabundance of citrullinated proteins in RA subjects relates smoking with increased citrullinated CRT, which in turn enhances SE-mediated signaling, explaining the effects of non-genetic factors associated to RA.

Citrullinated proteins are also neo-antigens, triggering autoantibody production that may account for the association of CRT autoantibodies with the SE, smoking and RA, even before the disease onset (Goëb et al., 2009; Uysal et al., 2010). The abundance of citrullinated CRT and anti-citrullinated CRT autoantibodies in serum of patients with bronchiectasis before developing RA suggests that citrullinated CRT is an early autoantigen in bronchiectasis patients prior to RA onset (Clarke et al., 2017). Association of CRT with the initial stages of RA may also explain the increased serum CRT levels found associated to RA and juvenile idiopathic arthritis that correlate with disease severity (Ni et al. 2013; Hashaad et al., 2017). Smoking also activates the aryl hydrocarbon receptor (AhR), an intracellular ligand-activated transcription factor known to mediate xenobiotic effects (Iqbal et al., 2013). Smoke-mediated AhR activation has been recently found to generate a pro-inflammatory response along with CRT-mediated SE signaling via synergistic activation of NF-κB (Fu et al., 2018). The NF-κB transcriptional effects conduce to Th17 differentiation, IL-17 production and osteoclast differentiation, leading to bone destruction, increased

susceptibility and arthritis severity in mouse models (Veldhoen et al., 2008; Fu et al., 2018).

The relevance of CRT interaction with the SE —the single most significant genetic risk factor associated to RA— relies upon a possible route for RA treatment. Diagnosis of the disease is largely based on symptoms and its current treatments are mostly palliative immunosuppressant therapies (Eriksson et al., 2010). A novel strategy may target the SE-CRT interaction by the administration of antagonistic drugs. The group of Dr. Holoshitz has also made advances towards the design of SE-antagonistic drugs based on the CRT-SE ligand hypothesis (Naveh et al., 2012; Ling et al., 2015). Synthetic linear peptides with the sequence DKCLA have been shown to bind CRT and specifically inhibit its interaction with SE-mimetic peptides, blocking SE-activated NO signaling (Ling et al., 2015). In particular, a stable backbone cyclic DKCLA analog was found even more potent than linear peptides, inhibited osteoclast differentiation at picomolar concentrations in mouse PBMCs, in human PBMCs and also reduced bone damage in collagen-induced arthritis mouse models (Ling et al., 2015). The SE-antagonistic peptide resulted a potent anti-osteoclastogenic and anti-arthritic agent.

SE-antagonists open a possible, plausible therapy for early RA in high-risk subjects by treating the underlying causes of the disease, rather than providing only palliative treatment. Although promising, competitive inhibitors of SE-CRT interaction by using CRT-binding peptides still require a thorough understanding given the multifunctional roles of extracellular CRT in autoimmunity.

4.5. Retroviral Disease: Spastic Paraparesis Associated with HTLV-1

Several authors have reported the interaction between CRT and glycoproteins of several RNA viruses that are important for infectivity, viral replication and maturation (Braakman and Anken, 2000; Nakhasi et al., 2001; Limjindaporna et al., 2009; Khadka et al., 2011; Martinez-Betancurt

et al., 2014; Wang et al., 2017b). However, the proposed role of CRT-glycoprotein association is related to CRT's chaperone activity required for the correct protein folding, with very few reported cases of extracellular CRT associated to viral infectivity and function. For example, the human immunodeficiency virus type 1 (HIV-1) is a lymphocyte-infecting retrovirus expressing proteins able to interact with host CRT (Otteken et al., 1996). The HIV-1 envelope glycoprotein gp160 transiently binds to CRT in the ER, where it requires to be N-linked to oligosaccharides. As with other viruses, it was proposed that CRT acts as a chaperone, binding gp160 folding intermediates during proper folding (Otteken et al., 1996). Western blot analysis of infected cells with HIV-1 showed that anti-CRT antibodies co-precipitate with gp160. The transient binding of gp160 to CRT during its processing together with the requirement of N-linked glycosylation sites suggests that CRT is acting as a molecular chaperone to facilitate its folding.

Proteins from a similar retrovirus, the HTLV-1, were also found to bind CRT, both in intracellular and extracellular environment (Alefantis et al., 2007). HTLV-1 was the first discovered human retrovirus (in 1980s), currently known as the etiologic agent of two human pathologies: the adult T-cell leukemia (ATL, an aggressive lymphoma) and the HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Nagai and Osame, 2003). Our group has been working with HAM/TSP patients characterized by a progressive central axonopathy associated with an axoplasmic transport disorder in long axons of the corticospinal tracts (Cartier et al., 2007). The virus does not infect neurons, despite the neurological failure observed in HAM/TSP patients. The main *in vivo* reservoir of HTLV-1 are CD4+ T lymphocytes; therefore, the viral effects in HAM/TSP must involve extracellular effectors (Grant et al., 2002; Jain et al., 2007; Alberti et al., 2011). The viral protein Tax, found in cerebrospinal fluid, serum and culture media of PBMCs from HAM/TSP patients, has been proposed as a possible extracellular cause of axonal damage (Cartier and Ramírez, 2005; Alefantis et al., 2007; Medina et al., 2016). Inside the cell, the multifunctional protein Tax is detected in the nucleus, cytoplasm, ER and Golgi apparatus of infected lymphocytes (Jain et al., 2007; Antell et al., 2014). Cytoplasmic Tax localizes in secretory-like vesicles (Jain et al., 2007;

Alefantis et al., 2007) and its C-terminal sequence has two putative secretory signals, DHE and YTNI, which could explain a secretion mechanism through the ER-Golgi pathway (Antell et al., 2014).

HTLV-1-infected T cell lines express higher levels of CRT, suggesting that CRT may play a role in HTLV-1 pathogenesis (Alefantis et al., 2007). CRT also interacts with Tax intracellularly, as observed by immunofluorescence co-localization and immunoprecipitation assays in infected cell lines (Alefantis et al. 2007; Medina et al. 2014). Medina et al. (2014) showed a correlation between CRT and Tax levels secreted from HTLV-1-infected PBMCs, suggesting a CRT-Tax secretion mechanism through the classical ER-Golgi pathway. Tax interaction with CRT in the ER may mask the ER retention signal of CRT, allowing its secretion to the extracellular medium. CRT-Tax interaction remains after secretion, as found by their co-immunoprecipitation from culture supernatants of infected PBMCs (Medina et al., 2014). Secreted Tax also binds soluble semaphorin 4D (sSEMA-4D), a protein released from PMBCs that might be associated to Tax enhancing the SEMA-4D-Plexin-B1 signaling pathway, accounting for the axonal degeneration (Quintremil et al., 2016). A possible scenario involving extracellular CRT in HAM/TSP pathology is that CRT could block the formation of the Tax-SEMA-4D complex. CRT has been observed selectively concentrated in Tax-containing cytoplasmic dotted structures outside the ER, so we can also hypothesize that CRT could also counteract the intracellular deleterious effects of Tax.

Furthermore, CRT additionally interacts with $p12^{I}$, an HTLV-1 protein localized in the ER and the cis-Golgi apparatus, required for *in vivo* lymphocyte infection (Ding et al. 2001, 2002). At the beginning of T cell infection by HTLV-1, $p12^{I}$ induces NFAT (nuclear factor of activated T cell) signaling associated to the viral replication via calcium release from the ER (Albrecht et al., 2002; Ding et al. 2002, 2003; Kim et al., 2003). NFAT activation ultimately allows T cell proliferation under environments with low levels of IL-2, an interleukin required for proliferation, and consequently, a larger number of infected T cells multiply *in vivo* (Rosadas and Puccioni-Sohler, 2015). The ER-resident $p12^{I}$ protein can be proteolytically cleaved to eliminate a non-canonical retention signal at the

N-terminal site, allowing the trafficking to the Golgi apparatus (Fukumoto et al., 2009). It has been shown that CRT expression blocks the $p12^{I}$-mediated NFAT activation by its action as calcium buffering protein (Ding et al. 2002). As in the case with Tax-CRT interaction, we could hypothesize that CRT counteracts the deleterious effects produced, in this case, by $p12^{I}$.

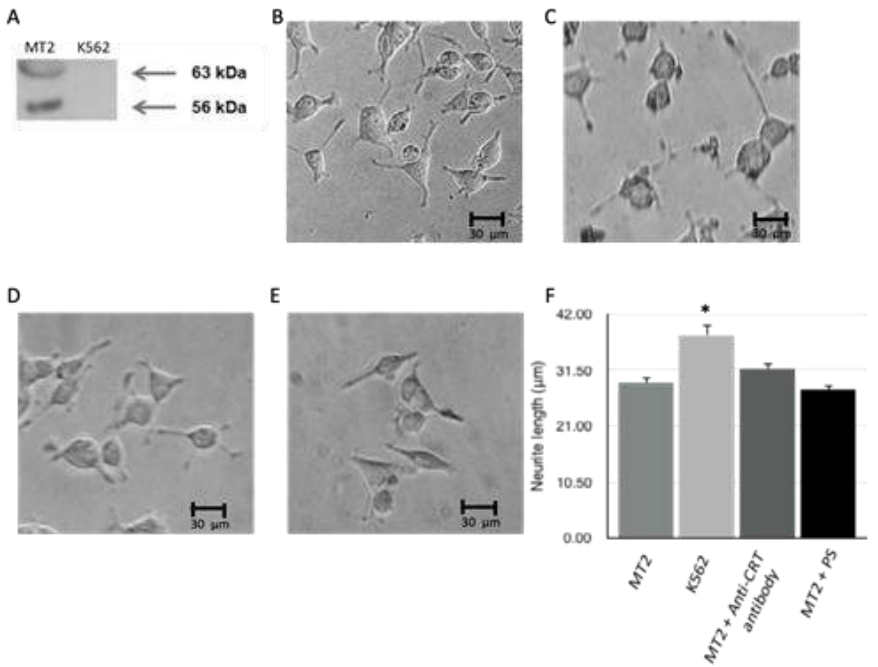

Figure 2. Extracellular effects of HTLV-1-infected lymphocytes on neural-differentiated PC12 cells. (A) Western blot detection of CRT in culture supernatants of MT-2 cells; (B) PC12 cells cultured with MT-2 culture supernatant; (C) K562 culture supernatant; (D) MT-2 culture supernatant with anti-CRT antibodies, and (E) MT-2 culture supernatant with rabbit pre-immune serum. Quantification of PC12 neurite length from all culture conditions (see Quintremil et al., 2016) is shown in (F). *$p<0.05$ compared to the MT-2 condition.

To understand why Tax secretion from infected cells causes deleterious effects in neurons, we have used an *in vitro* model to test the effects of extracellular Tax (Quintremil et al., 2016). The model includes PC12 cells during NGF-mediated neuronal differentiation and the culture supernatant of HTLV-1-infected MT-2 cells as a source of Tax. In western blot of MT-

2 culture supernatants it was shown the presence of extracellular Tax, CRT and SEMA-4D. The addition of MT-2 medium in PC12 culture significantly reduced the neurite length of PC12 cells compared to the addition of non-HTLV-1-infected K562 lymphocytes (Figure 2, Quintremil S, unpublished results). In the presence of anti-Tax antibodies, however, neurite length reduction was prevented, indicating that extracellular Tax causes, at least in part, the neuronal deleterious effects. Using PC12 cells, we also followed by western blot the presence of extracellular CRT in the culture supernatant of MT-2 cells. Figure 2 shows that the addition of rabbit anti-CRT immune serum to PC12 cell supernatants does not block the effect of MT-2 culture supernatant compared to a pre-immune serum control, indicating that CRT is not responsible for the deleterious effects of MT-2 secreted products.

5. Function of CRT in Wound Healing

Chronic wounds are a major cause of mortality, especially in patients with diabetes and other pathologies. Significant economic resources are spent on wound care and management worldwide (Nethi et al. 2019). Different reports have shown that CRT has beneficial properties for wound healing and, consequently, CRT has emerged as a candidate target for wound care therapy.

Wound healing is a dynamic and coordinated process that involves different phases and diverse cell types in communication with each other (Clark 1989; Gailit and Clark 1994; Clark et al. 2007; Nanney et al. 2008). After clot formation, there is an inflammatory phase characterized by leukocyte and macrophage migration into the wound, which remove bacteria and dead cells. After the inflammatory phase keratinocytes migrate over the wound and proliferate at the wound margins. During the tissue formation phase, cytokines and growth factors, such as transforming factor β3 (TGF-3β), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) are secreted from macrophages to control fibroblasts, connective tissue cells, and endothelial cell attraction. Extracellular matrix proteins are also produced to provide a

provisional matrix that assists continued keratinocyte migration and re-epithelization. Neovascularization, matrix deposition and protease secretion for clot dissolution occur before the complete wound closure.

Burd et al. (1991) isolated hyaluronic acid (HA) from fetal sheep and found that it was able to accelerate wound repair in a rat model. The wound repair rate was, however, not blocked by hyaluronidase. The responsible repair agent protein was named hyaluronan protein complex and later, after purification and amino acid sequencing, its active component was identified as CRT (Bakshandeh et al., 1992; Baksh et al., 1992; Cabrera et al., 1995). Gold et al. (2006) were pioneers in this field, showing that the use of recombinant CRT had beneficial properties in wound healing from porcine and murine models. CRT-treated (5 mg/mL) wounds in 12-week-old db/db mice, a type II diabetes mellitus model with impaired wound healing, showed anticipated wound closure (17.6 days post-wounding) compared to control mice treated with a saline solution (23.2 days post-wounding). CRT also promotes the proliferation and migration of dermal fibroblasts isolated from db/db mice and human dermal fibroblasts (Greives et al., 2012). The group lead by Gold et al. (2006) also tested rCRT effects in a porcine model (Yorkshire pigs), showing that granulation tissue formation, measured morphometrically as dermal depth, was increased in CRT-treated (5 mg/mL) wounds compared to Regranex gel (vehicle control) at 5 days post-wounding. The use of a 1.0 mg/ml dose versus a 5.0 mg/ml dose of CRT showed a dose-dependent effect (Gold et al. 2006).

Also in porcine model, CRT induced a dose-dependent increase in dermal thickness after 5 and 10 days of healing (Nanney et al., 2008). The dermal depths of the 5.0 mg/ml CRT-treated wounds were significantly smaller than negative controls treated just with buffer. Ten-day treatment with CRT resulted in more mature stratified epidermis when compared to buffer-treated wounds. Tissue staining comparison of adjacent unwounded skin and wounds treated with PDGF, which stimulate healing, shows a dynamic expression of CRT during wound repair and a drastic upregulation of CRT in the healing dermis of PDGF-treated wounds, suggesting a role of endogenous CRT in tissue repairing. Using different CRT concentrations (1, 5, 10, 50 mg/ml), Nanney et al. (2008) showed that CRT treatment of

wounds induced a dose-dependent increase in proliferating basal and suprabasal keratinocytes from the immature epidermis compared to control, buffer-treated wounds. In addition, CRT induced proliferation of primary human keratinocytes, fibroblasts and microvascular endothelial cells when increasing amounts of CRT were added to the cultures. Using scratch plate assays as an *in vitro* model of wound healing and chamber assays to measure migration, CRT was shown to induce migration of human monocytes, macrophages, primary keratinocytes and primary fibroblasts (Nanney et al., 2008).

Not only human CRT has beneficial effects on wound healing, but also CRT homologs from human parasites. CRT from *Trypanosoma cruzi* (TcCRT), a protozoan parasite that produces Chagas disease (discovered by Dr Carlos Chagas in 1909), is 50% homologous to human CRT and have been found extracellularly, where it modulates the host-parasite interaction (Ferreira et al., 2004; Ramírez et al., 2011). *In vitro* studies show that TcCRT is more effective than human CRT to promote migration and proliferation of human fibroblasts using the scratch plate assay. In addition, TcCRT was able to efficiently promote wound healing *in vivo* when delivered to rats in a chitosan matrix (Arias et al., 2012; 2018). Other studies have shown TcCRT inhibition of the complement system, promoting *T. cruzi* infection and, therefore, considering TcCRT as a virulence factor. However, the myriad of CRT functions also include benign effects, such as anti-tumoral and wound healing properties, suggesting a complex TcCRT-mediated host-parasite interaction promoting infectivity and, at the same time, protecting the host (Ramírez-Toloza and Ferreira 2017).

CONCLUSION AND FUTURE PERSPECTIVES

In some diseases, CRT levels found at the surface of cells and tissues, as well as CRT in serum and synovial fluid have predictive value in disease development and progression. Monitoring the expression of CRT could, therefore, serve as a relevant clinical biomarker for predicting successful therapy strategies, including immunological responses to tumoral cells. For

the search of novel cancer therapies, there are limited pathologies that respond to ICD inducer-mediated CRT exposure. However, there are currently several new ICD inducers under study, some of which are already in pre-clinical and clinical studies. The extracellular presence of CRT either soluble or as ecto-protein in the extracellular medium stimulates malignant cell (tumor and metastasis) removal and potentially achieves total eradication of cancer. Biomarkers, such as CRT, along to immune monitoring are important in clinical trials to identify the efficacy of ICD inducer treatments and predict the expected recovering (Garg et al., 2015). A better understanding of extracellular CRT effectors and associated pathways will ultimately help develop appropriate strategies in non-responder patients.

The pleiotropic functions of CRT make it a very interesting treatment candidate. Even though some developments of CRT treatment in different pathologies have been described here, studies on dosage, vehicles and characterization of active portions of CRT are still pending. There is already some focus toward the use of the CRT as a drug. For example, Čiplys et al. (2015) showed a large-scale production of biologically active human recombinant CRT in yeast. Additionally, Hernández et al. (2019) showed that gold nanoparticles functionalized with CRT had wound healing properties. The CRT nanocomposite was able to increase wound healing *in vitro* and *in vivo*, which could result effective to treat wounds in diabetic patients. We expect that extracellular CRT will provide new and effective treatment alternatives in the years to come.

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Chapter 2

CLINICAL IMPORTANCE OF SERUM TUMOR MARKERS ASSESSMENT IN PATIENTS WITH ADNEXAL MASSES

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ABSTRACT

Adnexal masses, including ovarian cancer, have increased in incidence during the last decade. Extensive introduction of screening programs and progress in diagnostic imaging methods have led to a progressive increase in adnexal masses detection, which can have

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gynecologic or nongynecologic etiologies, ranging from functional cysts to ovarian cancer, and intestinal masses. Appropriate discrimination between benign and malignant mass results in the correct choice between conservative and surgical management.

Pelvic examination has low sensitivity for detecting an adnexal mass; therefore detailed workup required employing different indicators. Currently, development and application of cancer biomarkers have significantly increased opportunities for improving cancer diagnosis and treatment. Early detection of malignancy can be achieved using serum levels of tumor markers such as Ca 125 combined with Ca 19-9, Ca 15-3, HE 4, and CEA. Nowadays, a multimodal approach including the evaluation of serum tumor biomarkers combined with imaging techniques seems to be the best strategy for adnexal tumor detection. Malignancy risk estimation of ovarian cysts could be also achieved by employment of new multivariate logistic regression models and scoring systems that have been introduced into clinical practice to improve the diagnostics. The most useful algorithms utilizing tumor markers are Risk of Malignancy Index (RMI), Risk of Ovarian Malignancy Algorithm (ROMA), OVA-1, and Triple screen. In this chapter, we provide an overview of the tumor markers and scoring systems as well as it's utilization for effective diagnosis of female adnexal masses.

Keywords: ovarian cysts, Adnexal mass, tumor markers, algorithm, scoring systems.

1. INTRODUCTION

The normal ovary size in women of reproductive age is in average 6.1-6.6 cm^3 [1], and contains epithelial, germ cell, and mesenchymal elements. Each of these cell types can undergo neoplasia, resulting in a wide variety of possible tumors arising from a single organ. An adnexal mass is a tumor in the uterine adnexa that can be quite variable in terms of their structure (cystic, solid, mixed consistency) and nature (benign, borderline and malignant) [2, 3].

Table 1. Types of adnexal masses

Benign ovarian tumors	Borderline ovarian tumors	Benign non-ovarian	Primary malignant ovarian	Other malignancies
Functional Cysts 1. Follicular cyst 2. Corpus luteum cyst 3. Theca lutein cysts. **Non-Functional** 4. Endometriomas 5. Serous cystadenoma 6. Mutinous cystadenoma 7. Cystic adenofibroma 8. Hemorrhagic cyst 9. Dermoid cysts (mature teratoma) 10. PCO Syndrome	1. Mucinous cystadenoma 2. Seromusinous cystadenoma 3. Serous cystadenoma	1. Paratubal cyst 2. Uterine myomas 3. Hydrosalpings 4. Pyosalpings 5. Tubo-ovarian abscess 6. Peritoneal pseudo-cysts 7. Appendicular abscess 8. Diverticula of intestines 9. Diverticula of ureter 10. Pelvic kidney 11. Obturator Nerve Schwannoma	12. Epithelial carcinoma 13. Sex-cord tumors 14. Germ cell tumors – Immature teratomas, yolk sac tumors and dysgerminomas 15. Adenocarcinoma of the ovary 16. -Serous cystadenocarcinoma 17. -Mucinous cystadenocarcinoma 18. -Papillary adenocarcinoma 19. -Solid-cribriform adenocarcinoma	20. Ovarian metastases form breast 21. Ovarian metastases from gastrointestinal tract 22. Gastrointestinal tumors 23. Fallopian tube malignancy

Different and multiple pathologic conditions can present as adnexal masses [4, 5, 6]. Differential diagnosis should take into consideration primary ovarian tumors, masses arising from the fallopian tubes or uterus, tumors from the gastrointestinal or urinary tract, pelvic kidneys, masses arising from remnants of embryological development, etc. (Table 1). More than 30 subtypes of ovarian neoplasm have been described. However, most are in one of 3 major categories: epithelial, germ cell, or stromal neoplasms.

Benign ovarian cysts are classified as functional (occurring during the normal menstrual cycle) or non-functional, i.e., organic (occurring regardless of the menstrual cycle). Simple ovarian cysts are common among both pre- and postmenopausal women [6, 7]. These cysts are mostly stable or spontaneously resolve within 6-12 weeks [7]. Available epidemiological data show that overall 7–14% of women present with simple ovarian cysts on regular gynecological examinations, whereas the one-year incidence of new simple cyst is 8% [2, 7]. Furthermore, the worldwide prevalence of benign ovarian cysts is estimated between less than 1% up to 18% for postmenopausal women and around 7% in asymptomatic women in the reproductive period.

Ovarian cancer remains the most lethal gynecological malignancy and it is the seventh most common malignant tumor in women, and the fourth most common cause of cancer death in the female population worldwide [2,6]. The incidence of has a peak in postmenopausal women [8]. Out of all primary ovarian malignant tumors papillary serous cystic adenocarcinoma is the most common type, followed by mutinous, endometrioid, yolk sac, dysgerminoma and adult granulosa cell tumor [9, 10].

According to numerous epidemiological data, risk factors for malignancy of ovarian cysts are nulliparity, low parity, delayed childbearing, early onset of menses, late menopause, postmenopausal estrogen use for 10 or more years, family history of ovarian or breast cancer, and any inflammation of the ovarian epithelium (caused by asbestos and talc exposure, endometriosis, and pelvic inflammatory disease). There is no consensus present in the literature about the influence of ovulation induction drugs on ovarian cancer development. According to the recent review, most studies show that fertility treatments do not increase the risks of invasive

ovarian cancer [11, 12]. However, some data support a small increase in the absolute risk of borderline tumors after fertility treatments; there is insufficient consistent evidence that a particular fertility drug increases the risk of borderline ovarian tumors [12]. Furthermore, higher body mass index (BMI) and postmenopausal status are associated with an increased ovarian cancer risk. However, some epidemiologic observations consistently suggest that suppression of ovulation; tubal ligation, tubal removal, and hysterectomy reduce the risk of ovarian cancer [13]. Researchers have determined that risk factors for benign ovarian cysts are early menarche, obesity, infertility, hereditary factors (for dermoid cysts), as well as Tamoxifen therapy (persistent ovarian cysts).

There is a wide variation in survival between histological groups, and stage at diagnosis remains an important factor in ovarian cancer survival [14]. More than two thirds of patients with ovarian malignancies survive only 1 year after the initial diagnosis. The overall 5-year survival rate for all stages of ovarian carcinomas is just 45% and it is worse for advanced stages [2]. In some studies, it was found that overall survival of women with ovarian epithelial carcinoma decreases from 9 years for stage II, to only 6 months for stage IV. The survival of patients with stage IV sarcomas was even shorter (just 2 months) [15].

Ovarian cancer has the worst prognosis among all gynecological malignancies, mainly due to predominantly an asymptomatic course of the disease. The symptoms of the ovarian cancer are very vague like bloating, pelvic or abdominal pain, poor appetite, feeling full quickly, and urinary urgency it is also known as “silent killer”. Thus, silent occurrence and slow progression, added to the fact that few effective methods for early diagnosis and no universal screening method for diagnosis of malignant ovarian tumor exists, made its mortality rate highest among gynecologic malignancies [16].

Over the last 15 years there have been numerous studies which have found that the majority of women have a constellation and pattern of symptoms that is somewhat unique to ovarian cancer [17, 18].

Ovarian cancer can be classified as either Type I or Type II. Type I OC includes low grade serous, endometrioid, clear cell, and mucinous carcinomas which are generally indolent, relatively genetically stable and

lack TP53 mutations. Type II OC includes high-grade serous and endometrioid, undifferentiated carcinomas and carcinosarcomas that are highly aggressive, evolve rapidly and display TP53 mutations in over 80% of cases [19].

As none of the individual risk factors is discriminatory between benign and malignant adnexal masses, malignant ovarian tumors are more often diagnosed at an advanced stage [10, 20]. More than two thirds of cases of ovarian cancer are diagnosed when the disease has progressed into the stage III or IV, involving peritoneal cavity or other organs, when the 5-year survival rate is as low as 10% [21].

In order to detect the disease in the very early stage, several approaches have been used to triage women with suspicious adnexal masses [22]. These attempts include the use of a single cut-off for serum Ca 125 and HE 4 level, ultrasound morphology and scoring systems, Doppler ultrasound parameters and complex statistical models developed from multivariate logistic regressions [23]. According to numerous guidelines [2, 6] for a newly diagnosed ovarian mass, patients are stratified on the basis of their menopausal status. Women in menopause, Ca 125 > 250 U/mL, HE4 > 150pmol/L, presence of ascites, evidence of the abdominal or distant metastasis (by examination or imaging studies) need to be referred to the gynecological oncologists, as each of these parameters have been shown to be significantly and independently related to the highly likelihood of malignancy [5,24]. However, none of these listed parameters examined alone have proven its absolute accuracy in predicting the adnexal tumor nature [3, 25].

2. Classification of Tumor Markers

An informative biomarker should have the potential to diagnose the disease prior to metastasis. Usually, early stage of the cancer reveals around 80% response rate to surgery and chemotherapy treatment. Overall, combination of panels of markers will eventually lead to effective screening strategies for personalized treatment by early detection of ovarian cancer

with high specificity and sensitivity. Tumor markers have a major role in the screening, diagnosis, and monitoring of most of the gynecologic cancers [26].

Tumor markers are the substances that can be detected in higher-than-normal amounts in the blood, urine, or body tissues of some patients with certain types of cancer. Tumor markers could be used for detection of tumor presence, follow-up of the disease and monitor response to the treatment:

- Screening – to identify early cancer risk;
- Diagnosing – to collaborate the diagnosis;
- Staging – to assess and stratify the risk;
- Prognosis – to predict outcomes;
- Localization – to locate the primary site of a malignancy;
- Therapy – to target the therapy;
- Surveillance – to detect recurrence in follow-up;
- Monitoring – to evaluate response to treatment.

However, diagnosis cannot be established based on tumor marker levels only due to following factors:

- Cancer heterogeneity;
- Lack of specificity of tumor markers leading to false positivity;
- Lack of sensitivity, leading to false negativity;
- Benign diseases could have positive CA 125 or CEA;
- Smokers have raised CEA;
- Higher levels only with large tumor volume.

Some markers could be elevated without tumor, there is no specific marker to a particular type of cancer, and not every patient with cancer has an elevated tumor marker level. Ideal tumor marker should have high sensitivity (SN) and specificity (SP), be cheap and simple method of determination [26].

Table 2. Classification of tumor markers

1. Oncoproteins/Oncogenes
2. Tumor suppressor genes
3. Adhesion molecules
4. Tumor associated proteins
a. Oncofetal antigenes
b. Epithelial antigens
c. Enzymes
d. Hormones

3. TUMOR MAKERS IN DIFFERENTIAL DIAGNOSIS OF ADNEXAL MASSES

The concept of tumor markers use as either screening or diagnostic test for ovarian cancer is based on identifying an abnormal level of a particular marker in serum, reflecting a systemic effect of disease [27]. Early detection of ovarian cancer can be achieved using tumor markers such as carcinoma associated antigen 125 (CA-125) combined with cancer/carbohydrate antigens CA 19-9, CA 15-3 serum levels and carcino-embryonic antigen (CEA) [26]. Other tumor biomarkers such as associated glycoprotein TAG 72, human chorionic gonadotropin (hCG), alpha fetoprotein (AFP), lactate dehydrogenase (LDH), and the most recently human epididymis 4 (HE 4) should be respected for early detection of ovarian cancer as well [26,28]. Some novel markers are still being investigated osteopontin, mesothelin, vascular cell adhesion molecule-1 (VCAM), Golgi phosphoprotein-3 (GOLPH3), kallikreins, human prostasin (PRSS8), mRNA etc. [29]. Different combination of multi-biomarkers like HE4, FOLR, Kallikrein, miRNA, ALDH1 and CA125 seems likely to be shown higher sensitivity at early diagnosis of ovarian cancer.

3.1. Cancer Antigen 125

Cancer antigen 125, also known as MUC16, is the first tumor marker of ovarian cancer, and the most commonly used gynecological tumor marker for evaluation of ovarian masses. It is a membrane glycoprotein antigen normally expressed in Mullerian and coelomic epithelial tissue derivatives [30]. Currently, it is used as the tumor marker of choice for routine monitoring of women treated for ovarian cancer and also to indicate patient survival rates [31, 32].

CA-125 is increased in some common benign gynecological diseases, or nongynecological conditions and also in malignancies of different origin resulting in a reduction of specificity [33]. In particular, CA-125 can be elevated by benign conditions like menstruation, pregnancy, endometriosis, adenomyosis, ovarian cysts, pelvic inflammatory disease, Meigs's syndrome, acute pancreatitis, peritonitis, pleuritic, peritoneal dialysis and chronic alcoholic hepatitis; and by non-ovarian malignancies like carcinomas of different tissue types (endometrium, endocervix, fallopian tubes, pancreas, lung, breast, colon, paratesticular), malignant mesotheliomas, immature teratomas, and lymphomas [33].

Elevated levels are detected in approximately 80% of women with epithelial ovarian cancer at the time of diagnosis, and in approximately 50% of women with Stage I disease [34]. CA-125 was found to significantly correlate with histopathological findings of carcinoma [8]. Elevated CA-125 levels have been reported in 12.7%-24% of patients with mature cystic teratomas [35].

A serum concentration of CA-125>35 U/ml is indicative of potential malignancies. At specificity of 90%, SN ranges between 50% and 60% in early stage postmenopausal women and 80–90% in advanced stage [36]. The level of CA-125 is also used in monitoring of recurrence of disease; postoperative serum of CA-125>65 U/ml is associated with worse 5 year survival.

Table 3. CA-125 diagnostic sensitivity by ovarian cancer stages

CA-125 diagnostic SN	**Ovarian cancer: by surgical laparotomy**	
Stage I-II: 50%	Stage I	Confined to ovaries
	IA	1 Ovary
	IB	2 Ovaries
	IC	1/2+capsule rupture/tumor at surface/malignant ascites
	Stage II	1/2 Ovaries + Pelvic extension
	IIA	Uterus/tubes
	IIB	Other pelvic organs
	IIC	A/B with malignant ascites
Stage III-IV: 80-90%	Stage III	1/2 Ovaries + Peritoneal metastases/regional lymphatic nodules
	IIIA	Micro peritoneal metastases
	IIIB	Macro < 2cm peritoneal metastases
	IIIC	Macro > 2cm/regional lymphatic nodules metastases
	Stage IV	Distant metastases beyond peritoneal cavity

However, serum CA-125 has been investigated for ovarian cancer screening with conflicting results [37]. The limitation of CA-125 lies in its absence in about 20% of ovarian cancers and elevations in other benign conditions of peritonitis, liver cirrhosis, endometriosis, menstrual cycle and pregnancy. CA-125 found to be increased in 0.2-5.9% healthy women and in 2.2-27.8% of benign disease [34]. Therefore, it can be seen that CA-125 has limitations when used to distinguish between benign and malignant ovarian masses. Women with malignant tumors had the highest levels of CA-125, but in numerous studies there were no significant differences between women with benign and borderline tumors [9, 38].

Thus, in order to increase the reliability of CA-125 testing even more, it was suggested that clinicians should use likelihood reference tables of CA-125 serum levels in order to better interpret preoperative CA-125 findings in patients with adnexal masses [27, 39]. Moreover, currently it is recommended that CA-125 serum levels are used to differentiate malignant and benign ovarian masses in combination with other diagnostic methods, especially in the form of different statistical models for preoperative prediction of the adnexal masses nature [27, 39].

3.2. Human Epididymis Protein 4

Human Epididymis Protein (HE4) is a novel serum marker, glycoprotein, product of the WFDC2 gene. HE4 is more sensitive in the prediction of the risk of ovarian malignancy than CA-125 alone in patients with a pelvic mass, and is frequently over expressed by epithelial ovarian cancer, especially serous and endometrial ovarian carcinomas. As a single tumor marker, HE4 has the highest SN for detecting ovarian cancer, especially Stage I disease [40]. Compared with CA-125, HE4 has an increased SP to discriminate patients with gynecological malignancies from those with benign gynecological disease, however the combination of HE4 and CA-125 are more sensitive than either marker alone [40].

The main advantage of this marker is the fact that levels of the protein HE4 are not elevated in benign gynecologic conditions, but have been shown to be elevated in women with ovarian cancers [37,41] and up to now, the most promising new diagnostic tool in ovarian cancer seems to be HE4 [42, 43].

HE4 as a tumor marker for epithelial ovarian cancer has received authorization from the United States Food and Drug Administration (FDA) to monitor disease progression or recurrence of disease (follow-up) together with CA-125, even if few studies are available to date about its use in this setting [44]. In some study, analysis of urine and serum samples at 95% specificity show SN of 72.9% that is enhanced to 76.5% in combination with CA-125 [40]. HE4 demonstrates the highest SN for stage I diagnosis with 45.9% SN at 95% SP but without substantial difference on combination with CA-125. In contrast, diagnostic SN from sera of ovarian carcinoma was 82.5% for HE4 and 76.6% for CA-125, at 95% SP [40]. Benign pelvic mass of HE4 levels show only 8% elevation compared to 29% of CA-125. In urine, 86.6% SN for stage I/II and 89.0% for stage III/IV, at 94.4% specificity were observed and expression was seen prior to clinical recurrence [41]. Serum levels of HE4 however seem likely to be improbable for ovarian cancer diagnosis and need further assessment.

However, HE4 is not specific for ovarian cancer and some expression has also been found in other malignancies, mainly pulmonary and

endometrial adenocarcinomas [45]. Moreover, significant elevation of HE4 was found in patients with renal failure, while slight increase was detected in liver disease and pleural effusions [46]. Moreover, if used alone, HE4 has about 20% of false negative results. Therefore, it is suggested to use HE4 serum levels together with other diagnostic methods. Assessment of HE4 in combination with CA-125 serum levels appears to be the most effective tool for the early diagnose of ovarian carcinoma [40].

Table 4. Reliability of tumor markers Ca 125 and HE4 for predicting malignancy of adnexal masses (range of mean values according to available literature data)

Tumor markers	Premenopausal women				Postmenopausal women			
	SN %	SP %	PPV %	NPV %	SN %	SP %	PPV %	NPV %
CA-125	70 – 76	67 – 71	45 – 85	93 – 97	83 – 89	76 – 79	63 – 88	78 – 80
HE4	65 – 70	87 – 100	90 – 100	95 – 98	85 – 88	94 – 97	94 – 98	80 – 82

Legend: SN – sensitivity, SP – Specificity, PPV – positive predictive value, NPV– negative predictive value.

Studies focusing on the potential use of HE4 as a biomarker of ovarian cancer suggest that it is elevated in over 50% of ovarian cancer patients whose tumors do not express CA-125 [40]. Investigations into the use of HE4 as an ovarian cancer biomarker have proceeded both in the area of population based screening and in the differential diagnosis of a pelvic mass. Despite a number of promising reports, it has become apparent that HE4 is not sufficiently sensitive or specific to function effectively as a standalone test. However, the combined use of CA-125 and HE4 in the differential diagnosis of pelvic masses has received a considerable amount of attention.

3.3. Cancer Antigen 19-9

Another commonly used tumor marker, Cancer antigen 19-9 (CA 19-9), is a carbohydrate chain antigen and associated with different types of malignancies: ovarian carcinoma, hepatocellular carcinoma, pancreatic

adenocarcinoma, and other types of cancer. CA 19-9 has normal values < 35IU/ml. With an upper limit of normal of 37 U/ml, the assay's overall SN is approximately 25% and its SP is 90%, positive predictive value (PPV) 47%, negative predictive value (NPV) 70% and accuracy 75% [47].

Some researchers results suggest that elevated levels of CA 19-9 are associated with tumor size in women aged 26-35, but not in adolescents/young adults [48]. It has been found that CA 19-9 is secreted into the cystic cavity by the mucinous epithelium of the mature teratomas and large size or any inflammation of cyst tissues could be possible causes of CA 19-9 leakage into the bloodstream [49]. Elevated levels of CA 19-9 have been found in up to 50% of patients with dermoid cysts [50]. It has been suggested that CA 19-9 is more commonly elevated than CA-125 and it is a more useful tumor marker for diagnosing mature teratomas and elevated serum CA 19-9 levels were correlated with larger tumor size but not with bilateral localization [51]. Generally, the combined detection by CA-125 and CA 19-9 improve the diagnosis rate of ovarian epithelial cancer [52].

3.4. Carcinoembryonic Antigen

More than fifty years has passed since Gold and Freedman first described the tumor associated carcinoembryonic antigen (CEA) in human colon cancer tissue extracts. Twenty-nine different genes/pseudogenes have now been identified in the human CEA gene family [53]. CEA is one of the most used tumor markers in clinical application. It has important diagnostic value for gynecology malignant tumors and other localization masses [54]. In particular, elevated serum CEA levels were demonstrated in about 50% of patients with ovarian adenocarcinomas and up to 87%-88% in mucinous histotypes. Several immunohistochemical studies have reported the ability of anti-CEA monoclonal antibodies to differentiate gastrointestinal metastasis to the ovary from ovarian adenocarcinomas, as the former show significantly higher immunoreactivity in tumor tissues [55]. Serum CEA levels were also shown to be elevated in nongynecological ovarian carcinoma compared with primary ovarian tumors [56].

However, single detection CEA has little value for epithelial ovarian cancer detection with low sensitivity or specificity. CEA levels above 5 ng/mL have sensitivity of 10.1% and specificity of 95% in differentiating malignant from benign cases [57].

Several published studies has demonstrated that combination detection serum CA-125, CA 19-9 and CEA can provide satisfactory diagnostic value for epithelial ovarian cancer [58].

The main reasons why CEA is useful as a serum tumor marker for colorectal and gynecological cancers [59] are probably that CEA is a stable molecule, has a restricted expression in normal adult tissue and is expressed at high levels in positive tumors. However, the diagnostic sensitivity or specificity of CEA is not high enough for diagnosis of epithelial ovarian cancer. Then, combination detection several serum biomarkers may increase the diagnostic value [60]. Although the benefit of the application of CEA is still controversial, several studies have shown its importance in, for example, prediction and early detection of disease progression and metastasis [61].

Results of studies on CEA Sn and SP comes controversial. Some of the studies have recommended using the Ca125/CEA ratio, since values exceeding 25 showed a high sensitivity (73%-100%) and specificity (62%-100%) for ovarian malignancy diagnosis [61]. The other researchers found that the combination of the tumor markers CEA and Ca125 did not contribute significantly to the detection of malignant adnexal masses compared with CA-125 alone. Higher CEA levels could be useful in differentiating metastatic tumors from primary ovarian malignancy and in diagnosis of mucinous histology, this issue should be investigated in large, well-designed, prospective cohort trials [57].

On the other hand, results of some recent study indicated that combination detection serum CA-125, CA 19-9 and CEA was promising biomarker for epithelial ovarian cancer with relative high sensitivity and specificity [62]. According to the authors conclusion, after review of twelve prospective diagnostic publications, the pooled diagnostic sensitivity specificity, positive likely hood ratio, negative likely hood ratio, diagnostic odds ratio, and area under the curve (AUC) were 0.90 (95%CI: 0.80 to 0.92), 0.83 (95%CI: 0.80 to 0.86), 5.35(95%CI:3.90 to 7.33), 0.13 (95%CI: 0.10 to

0.16), 48.53 (95%CI: 29.91 to 78.72) and 0.92 (95%C: 0.89 to 0.94) respectively by fixed or random effect model [62].

According to the authors' conclusions cited above, CEA along does not contribute to the precision diagnostics for ovarian cancer and recommended to be used in combination with the other cancer antigens. As the opinions are controversial, this subject should be further investigated in large, well-designed, prospective cohort trials.

3.5. Cancer Antigen 15-3

A number of biochemical parameters and tumor markers have been intensively evaluated to distinguish malignant masses from benign. Among these parameters cancer antigen 15-3 (CA 15-3), which is also known as MUC1 or EMA. CA 15-3 is the serum marker most widely used in diagnostics of breast cancer with normal range: 0–25 U/mL [63]. Like CA 125, MUC1/CA 15-3 is a member of the mucin family and is a membrane-associated O-glycoprotein with a large extracellular domain. Ca 15-3 is expressed on the apical membrane of almost all glandular epithelia, exhibiting strong expression in the mammary gland and the female reproductive tract during pregnancy and lactation. CA15.3 is over-expressed in a wide variety of cancers, including breast and ovarian [64]. Although CA 15-3 is well known as tumor markers, evidence suggests that it may play a role in cancer metastasis, tumor growth and survival, inhibition of immune response, and prognosis [65]. It is well known that CA15-3 levels significantly higher among postmenopausal women. Studies on CA15-3 observed a modest positive association between age and level of the antigen [65]. CA 15-3 has a role in cell adhesion and cellular polarity and through its intracellular domain is implicated in signal transduction, interacting with the EGF receptor, and activating the mitogen-activated protein (MAP) kinase pathway. Immunohistochemical studies have shown that CA 15-3 is upregulated in renal carcinoma cells and that overexpression is associated with a higher tumor grade and stage [66,67]. CA 15-3 has been shown to be essential for ovarian cancer tumorigenesis in mouse models and is over

expressed in approximately 90–100% of serous carcinomas [68]. In some studies, CA 15-3 together with CA-125 had the higher positive rate in ovarian cancer and also showed significant differences in serum levels between tumors with and without lymph node metastasis [69]. The positive rates of CA 19-9, CA15-3 and CEA in serous ovarian carcinomas were found to be around 8%, 8% and 16%, respectively.

3.6. Other Serum Tumor Markers

Although clinicopathological data support the epithelial ovarian cancer types diversity, the histological differences does not significantly affect the clinical supervision of ovarian carcinoma. Thus, understanding the panel of different biomarkers are crucial for developing effective approaches to deal with the ovarian cancer at the level of screening, diagnosis, prognosis, prevention and therapy. Some of the novel biomarkers are reviewed below.

Human kallikrein-related peptidases (KLKs) comprise 15 members and are the single largest family of serine proteases. KLKs participate in growth, angiogenesis, apoptosis, and metastasis in tumor cells [70]. Ovarian cancer effusions analysis show high expression of KLK6-10, increased expression of KLK6 and KLK10 are found even in negative or low CA-125 level [71]. Some KLKs are increased in ovarian cancer and related to progression of ovarian cancer predominantly in late stage of serous ovarian carcinomas including KLK4-8, KLK10-11, and KLK13-15. KLK4 is [72].

Osteopontin – biological substance, an acidic, calcium-binding glycoprotein constituting the extracellular matrix, synthetized by osteoblasts and vascular endothelial cell. Researchers identified osteopontin in tissue as early detection biomarkers of ovarian cancer. Epithelial ovarian cancer display high osteopontin expression with higher expression in borderline and invasive ovarian cancer than in benign tumors. Osteopontin sensitivity yielded to 81.3%, but raised to 93.8% in combination with CA-125 at specificity of 33.7%. No significant differences observed among different histological types, grades, or stages of ovarian cancer [73]. Diagnosis at cut-off value of 252 ng/ml produces 80.4% specificity with sensitivities of stage

I/II and stage III/IV ovarian cancers as 80.4% and 85.4%, respectively [74]. Osteopontin function in metastasis and tumor progression and its potential role in prognosis is still under investigation.

Vascular Cell Adhesion Molecule-1 (VCAM) is a cell surface receptor expressed on activated mesothelial and endothelial cells. It functions in regulation of leukocyte attachment and extravasation at sites of inflammation [75]. VCAM-1 is preferentially expressed on mesothelium of ovarian cancer and contributes to mesothelial migration; therefore, high level of VCAM-1 is suggestive about malignancy. However, low concentration of VCAM-1 is also found in serum of ovarian cancer patients. A combination of four serum proteins, CA-125, CEA, HE4, and VCAM-1 produce sensitivity of 86% at 98% specificity for diagnosis of early stage ovarian cancer [76].

Mesothelin is a surface glycoprotein on mesothelial cells lining the peritoneum, pleura and pericardium. According to the researchers' data, cancer cells with high mesothelin is involved in metastasis, and along with CA-125 shows possibility of peritoneal metastasis [77]. Early stage ovarian cancers have shown 42% elevation of urine mesothelin level with 98% specificity [78].

Apolipoprotein A1 (ApoA1) is a major protein component in high-density lipoprotein with anti-atherogenic, anti-inflammatory and antioxidant properties. Ovarian cancer patients show decreased serum ApoA1 levels [79]. Detection of early stage cancers at high-risk population has been particularly difficult and use of multiple markers might improve sensitivity. Germ cell cancers are associated with the increased levels of *alpha-fetoprotein* (AFP), hCG and LDH. Serum level of LDH is elevated in up to 95% of dysgerminomas. Alpha-fetoprotein is significantly elevated in almost all patients with yolk sac tumors [80]. Therefore, as these carcinomas are more frequent in younger women, these markers should be evaluated along with CA-125 in young patients with adnexal masses [25, 28].

Nevertheless, results from different studies are conflicting [9, 28]. Although numerous substances were investigated and found to be elevated in women with ovarian carcinomas, few significant correlations with tumor nature (benign, borderline, malignant) as well as exact histopathological

type were found, indicating their inappropriate accuracy in prediction of ovarian tumor nature, therefore it is still the field for further investigations [81,82].

3.7. Novel Biomarkers in Development

3.7.1. Tumor Protein p53

The tumor protein p53 gene prevents cancer formation and functions as a tumor suppressor. Mutations in tumor protein p53 with overexpression or loss of protein expression are highly prevalent in high-grade serous ovarian carcinoma (96–100%) and uncommon in other ovarian carcinoma subtypes, including low-grade serous ovarian carcinoma (<10%) [83].

The prognostic value of tumor protein p53 is still the subject of debate, although some literature evidence suggests that the presence of tumor protein p53 mutations is related to a worse prognosis [83]. Other studies, however, link the presence of mutated tumor protein p53 to a greater response to chemotherapy [84]. Detecting anti-p53 antibodies in serum has been proposed as a potential biomarker for the detection and prognosis of ovarian cancer, with very limited results currently [85].

3.7.2. Folate Receptor

Folate is essential for nucleotide synthesis and DNA replication. It must be transported into the cell either by the reduced folate carrier or via its own receptor (FR). FR is a transmembrane glycoprotein that permits one-way transport of folate into the cell. A FR is normally expressed in areas of the body that need to retrieve folic acid prior to excretion (kidney proximal tubules) or to concentrate it in the cerebrospinal fluid (choroid plexus). It is frequently highly overexpressed in several epithelial tumors, including breast cancer and ovarian cancer cells (80%) [86, 87]. It's overexpression has also been regarded as a poor prognostic factor associated with a sub-optimal response to chemotherapy. Accordingly, FR has been considered a target for new drug development [88].

3.7.3. Angiogenesis-Related Biomarkers

Angiogenesis represents a hallmark phenomenon in cancer, and it is responsible for tumor spread and metastasis in ovarian cancer, among other tumor types, as it leads to new blood vessel formation. In recent years angiogenesis has been given considerable attention in order to identify targets for developing effective anti-tumor therapies. Growth factors have been identified to play key roles in driving angiogenesis and, thus, the formation of new blood vessels that assist in cancer cell nutrition. The prognostic value of vascular endothelial growth factor (VEGF) expression in patients' serum currently is still under exploration and discussion. Elevated VEGF levels are related to greater vascular permeability and increased tendency towards peritoneal progression and ascites [89]. A meta-analysis including over 1000 ovarian cancer patients confirmed that elevated serum VEGF levels had a negative impact on patient survival at early stages [90].

These angiogenesis-related biomarkers are key players in complex molecular pathways within the tumor cell and they have been in the spotlight of the development of anti-angiogenic molecules that may act as stand-alone therapeutics, or in concert with standard treatment regimens such as chemotherapy.

3.7.4. Genetic Testing

Modern approaches to tumor diagnostics are assessment of DNA, RNA, and proteins to form a detailed molecular map to guide more precise and individualized treatment decisions. The presence of pathogenic mutations in BReast CAncer gene (BRCA)-related genes identify an important subset of high-grade serous epithelial ovarian cancers. The spectrum of abnormalities in this category includes mutations in BRCA1, BRCA2, RAD51C, RAD51D, BARD1, BRIP1, PALB2, MLH1, MSH2, MSH6, PMS2, and STK11 [91].

4. Scoring Systems Based on Tumor Markers in Differential Diagnosis of Adnexal Masses

In September 2016, the Food and Drug Administration proposed that references be avoided while performing screening tests for ovarian cancer due to concerns about unreliable sensitivity, faulty reliability and high numbers of inaccurate results [92-94]. Clinicians have claimed that only an early diagnosis and an appropriate management plan of the disease are successful in reducing mortality [95]. New serum tumors markers and scoring models have been proposed and even introduced into clinical practice from 1990 (Table 1), such as the risk of malignancy index (RMI), OVA1, and the Risk of the Ovarian Malignancy Algorithm (ROMA) [96].

Table 6. Algorithms and study models in discrimination of adnexal masses

Algorithm/Study Model name	Abbreviation	Year of introduction
Algorithms		
Risk of Malignancy Index	RMI	1990
Risk of Ovarian Cancer Algorithm	ROCA	1996
Risk of Ovarian Malignancy Algorithm	ROMA	2010
In Vitro Diagnostic Multivariate Index Assay (OVA1)	IVDMIA	2011
CoPenHagen Index	CPH-I	2015
OVA2	Overa®	2016
Triple screen		2017

4.1. Risk of Malignancy Index

Risk of malignancy indices (RMIs) are specifically designed scoring systems used for the presurgical classification of adnexal tumors. RMI 1 was first proposed by Jacobs et al. in 1990 and was meant to help with differential diagnoses of benign and malignant adnexal mass [97]. It is a combination of ultrasound findings (U), the menopausal status (M), and serum CA-125 levels (RMI = U x M x CA-125). The original RMI (RMI-1) was slightly altered in 1996 by Tingulstad et al. [98] and then finally named RMI 2, which in turn was modified again and then introduced into everyday clinical

practice in 1999 as RMI 3 [99]. Several years later, in 2009, Yamamoto et al. [100] proposed RMI 4 by including an additional ultrasound parameter in the RMI 1 formula - the parameter of tumor size score (S). Finally, a new RMI 5 was designed by adding Doppler blood flow of the ovarian mass to the calculation of the previous RMI to increase specificity of RMI 1 in discriminating malignant ovarian masses [101].

Many studies have reported RMI to be the best available test for ovarian malignancies diagnosis [102]. Studies have also shown that RMI can differentiate malignant from benign as well as tumors with nonspecific histopathologic characteristics [103]. Furthermore, RMI has proven to have the most importance for prediction of malignancy among all other parameters of patients' like history data, symptoms, imaging and biomarkers [104-106].

In their original study, Jacobs et al. described a sensitivity (SN) of 85% and a specificity (SP) of 97% for a RMI cut-off level of 200 [96].

Many investigations were performed to identify efficacy of five listed RMIs [28, 97-101]. Yamamoto et al. [100], confirmed the high sensitivity and specificity of RMI 2 and RMI 3 at an optimal cutoff level of 200. The SN and SP for RMI 2 were 90.0% and 80.0%, respectively; and for RMI 3 - 82.6% and 86.4%, respectively. These results are comparable to those of Tingulstad et al. [99]. The positive predictive values (PPV) of RMI2 and RMI 3 were 49.3% and 52.5%, respectively [100]. These mentioned predictive values are lower than those reported in previous studies. The RMI 4 at a cutoff level of 450 had SN of 86.8%, a SP of 91.0%, a PPV of 63.5%, and a negative predictive value (NPV) of 97.5% [100].

Several authors compared RMI 1, RMI 2, RMI 3, and RMI 4 and confirmed that there is no statistically significant difference between these indices in discriminating benign and malignant ovarian masses [107]. Comparison of five RMIs (RMI 1-5) by Hayam et al. [101] confirmed the RMI 1 is the gold standard for preoperative differentiation between benign and malignant ovarian masses.

Other researchers tried to replace CA-125 with HE4 to check for efficacy of this new tool, but found that this manipulation did not improve the overall performance of RMIs [108]. Studies showed that ultrasound

parameters are more informative and important than tumor markers. RMI can be used for the triage of patients in a low resource setting when complicated radiological and biochemical tools might not be available. It helps to facilitate proper diagnosis and referral to a higher center [109].

4.2. Risk of Ovarian Cancer Algorithm

Risk of Ovarian Cancer Algorithm (ROCA) is a screening method that designates intermediate and elevated levels of risk. ROCA was developed utilizing data from prospective screening trials for postmenopausal women that included more than 22,000 women in the United Kingdom and more than 5000 women in Sweden. Statistical breakdown of these data indicated that majority of women without ovarian cancer had a constant level of CA-125. In contrast, women with incident cases of ovarian cancer had a baseline level followed by a sharp increase in CA-125 values significantly above her baseline, called a change-point CA-125 profile, which could not be explained by background CA-125 fluctuations [110]. Elevated ROCA risk was a signal to refer a woman to a transvaginal scan (TVS). After each new CA-125, the risk is recalculated and the woman retriaged. Thereby, ROCA contributes to the efficient and rapid triage of women with significantly rising CA-125 levels to TVS while assigning normal testing frequency to women with high but stable CA-125 levels [111].

ROCA identified early stage incident cases with excellent specificity - 99.9% (95% CI 599.7%, 100%), PPV of 40% (95% CI 512.2%, 73.8%), and highlights the opportunity for implementing effective strategies for the early discrimination of ovarian cancer in postmenopausal patients [111].

In the recent study done by Naumann et al. [112], the ROCA test was found to be useful in order to improve the detection of early ovarian cancer. However, to become useful in general practice, the cost for ROCA should be reduced ten-fold and this model supports the FDA's criticism of the ROCA test.

4.3. Risk of Ovarian Malignancy Algorithm

The Risk of Ovarian Malignancy Algorithm (ROMA) is a qualitative serum markers test for ovarian mass discrimination. It combines serum concentrations of the biomarkers (CA-125 and HE4) along with menopausal status. The diagnostic potential of the CA-125/HE4 combination was first recognized by Moore et al. [113]. CA-125/HE4 tests data being analyzed in a logistic regression model helps to divide patients with a pelvic mass into high/low-risk groups for ovarian malignancy.

A recent study revealed that ROMA achieved a higher sensitivity for identifying women with epithelial ovarian cancer compared to RMI (SN 94.3% vs. 84.6% at a SP of 75%) [113]. This finding was specifically obvious among stage I and II of cancer, where ROMA detected 85% and RMI 65% of cases. However, the comparative performance of ROMA vs RMI remains upon question [114].

Recent studies of HE4 and ROMA reported mixed results. Substantial number of them reaffirmed the complementary performance of HE4 to CA-125 and the superb diagnostic potential of the HE4/CA-125 combination or ROMA over CA-125 alone [115]. In the study of Holcomb et al. [115] the SN of CA-125 and HE4 for epithelial ovarian cancer discrimination was 83.3% and 88.9%, respectively; the SP of CA-125 and HE4 was 59.5% and 91.8%, respectively. However, several groups reported completely contrary data. For example, based on a large prospective study of women diagnosed with a pelvic mass, Van Gorp et al. [116] came to conclusion that HE4 or ROMA did not improve utilization of CA-125 alone. Thus, variations in the groups of the target population has an impact on the performance of the CA-125/HE4 combination.

4.5. In Vitro Diagnostic Multivariate Index Assay (OVA1)

The OVA1 test is an *in vitro* Diagnostic Multivariate Index Assay (IVDMIA) of Proteomic Biomarkers. In September 2009, the FDA passed OVA1 (Vermillion) as the first in vitro diagnostic multivariate index test

[117] for evaluation of ovarian cancer risk in women with ovarian masses. Although, the use of OVA1 is restricted to deciding on the type of surgery that medical professionals need to apply [118].

The biomarkers included in the OVA1 (except CA-125) were detected through multicenter proteomic studies [117]. The test has an overall SN of 92.2% as a stand-alone test and rises to 98.1% when accompanied by ultrasound imaging and physical examination. The SN and NPV of OVA1 are considerably higher for patients with early-stage ovarian cancer [119]. The NPV for OVA1 ranges from 92.0% to 96.9 [120]. The SN of OVA1 in premenopausal women is 88.2% when combined with ultrasound imaging and physical evaluation, whereas CA-125 only reaches 47.1% [119]. Therefore, premenopausal women are at an increased risk for failure to be diagnosed at early-stage disease, resulting in an increased mortality risk associated with advanced disease [121].

Opinions regarding OVA1 are controversial as a recent evaluation of the assay, suggested that the markers utilized in the test, did not improve upon the performance of CA-125 in prediagnostic samples [122]. This data may indicate a potential limitation in the usefulness of the OVA1 test.

4.6. Copenhagen Index – 1

The Copenhagen Index (CPH-I) is based on serum CA125 level, serum HE4 level and the patient's age [123].

CPH-I may be a test to contribute in the discrimination of patients that might possibly benefit from a referral to a specialized cancer center. CPH-I can be easily performed in primary and secondary health services in the case of suspicious masses visualized on ultrasound or another imaging. However, caution is necessary since, in practical situations, the sensitivity of CPH-I may be as low as 70% and incorrect interpretation of results can in turn lead to a delayed referral of a woman to a specialized cancer center [124]. CPH-I was highly significant in discriminating benign from malignant ovarian disease in the research of Karlsen et al. [125]. At the defined cut-off of 0.070

for CPH-I the sensitivity and specificity were 95.0% and 78.4% respectively in the training cohort and 82.0% and 88.4% in the validation cohort. Results of recent investigation carried by Høgdall et al. [125] showed that ROMA, RMI and CPH-I are useable in the differentiation between women with a benign and malignant ovarian tumor [125]. Furthermore, a Brazilian study independently applied ROMA and CPH-I in a population of 384 patients, where 87 patients were diagnosed with ovarian cancer [123]. It was concluded that CPH-I and ROMA performed equally well for the discrimination of ovarian cancer from benign ovarian tumors [126]. The CPH-I is helpful for everyday use, as it does not require ultrasound examination. The model only requires a blood sample and the patient's age without considering the menopausal status.

4.7. Triple Screen

A Triple screen is a newly designed algorithm, which relies on symptoms and abnormal serum tumor markers in postmenopausal women. It was designed as a test to help clinicians discriminate between benign and malignant ovarian masses. The test measures three specific variables: patient symptoms, serum CA 125 and HE4 level. Given that symptoms are an important component of ovarian cancer diagnosis, the authors developed very simple algorithm, including a self-administered symptom index (SI) with serum CA 125 and HE4 to define ovarian cancer [127]. The SN of this triple screen is similar to risk of ovarian malignancy index or CA 125 alone but with higher SP and PPV [128]. A triple screen was considered positive if at least two out of the three markers were abnormal (positive SI, CA 125 ≥35U/mL, HE4 ≥140pmol/L) [127]. Triple screen is a test that any practitioner can offer without any special calculations or scoring systems.

CONCLUSION

Adnexal masses of different nature are a relatively common finding in women of all ages. Proper approach to patients with adnexal masses depends

on appropriate clinical/preoperative discrimination between benign and malignant ovarian tumors. Available methods for diagnosis and evaluation of adnexal masses are bimanual pelvic examination, imaging methods and serum tumor markers. However, there are still many questions and circumstances in the natural history of adnexal masse that need to be investigated for successful diagnosis and management and tumor markers alone even in complex with different imaging modalities cannot provide precise diagnosis of ovarian cancer. Therefore, many different scoring systems/algorithms that employ multiple clinical, laboratory, and radiologic findings were developed by researchers. Current guidelines suggest that scoring systems such as RMI, ROMA or ROCA should be applied for triage of women with adnexal tumors.

Competing Interests

The authors declare that they have no competing interests.

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Author's Contributions

GA compiled, analyzed and reviewed data and prepared the manuscript. MT provided intellectual input to contribute towards manuscript preparation and edited the manuscript. Both authors reviewed and approved the final manuscript.

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Chapter 3

IMMUNOMODULATING GLUCANS CAN INFLUENCE INNATE IMMUNITY AND PREVENT INFLAMMATORY DISEASE

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ABSTRACT

Immunomodulating β-glucans show potent and unique properties as biological response modifiers (BRM) within the immune system. Interest in the medicinal properties of β-glucans has increased due to their immunomodulating activities and prevention of pathological effects for many diseases. Recently, these glucans have been made available as alternative medicine for the inhibition of inflammation and alleviation of

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various disorders, such as inflammatory bowel disease (IBD). In this chapter we describe two β-glucans of mushroom origin, lentinan (LTN) from *Lentinula edodes* and *Pleurotus citrinopileatus* glucan (PCPS), which inhibit inflammatory responses and prevent related diseases such as IBD. Various immunocompetent cells and active molecules have recently been identified within the innate immune system, and the concept of pathogen-associated molecular patterns (PAMPs) pattern recognition receptors (PRRs) was developed. Toll-like receptors (TLRs) are already recognized as typical receptors within the scope of innate immunity. Moreover, regarding the specific receptor for β-glucan, Dectin-1 has been discovered as a PRR playing an important role. Dectin-1 and several TLRs are synergistically involved within glucan's effect on immunocompetent cell modulation, including for monocytes, macrophages and dendritic cells. We show here that two β-glucans play a vital role in innate immunity modulation, and are therefore predicted to be candidates for alternative therapies against inflammatory diseases.

Keywords: anti-inflammation, β-glucans, inflammatory bowel disease, macrophage differentiation, lentinan, *P. citrinopileatus* glucan (PCPS)

INTRODUCTION

There have been many reports that glucans originating from mushrooms and sea weeds possess clinical benefits, such as: anti-inflammatory, anti-allergic and anti-carcinogenic activities [1-5]. β-glucans within mushrooms are well-known for their pharmacological values as pivotal immunomodulators. It has been shown that β-glucans can activate leukocytes and other immune cells, and can promote their phagocytic, cytotoxic and antimicrobial activities. Many scientific reports have shown that the use of such glucans may be employed for the treatment of immune system-related diseases, including: allergic asthma, food allergy, atopic dermatitis, inflammation, autoimmune joint inflammation, atherosclerosis, hyperglycemia, thrombosis, human immunodeficiency virus infection, listeriosis, tuberculosis, and septic shock [6]. Hamuro et al. reported that lentinan (LNT), a β-(1, 3)-glucan from *L. edodes*, mediated the augmentation of alloreactive murine cytotoxic T-lymphocytes and enhanced

the generation of cytotoxic T-lymphocytes [7]. In addition, LNT stimulates natural killer (NK) cell activity [8] as well as several macrophage functions [9, 10].

Inflammation plays a critical role in the development of several diseases, including inflammatory bowel diseases, asthma, rheumatoid arthritis, atherosclerosis, and various human cancers. IBD, including Crohn's disease (CD) and ulcerative colitis (UC), are characterized by chronic inflammation of the gastrointestinal tract [11, 12]. Their incidence and prevalence has increased steadily worldwide [13]. IBD causes inflammation with uncertain cause within the intestinal tract, and patients diagnosed with IBD at a young age are also at increased risk of developing colon cancer later in life [14-16]. Within CD, inflammation frequently occurs anywhere along the digestive tract from the mouth to anus, and may be discontinuous in one or more organs. The colon wall thickens and inflammation invades the muscular coat from the mucosal membrane. In UC, inflammation occurs only in the large intestine, the colon wall become thinner and inflammation is present in the mucosal membrane, which begins in the rectum or sigmoid colon and spreads up throughout the entirety of the large intestine [17]. While the precise etiology of these diseases remains unknown, understanding of IBD pathophysiology has advanced. The typical features of these disorders have been examined in various studies, especially within intestinal immune cells and intestinal epithelial cells (IECs) in IBD patients. It has become apparent that IECs and macrophages secrete large amounts of pro-inflammatory cytokines in the gastrointestinal tract of IBD patients. It is reported that IECs, macrophages, and T cells secrete large amounts of chemokines - such as IL-8 - and pro-inflammatory cytokines including: TNF, interleukin (IL)-6, IL-12, IL-17, IL-23, and interferon (IFN)-γ within the inflamed intestines of IBD patients [18]. It is well-known that secretion of IL-8 and TNF plays an important role in the immune response, and that the dysregulation of these cytokines is implicated in the pathogenesis of IBD [19]. IL-8, a member of the CXC chemokine family, is secreted excessively by a variety of cells - such as IECs - at sites of inflammation in IBD [20]. IL-8 causes excessive recruitment and transmigration of neutrophils into inflamed tissues after injury to epithelial cells. Mononuclear cells from the gut lamina propria of

CD patients spontaneously secreted TNF and induced epithelial destruction [21]. To study the effect of LNT on the innate immune response during colonic inflammation, an innate immune-mediated model of colitis induced by DSS was performed. DSS is a sulfated polysaccharide that can disrupt the mucosal epithelial barrier, thus exposing local macrophages to stimuli from intestinal microflora [22].

We have shown that the β-(1, 6)-glucan from *Pleurotus citrinopileatus* mushroom, termed PCPS, induced the Dectin-1 signaling pathway through Syk and Raf-1 activation in innate immune cells, such as monocytes, dendritic cells (DCs) and macrophages [23]. The basidiomycete fungi *P. cornucopiae* var. *citrinopileatus*, also known as 'Tamogitake' is a palatable edible mushroom. A 450 kD polysaccharide fraction of *P. citrinopileatus* was prepared from a hot water extract as previously described [24]. Our data showed that this glucan contains β-1, 6-glucopyranoside main chain residues as indicated by GC-MS analysis, with NMR data indicating that β-1, 3-glucopyranoside residues are attached to the main structure. Moreover, we have established how this glucan affects the differentiation of monocytes to macrophages [25].

Macrophages are critical, dynamic, and heterogeneous immunocompetent cells contributing to regulation of inflammatory responses within innate immunity [26]. Several tissues contain matured monocyte-derived macrophages, such as the dermis and intestine [27-29]. Moreover, tissue-resident macrophages possess highly heterogeneous properties as a result of their adaptation to varying local tissue environments, such as within the peritoneal cavity, lung, spleen, intestine and liver [30, 31]. Their characteristics are dependent on the extracellular microenvironment. Macrophages activated by IFNγ/LPS are polarized to be inflammatory, which are referred to as M1 or classical activated macrophages. M1 macrophages express mediators such as TNF and IL-6, and possess microbicidal and inflammatory functions. In contrast, M2 or alternative activated macrophages are characterized by the expression of several anti-inflammatory markers, such as IL-10 or IL-13, and play an important role in the suppression of inflammation. It is generally considered that maintaining a balance of M1 and M2 macrophages could prevent development of

immunological disease, such as chronic inflammation, allergies, or cancer [32, 33]. Within inflammation activated monocytes in the blood migrate to inflamed tissues and differentiate into specific monocyte-derived macrophage populations contributing to restoration of tissue integrity and elimination of pathogens [34]. These cells can differentiate from peripheral monocytes via stimulation with various cytokines and growth factors. More insight into how functional β-glucan affects the generation of a variety of macrophage subtypes, either with pro-inflammatory and/or anti-inflammatory properties, is important to maintain such a balance. We explored the functional effects of PCPS on human monocyte-to-macrophage differentiation. We show here that PCPS can inhibit the secretion of pro-inflammatory cytokines and chemokines within inflamed macrophages by LPS/IFNγ, and promote the differentiation into anti-inflammatory macrophages.

In this chapter the properties of immunomodulating β-glucans, LNT and PCPS, are described in relation to their anti-inflammatory actions.

ANTI-INFLAMMATORY ACTION OF LENTINAN (LNT) IN INFLAMMATORY BOWEL DISEASE

Lentinan (LNT) is one of the most well-known immunomodulating β-glucan in *Lentinula edodes*, the shiitake mushroom. LNT has two branches at O-6 for every five β-(1, 3)-linked D-glucopyranosyl residues, with an average molecular weight of approximately 400 kDa. Xu et al. [35] reported that treatment with LNT resulted in striking inhibition of nitric oxide (NO) and TNF production in inflamed macrophages via LPS-priming. NO, as well as TNF, exert a key role in the pathogenesis of many infectious and inflammatory diseases [36-38]. NO production induces tissue damage associated with acute and chronic inflammations, and it has been previously reported that NO inhibition markedly increased the survival rate of puncture model mice for cecal ligation [39]. Therefore, identification of novel pharmacological reagents that have the ability to suppress NO

overproduction is of considerable medical interest. TNF is also a key molecule within immunopharmacology, with beneficial biological effects on a variety of normal cells, mostly within inflammatory processes. Xu et al. [35] discovered that LNT could control inflammatory diseases associated with NO and TNF overproduction. Moreover, they suprisingly showed that LNT enhanced LPS-induced NF-κB p65 nuclear translocation and NF-κB activity, and however did severely inhibit the phosphorylation of JNK1/2 and ERK1/2. Taken together, it is suggested that suppression of LPS-induced inflammation associated with NO and TNF production was at least partially attributable to the inhibition of JNK1/2 and ERK1/2 activation.

ANTI-INFLAMMATORY EFFECT OF LNT WITHIN GUT INFLAMMATION

We will introduce here the suppressive effect of LNT on gut inflammation using in vivo model. To assess the inhibitory effect of LNT on colonic inflammation, we used an innate immune-mediated model of DSS-induced colitis. Mice treated with DSS developed significant signs of IBD-like colitis, bloody diarrhea and wasting conditions accompanied with sluggish, weak movements, as well as a decrease in water and food intake. In addition, a DSS-treated group exhibited decreased mean body weight in contrast to a vehicle-treated control group. However, oral administration of 100 μg/mouse LNT to DSS-induced colitis mice improved mean body weight, which was significantly higher than that of the DSS colitis mice. A similar result was observed in colon length, which was significantly longer in DSS-treated mice with LNT at a concentration of 100 μg/mouse (6.0 cm) versus that within the DSS-treated mice (4.9 cm). These results suggested that oral administration of LNT exhibited inhibitory activity on body weight loss and shortening of the colon in DSS-induced colitis. Moreover, for histological examination of murine intestinal tissue within DSS colitis, oral administration of 100 and 200 μg LNT/mouse significantly inhibited increase of histological score in DSS-induced colitis mice. These results

indicated that oral administration of LNT promoted intestinal anti-inflammatory activity through alleviation of the severity of inflammation and infiltration of inflammatory cells to colonic mucosa, as well as prevented some epithelial damage. The DSS-induced colitis model has been associated with an increase in Th1 responses, and therefore mRNA expression levels for several cytokines and chemokines related to inflammation in whole colon tissue were analyzed using qPCR in our study. Treatment with LNT could improve aberrant mRNA expression induced by DSS with significant reduction in the Th1 type pro-inflammatory cytokine IFNγ (100 and 200 μg LNT/mouse) and IL-1β (200 μg LNT/mouse) mRNA expression. These results suggested that oral LNT administration might exhibit anti-inflammatory activity via modulation of pro-inflammatory cytokine mRNA expression within the gut of DSS-induced colitis mice.

LNT SUPPRESSES IL-8 EXPRESSION IN INTESTINAL EPITHELIAL CELLS (IECS)

The gastrointestinal tract of higher organisms is lined with a single layer of IECs. This physical barrier separates subepithelial mucosal immune cells such as lymphocytes, macrophages, and dendritic cells, from a variety of antigenic substances including bacteria and food antigens present in the intestinal lumen [40, 41]. Integrity of the epithelial barrier is essential for the maintenance of host homeostasis, as it can prevent the dysregulated uptake of luminal antigens. As shown in Figure 1, to precisely assess the bioavailability of functional food factors through the gastrointestinal tract, Tanoue and Mizuno et al. [42] previously established an in vitro gut inflammation model composed of intestinal epithelial Caco-2 cells and macrophage RAW264.7 cells. In this model the environment surrounding the gut was mimicked using Caco-2 cells grown as monolayers on the underside of the transwell inserts (inverted position) and effector cells (such as macrophages etc.) on the basal side. This gut in vitro model was used to investigate anti-inflammatory factors acting against intestinal inflammation;

their study showed that LPS addition into the lower chamber did not induce IL-8 mRNA expression in Caco-2 cells despite that the TNF production from RAW264.7 cells was relatively normal. These results suggested that TNF in the basolateral compartment was essential for the upregulation of IL-8 mRNA expression within Caco-2 cells.

On the other hand, Nishitani et al. [43] also reported that LNT exhibits intestinal anti-inflammatory activity through inhibition of IL-8 mRNA by use of the same in vitro model employed by Tanoue and Mizuno et al. This group has shown that LNT treatment (500 μg/ml) significantly reduced IL-8 mRNA expression in Caco-2 cells. Interestingly, their report has also shown that LNT stimulation may have induced endocytosis of TNF receptor-1 (TNFR1) in Caco-2 cells, resulting in inhibition of IL-8 mRNA expression. Furthermore, while IL-8 mRNA expression was increased in Caco-2 cells in the presence of TNF in the basolateral compartment, TNF secretion could not be detected in the apical compartment (IECs side). Moreover, they showed that anti-mouse TNF antibody (Ab) treatment applied to the basolateral compartment (macrophage side) in that model completely suppressed IL-8 mRNA expression [42]. However, LNT treatment of Caco-2 cells did not reduce secretion of TNF in RAW264.7 cells (effector cells on basal side) when stimulated by LPS [44]. Interestingly, LNT was not detected in the basolateral supernatant, indicating that it was not able to penetrate the Caco-2 monolayer in this model. These results suggested that the LNT inhibitory activity on IL-8 mRNA expression acted through the interaction between LNT and the surface of Caco-2 cells (IECs), but not between LNT and RAW264.7 cells (i.e., effector cells in gut lamina propria).

Transcriptional activity of the human IL-8 promoter is known to be regulated by various factors, such as NF-κB [45]. In the Caco-2/RAW264.7 gut inflammation model, an increase in IL-8 mRNA expression in Caco-2 cells was observed in association with TNF production from LPS-stimulated RAW264.7 cells [44]. Therefore, NF-kB p65 level in the nuclei of Caco-2 cells was examined. As expected, the nuclear NF-κB p65 levels increased in association with TNF production. Subsequently, it was shown that LNT treatment (500 μg/ml) significantly attenuated the increase of NF-kB p65

protein levels. These results suggest that LNT addition suppressed NF-kB activation within intestinal cells in gut inflammation.

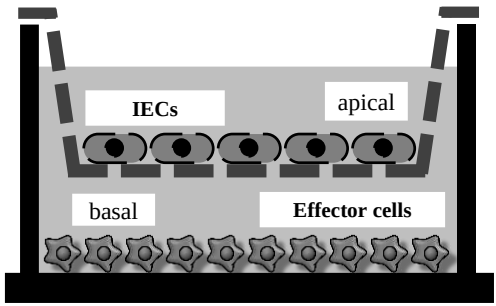

Figure 1. The in vitro gut inflammation model. Co-culture system constructed with IECs (Caco-2) and effector cells (e.g., RAW264.7 cells).

LNT IS RECOGNIZED BY INTESTINAL EPITHELIAL CELLS (IECS) VIA DECTIN-1 AND TLR2

Here we will discuss how intestinal cells recognize LNT. To assess this mechanism, the effect of anti-LNT Ab on IL-8 mRNA expression inhibition by LNT in Caco-2 cells was investigated by use of the co-culture system with Caco-2/RAW264.7 (Figure 1). Treatment with anti-LNT Ab did not have a significant effect on TNF production from RAW264.7 cells within this in vitro gut inflammation model. However, Ab addition was capable of canceling inhibition of IL-8 mRNA expression within Caco-2 cells. These results suggest that Caco-2 cells might recognize the LNT structure via the cell surface receptor. Further studies investigating the association between LNT and receptors on the cell surface are necessary to fully understand the anti-inflammatory activity mechanism of LNT. It was revealed that neutralizing antibodies against anti-Dectin-1 and anti-TLR2 affected the inhibition of NO production. Dectin-1 has been extensively identified as a zymosan β-glucan receptor [46-49], which is primarily expressed by cells of myeloid origin, including murine macrophages, dendritic cells, and neutrophils. Rogers et al. reported that both Dectin-1 and TLR2 were

activated by zymosan, but the TLR2 ligand remains relatively unknown [50]. To determine whether LNT binds to Dectin-1 or TLR2, Xu et al. determined the interactions between LNT and these two receptors [35] by using anti-Dectin-1 and anti-TLR2 Abs to neutralize Dectin-1 and TLR2, respectively, and subsequently examined NO production. Addition of anti-Decin-1 Ab barely affected inhibition of LPS-induced NO production, but anti-TLR2 Ab suppressed NO production. Those results suggested that there was a high affinity interaction between LNT and Dectin-1, but little binding to TLR2 was observed.

The mainstream treatments used to manage IBD are largely based on immunosuppressive approaches with broad acting agents such as prednisone, cyclosporin A, and tacrolimus [51]. Although these treatments are relatively effective, a number of patients develop significant side effects and/or become unresponsive. The perception that alternative medicine is healthier than classical therapeutic options has led a growing portion of the population to seek alternative treatments to ameliorate various disorders, including chronic intestinal inflammation [52]. However, the absence of empirical data showing the efficacy and mechanistic action of these treatments prevented their incorporation into mainstream medicine. Although it has been reported that mushroom-derived β-glucans exhibit immune-activating properties [53], it remains unknown how these glucans induce their anti-inflammatory effects. Mizuno et al. have provided evidence for the anti-inflammatory activity of LNT using in vivo and in vitro models of gut inflammation, as mentioned above. LNT significantly improved weight loss, shortening of the colon, and histological scores, which were used to assess the degree of gut inflammation in DSS-induced colitis mice. It was also shown that LNT treatment in DSS mice attenuated the increase in the pro-inflammatory cytokine IL-8 in colon segments. Elucidating the entire mechanism of the LNT suppressive effect on intestinal inflammation would provide valuable information concerning the establishment of novel therapies for treating IBD patients.

P. CITRINOPILEATUS POLYSACCHARIDE (PCPS) SUPPRESSES DIFFERNTIATION DIFFERENTIATION INTO PRO-INFLAMMATORY MACROPHAGES

Macrophages originate from circulating monocytes, and their characteristics are dependent on their extracellular microenvironment. Those activated by IFNγ/LPS are polarized toward inflammatory macrophages (M1), whereas M2 macrophages are characterized by expression of several anti-inflammatory markers. The anti-inflammatory properties of M2 (i.e., alternative activated macrophages) play an important regulatory role in the innate immune system. We discuss here that PCPS can modulate monocyte-to-macrophage differentiation. The chemical structure of the glucan, which is primarily composed of β-1, 6-O-linked glucose [54, 55], was inferred by the NMR spectra and MS data as previously described [56, 57]. This glucan induced a tendency for increased secretion levels of the anti-inflammatory cytokine IL-10. Moreover, it enhanced the expression levels of the anti-inflammatory chemokines CCL2 and CCL8 mRNA and inhibited expression of CCR2 mRNA, the latter of which is a pro-inflammatory chemokine, in IFNγ/LPS activated macrophages. In addition, treatment of monocytes with laminarin and antibodies against Dectin-1 and TLR2 during PCPS treatment affected the glucan-modulated macrophage differentiation. The results in our study indicated that the glucan directs the differentiation of monocytes towards a macrophage cell population with reduced pro-inflammatory capacity via Dectin-1 and TLR2 [26, 55].

PCPS SUPPRESSES THE PRODUCTION OF PRO-INFLAMMATORY CYTOKINES AND CHEMOKINES IN IFNγ /LPS-ACTIVATED MACROPHAGES AT AN EARLY STAGE

We determined the levels of cytokines secreted by activated THP-1 cells to assess whether the *P. citrinopileatus* glucan PCPS modulates macrophage

differentiation. THP-1 cells were stimulated with PMA for 48 h, followed by rest for 24 h in medium without FBS to direct differentiation to naïve macrophages [25]. To activate cells to pro-inflammatory macrophages, cells were subsequently incubated with IFNγ and LPS for 24 hours. PCPS was included in the cell culture throughout the entire differentiation period (4 days). Our data showed that secretion of the pro-inflammatory cytokine TNF by cells was significantly reduced in the presence of PCPS, compared to the cells primed with IFNγ/LPS only, which indicated that PCPS affects cellular activation by IFNγ/LPS. These results suggested that the PCPS glucan can affect differentiation of THP-1 derived macrophages.

We subsequently subdivided the THP-1 macrophages differentiation into the following 3 stages: (a) treatment of cells with PMA, (b) incubation in medium without FBS to obtain naïve macrophages, and (c) activation of naïve macrophages with LPS and IFNγ. To determine at which stage the PCPS glucan modulated the macrophage differentiation, we added PCPS to the cell culture at varying time points as outlined in Figure 2A. We exposed the cells to PCPS only within the first 48 h (i), or PCPS was added simultaneously with IFNγ/LPS treatment (ii). In addition, PCPS was present throughout the entire differentiation period (iii). In all three experiments, the secreted levels of TNF, IL-6 and IL-10 were determined in the culture media after 4 days. Our results showed that PCPS treatment during the early stage (i.e., stage i) could significantly inhibit the secretion of TNF and IL-6 (Figure 3) following IFNγ/LPS activation of macrophages. However, secretion of the anti-inflammatory mediator IL-10 was increased. These data suggest that PCPS counteracted monocyte differentiation into pro-inflammatory M1 macrophages. On the other hand, glucan PCPS treatment during IFNγ/LPS activation interestingly showed a tendency towards increased secretion of TNF and IL-6, compared to early PCPS treatment, but did not affect IL-10 expression. These results suggest that PCPS modulated the capacity of naïve macrophages to develop into a macrophage subtype with reduced pro-inflammatory properties.

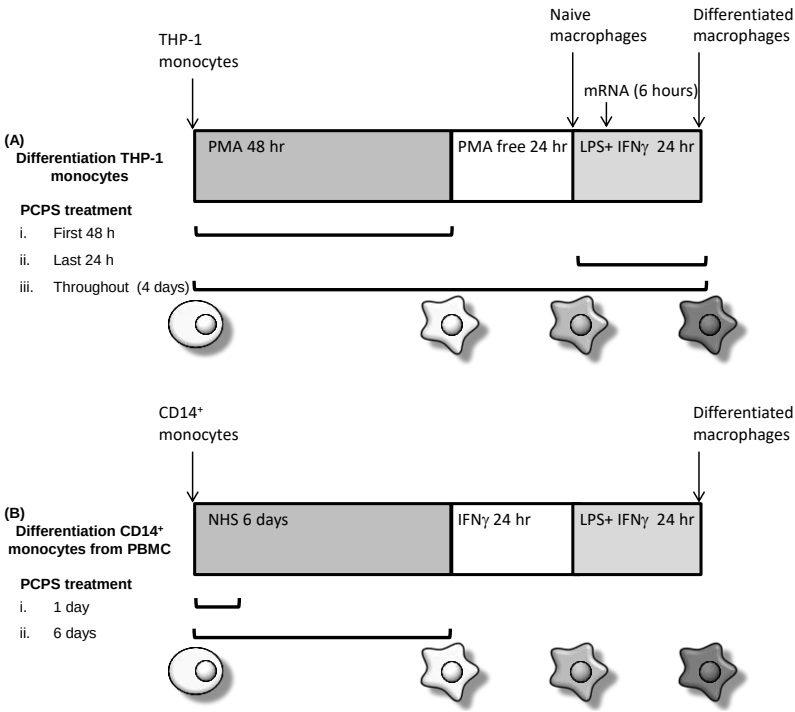

Figure 2. Outline of the PCPS treatment at different periods during differentiation of the macrophages. (A) PCPS treatment for THP-1 monocyte to macrophage differentiation; (i) THP-1 cells were differentiated into macrophages by PMA (160 nM) and PCPS (1 μg/ml) for 48 h. The cells were washed to remove the PMA and PCPS, and incubated for another 24 h in RPMI medium without FBS. Subsequently the cells were stimulated with PCPS, LPS (10 ng/ml) and IFNγ (20 ng/ml). (ii) The cells were differentiated into macrophages by PMA for 48 h, washed to remove PMA and incubated for another 24 h in RPMI 1640 medium without FBS. Subsequently the cells were stimulated with PCPS, LPS and IFNγ. (iii) PCPS is present during the entire differentiation period. After washing to remove PMA, PCPS is added again. After washing the naïve macrophages, PCPS is added again together with LPS and IFNγ. After the entire period (4 days) levels of cytokines are measured in the culture supernatants. When indicated, cells were harvested for mRNA determination 6 h after adding IFNγ and LPS. (B) PCPS treatment for $CD14^+$ monocyte-to-macrophage differentiation; $CD14^+$ monocytes from PBMC were differentiated into macrophages with 5% normal human serum (NHS) in the course of 6 days in the presence or absence of PCPS (0.5 μg/ml) for 1 day (1d) or 6 days (6d). Polarized macrophages were obtained by culturing the macrophages in the presence of 100 U/ml IFNγ for 24 hrs and for an additional 24 hrs also in the presence of 10 ng/ml LPS. After the entire period (8 days) levels of cytokines were measured in the culture supernatants. This figure is reprinted from Minato, K. et al. *Pleurotus citrinopileatus* polysaccharide stimulates anti-inflammatory properties during monocyte-to-macrophage differentiation. *Int. J. Biol. Macromol*, 122, 2019.

In addition, to assess whether PCPS stimulation at the commencement of macrophage differentiation (initial 48 h) influences chemokine expression as well CCL2, CCL8 and CCR2s mRNA levels were determined. CCL2 and CCL8 mRNA expression was dramatically upregulated by PCPS treatment at the initiation of differentiation (Figure 4). By contrast, glucan stimulation inhibited the expression of CCR2s mRNAs, which are known to be pro-inflammatory chemokines, within matured macrophages. Furthermore, we showed that expression of the gene coding for the chemokine CCL2 (MCP-1; monocyte chemotactic protein-1) was strongly upregulated by PCPS during early monocyte-to-macrophage differentiation. CCL2 promotes the expression of negative regulators of macrophage activation and pro-inflammatory cytokine production. Moreover, CCL2 plays a key role in Th2 polarization [58]. Sierra et al. [59] showed that CCL2 expression was enhanced in anti-inflammatory macrophages, and also enhanced LPS-induced production of IL-10. Those findings indicated that M2 macrophages might be the primary source of CCL2. It was also suggested that anti-inflammatory macrophages consistently produced CCL8 protein [59]. Both CCL2 and CCL8 act via the receptor CCR2, and CCR2A and CCR2B isoforms are expressed by monocytes [60]. It was suggested that M1 macrophages displayed higher expression of CCR2 compared to M2 macrophages [59]. Interestingly, a recent study suggested that IL-6, which was previously considered to be a classical pro-inflammatory cytokine, might promote M2 polarization [61]. In summary, our recent data supported the conclusion that the glucan PCPS possesses the ability to bias the differentiation of monocytes into a macrophage subtype with anti-inflammatory properties. However, there remains controversy concerning the issue of how CCL2/8 and CCR2 can contribute to the macrophage differentiation. Further study on the relationship between cytokines/chemokines and macrophage profiles are necessary to more completely understand how these mediators modulate macrophage differentiation.

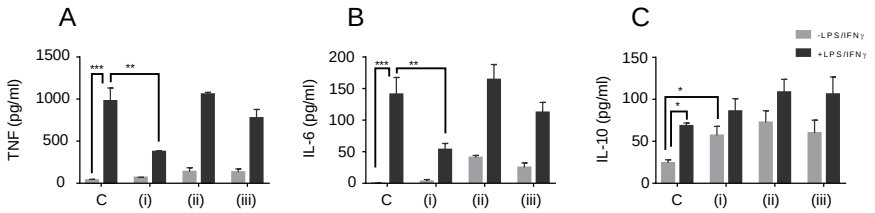

Figure 3. Pre-treatment of *P. citrinopileatus* polysaccharide PCPS inhibits secretion of pro-inflammatory cytokines in IFNγ/LPS activated macrophages. Human THP-1 cells were stimulated with the PCPS (1μg/ml) in different stages of macrophage development, as outlined in Figure 2A. (1) Cells were incubated with PMA (160 nM) and PCPS (1 μg/ml) or RPMI medium as control (C) for 48hr. Then, the cells were free of PCPS until the end of differentiation. (2) PCPS was added after 3 days, together with LPS (10 ng/ml) and IFNγ (20 ng/ml). (3). PCPS (1 μg/ml) was present throughout the entire differentiation period. After 4 days, TNF (A), IL-6 (B) and IL-10 (C) levels in the cell supernatant were determined by ELISA. The depicted error bars represent the SEM of these 3 experiments. For statistical analysis, a one-way ANOVA followed by the Tukey test was used; $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. This figure is reprinted from Minato, K. et al., *Pleurotus citrinopileatus* polysaccharide stimulates anti-inflammatory properties during monocyte-to-macrophage differentiation. *Int. J. Biol. Macromol*, 122, 2019.

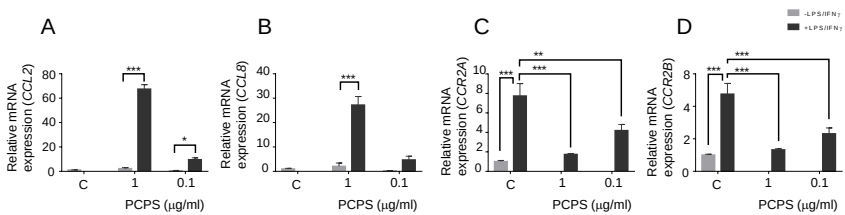

Figure 4. *P. citrinopileatus* polysaccharide PCPS affects the expression of selected chemokines and their receptors in IFNγ/LPS activated macrophages. Human THP-1 cells were stimulated with PCPS during PMA treatment or in RPMI as control (C) for 48 h, as indicated in Figure 2A (i). Polarized macrophages by IFNγ/LPS were harvested after 6 h of IFNγ + LPS stimulation, and the mRNA levels of CCL2 (A), CCL8 (B), CCR2A (C) and CCR2B (D) were determined in the cells by using quantitative real-time PCR. Expression was determined relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and then each expression level was compared to the level of the RPMI medium control. The depicted error bars represent the SEM of these 4 experiments. For statistical analysis, a one-way ANOVA followed by the Tukey test was used; $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. This figure is reprinted from Minato, K. et al. *Pleurotus citrinopileatus* polysaccharide stimulates anti-inflammatory properties during monocyte-to-macrophage differentiation. *Int. J. Biol. Macromol*, 122, 2019.

PCPS SUPPRESSES SECRETION OF PRO-INFLAMMATORY CYTOKINES IN IFNγ/LPS-ACTIVATED HUMAN MONOCYTE-DERIVED MACROPHAGES

To assess whether the glucan PCPS can influence the differentiation of primary human monocytes into macrophages in a similar way as THP-1 monocytes, we isolated $CD14^+$ monocytes from commercially obtained buffy coats and cultured them in the presence of human serum for 6 days to generate naïve macrophages. After 6 days the cells were incubated with IFNγ for 24 h, and subsequently with LPS for 24 h, to generate pro-inflammatory macrophages. These human $CD14^+$ monocytes were incubated with PCPS for 1 or 6 days prior to IFNγ stimulation (Figure 2B), and the secreted levels of different cytokines were measured in the culture supernatants after 8 days. The data showed that secretion of both TNF and IL-6 were dramatically suppressed in the differentiated macrophages after 1 day of pretreatment with PCPS (Figure 5). In addition, secreted IL-10 levels increased following pre-stimulation with PCPS for 24 h. These data showed that monocyte activation with PCPS for 24 h reduced TNF secretion by IFNγ/LPS-activated human macrophages after 7 days.

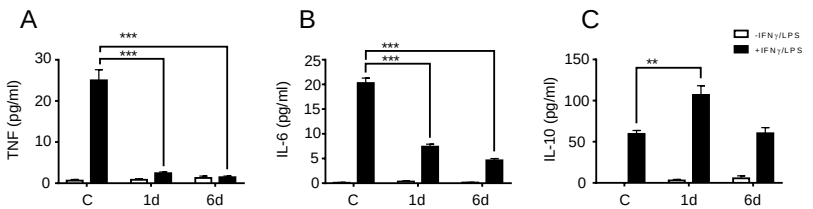

Figure 5. *P. citrinopileatus* polysaccharide PCPS suppresses the secretion of pro-inflammatory cytokines in human IFNγ/LPS-activated macrophages. $CD14^+$ monocytes were cultured at a concentration of 2×10^6 /ml in DMEM medium supplemented with 5% normal human serum. PCPS (0.5 μg/ml) was added to the immature monocytes for 1 day (1d) or 6 days (6d), respectively (Figure 2B). The levels of TNF (A), IL-6 (B) and IL-10 (C) in the cell supernatants were determined by ELISA. The depicted error bars represent the SEM. For statistical analysis, a one-way ANOVA followed by the Tukey test was used; $^{**}p < 0.01$ and $^{***}p < 0.001$. This figure is reprinted from Minato, K. et al., *Pleurotus citrinopileatus* polysaccharide stimulates anti-inflammatory properties during monocyte-to-macrophage differentiation. *Int. J. Biol. Macromol*, 122, 2019.

This suggested that short-term triggering of monocytes with glucan during early monocyte-to-macrophage differentiation leads to induction of long-lasting changes.

PCPS MODULATES THE PHENOTYPE OF MACROPHAGES VIA DECTIN-1 SIGNALING

Several pattern recognition receptors, such as lectins - including Dectin-1 and Toll-like receptors (TRLs) - have been implicated in the recognition of glucans and their downstream signaling pathways [62]. The C-type lectin Dectin-1 is a major fungal receptor that is widely expressed on myeloid cells [63, 64], and recognizes β-1,3- and/or β-1,6-glucans [65]. Dectin-1 is expressed on monocytes as well as on matured macrophages and DCs [66]. We recently showed that Dectin-1 played a major role in the PCPS-mediated activation of immature human DCs. That study demonstrated that anti-Dectin-1 antibodies inhibited glucan PCPS-induced secretion of several pro- and anti-inflammatory cytokines, such as TNF, IL-12, IL-10 and IL-6 [23]. Therefore, we have hypothesized that the effect of the glucan PCPS on monocyte differentiation could be due to the activation of Dectin-1 on monocytes. To test our hypothesis, we explored whether the anti-Dectin-1/CLEC7A Ab could recover a decrease in TNF secretion in IFNγ/LPS-activated macrophages after PCPS-treatment (48 h during PMA treatment) of the monocytes. Our data indicated that incubation of the monocytes with anti-Dectin-1 Abs (Figure 6A) during PCPS treatment could partly prevent the decrease in secreted TNF and IL-6 levels after day 4 within IFNγ/LPS-activated macrophages. This result indicates that Dectin-1 is involved in the modulation of monocytes by PCPS, leading to the generation of macrophages with reduced pro-inflammatory properties.

Moreover, interfering with Dectin-1 action did not completely abolish PCPS-induced effects, which could suggest involvement of other receptor(s) in monocyte modulation. Studies by Gantner et al. [67] showed that the signaling pathways via one or more TLRs could contribute to the activation

of Dectin-1 signaling in immune cells. It was demonstrated that crosstalk between Dectin-1 and specific TLRs was required for activation of NF-κB and regulation of cytokine and chemokine expression in macrophages, suggesting that collaboration between Dectin-1 and TLRs plays an important role in various immune responses. In addition, using HEK293 reporter cell lines expressing TLR2, 3 or 4, we recently showed that the activation of Dectin-1 signaling via glucan was synergistically triggered by stimulation of the TLR2 pathway in dendritic cells, whereas the stimulation of Dectin-1 signaling by PCPS was additively augmented by TLR4 activation [23]. To further identify which monocyte receptor candidate was potentially involved in PCPS-modulated macrophage generation, we explored the possibility that PCPS influenced macrophage differentiation via a TLR-dependent signaling by use of anti-TLR4 and TLR2 antibodies (Figure 6B). Anti-TLR4 and TLR2 Abs could inhibit secretion of TNF in the IFNγ/LPS-activated macrophages, as expected. In the presence of PCPS, the anti-TLR4 Ab could additively inhibit TNF secretion, indicating that anti-TLR4 Abs did not inhibit PCPS action. These results suggested that the signaling pathways via Dectin-1 and TLR4 were independently activated. By contrast, in the presence of anti-TLR2 Abs, PCPS had no additional effect on TNF secretion inhibition, which suggested that Dectin-1 and TLR2 acted synergistically, or TLR2 signaling might advance Dectin-1 signaling activation.

In our study, monocytes treatment with PCPS could inhibit the expression of TNF and IL-6 in IFNγ/LPS-activated macrophages. Simultaneously, the secretion of the anti-inflammatory cytokine IL-10 was promoted by the glucan PCPS priming the monocytes. Those data suggested that the glucan acted early during differentiation by modulating monocyte properties, and induced a long-lasting effect. Since PCPS also induced long-lasting changes for suppression of TNF and IL-6 during monocyte-to-macrophage differentiation, these effects could be due to epigenetic remodeling. Taken together, these findings suggest that monocytes stimulation early during macrophage differentiation could contribute to restoration of disturbed M1/M2 balance. Moreover, our data indicated that the glucan predominantly primed Dectin1 signaling in monocytes. On the other hand, the blocking of TLR2 inhibited TNF secretion in IFNγ/LPS-

activated macrophages, whereas it further enhanced the inhibition of TNF secretion during blockage of TLR4. Mechanistically, our data show that both Dectin-1 and TLR2 are involved in glucan PCPS-mediated macrophage modulation. These results could suggest a reduced capacity of monocytes to differentiate towards a pro-inflammatory M1 macrophage subtype under inflammatory conditions.

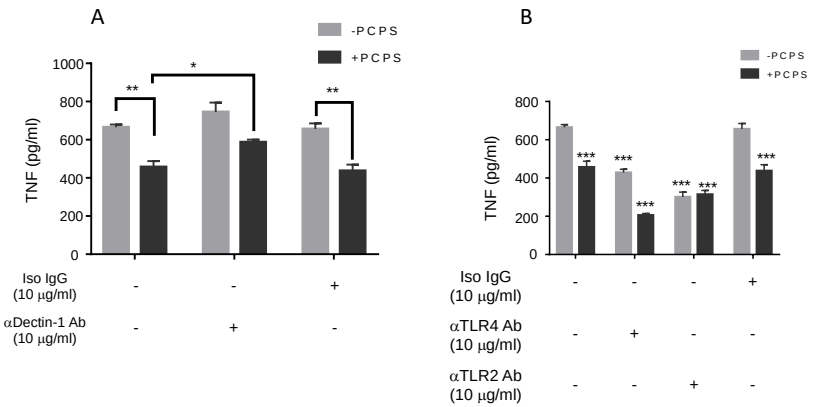

Figure 6. (A) The *P. citrinopileatus* polysaccharide PCPS acts via Dectin-1 in macrophage differentiation. (B) The *P. citrinopileatus* polysaccharide PCPS stimulates Toll-like receptor (TLR) 2, not TLR4. Human THP-1 cells were pre-incubated with anti-CLEC7A or anti-TLR4 or anti-TLR-2 Ab antibody (Ab, 10 μg/ml each), or an isotype control Ab (10 μg/ml). After 1 h, cells were treated with PCPS (1 μg/ml) and PMA (160ng/ml) for 48 hours, washed and cultured for another 24 h in RPMI 1640 medium without FBS and PCPS. Finally, the cells were stimulated with LPS (10 ng/ml) and IFNγ (20 ng/ml) without PCPS. After day 4, the levels of secreted TNF in the supernatants were determined by ELISA. The depicted error bars represent the SEM of 3 experiments. For statistical analysis, a one-way ANOVA followed by the Tukey test was used; $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ when compared to the RPMI medium control in the absence of PCPS. This figure is reprinted from Minato, K. et al., *Pleurotus citrinopileatus* polysaccharide stimulates anti-inflammatory properties during monocyte-to-macrophage differentiation. *Int. J. Biol. Macromol*, 122, 2019.

CONCLUSION

It is suggested that inflammation could give rise to the development of several critical diseases, such as: IBD, cardiovascular and

neuroinflammatory diseases, and cancer. We are exploring potent compounds that could prevent many kinds of inflammatory diseases. Inflammation and its related diseases place a heavy burden on our health care system. Many studies have previously shown the possibility for β-glucans to prevent inflammation. In this chapter, we discussed how lentinan (LNT) and P. citrinopileatus mushroom glucan (PCPS) play important roles as immunomodulators at inflammatory sites, and can regulate the activity of several pivotal immune cells, such as macrophages and intestinal cells. Our data suggest that these two glucans could be important in ameliorating several inflammatory conditions, therefore may influence the maintenance of human health.

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Chapter 4

POLYACRYLAMIDE HYDROGEL BASED FILLER FOR HIV-RELATED FACIAL LIPOATROPHY REHABILITATION

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ABSTRACT

The highly active antiretroviral therapy (HAART) against HIV infection has been showed as an efficient treatment, and in recent years guaranteed a longer life for infected patients, however this therapy is not without side effects; patients receiving HAART may develop a typical syndrome, the so-called HAART-related lipodystrophy. This syndrome is both characterized by metabolic alterations and abnormal distribution of fat in the body, such as loss of subcutaneous fat of the face (also known as

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facial wasting) and extremities, and relative increase of the fat of the trunk. Medical literature shows how facial wasting is recognized as a stigma of the infection for the patient-self, due to this reason facial features restoring is a keypoint in social life for HIV positive patients. In this clinical situations polyacrylamide hydrogel based fillers can be suitable to restore facial features. The authors present indications, techniques, complications and their treatment regarding the use of polyacrylamide for facial wasting rehabilitation.

Keywords: facial wasting, HIV, lipodystrophy, facial lipoatrophy, antiretrovirals, lipofilling, lipodhystrophy, facial rehabilitation, nonabsorbable filler

INTRODUCTION

The use of highly active retroviral therapy (HAART) with protease inhibitors, first described in the United States in 1997, has demonstrated a significant clinical benefit in the treatment of HIV infection [1-2]. Within one year of its introduction, mortality rates dropped by 45%, and there was a significant decline in the number of reported AIDS-defining diseases. Protease inhibitors in combination with nucleoside analog reverse-transcriptase inhibitors are recommended as standard-of-care antiretroviral therapy [3].

But, as the most part of chronic pharmacologic therapies, there are several side effects such as: diarrhea, renal calculi, nausea, and perioral paraesthesias. One medication-associated condition that has become prevalent among HIV-infected patients is HIV-associated lipodystrophy, a syndrome characterized by abnormal fat metabolism and deposition [4]. Broadly speaking the lipodystrophy syndrome is composed of three components [5]:

1) Lipoatrophy (subcutaneous fat loss in the face, limbs, and buttocks).
2) Lipohypertrophy (fat accumulation in the abdomen, and dorso-cervical fat pad).

3) Metabolic disturbance (insulin resistance, hypercholesterolemia, and hypertriglyceridemia [6].

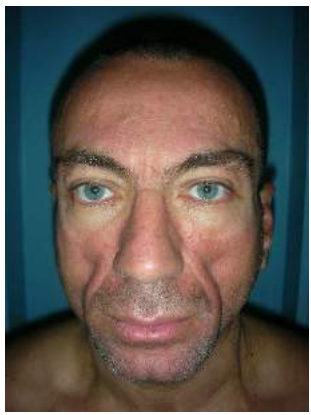


Figure 1. An example of lipoatrophy of the face (facial wasting) induced by HAART therapy.

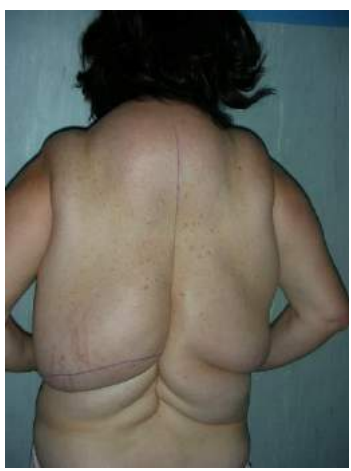


Figure 2. An example of lipohypertrophy of the back and dorso-cervical fat pad (buffalo hump deformity) induced by HAART therapy.

Despite the initial reports of an association between protease inhibitors and lipodystrophy, it soon became apparent that other drugs were implicated. In 1999, an association between thymidine analogues, particularly d4T, and fat wasting was reported [2], supported by

improvements in subcutaneous fat and serum triglyceride levels after switching d4T to zidovudine (AZT) or abacavir (ABC). Over the course of time it has been realized that the components of lipodystrophy syndrome are, at least partially, independent processes, different antiretrovirals are associated with different types and degree of toxicity, and lipodystrophy syndrome is the result of a complex interaction between a variety of factors [7]. In general thymidine analogues, especially d4T, are associated with lipoatrophy and protease inhibitors with lipohypertrophy and dyslipidaemia.

Lipoatrophy is common on HAART-treated subjects [7-9]; cross-sectional studies reveal prevalence between 13% and 38% [10-11]. Differences in estimates of lipoatrophy prevalence are likely secondary to differences in defining/assessing lipoatrophy, duration of antiretroviral therapy, and duration of thymidine analogue use [12].

It is therefore impossible to compare rates of lipoatrophy across the different studies.

FACIAL LIPOATROPHY

Facial lipoatrophy, also known as facial wasting (FW), is the most distressing manifestation for HIV patients. It can be stigmatizing, severely affecting quality of life and self-esteem, and it may result in reduced antiretroviral adherence [13]. Defining the prevalence for FW is hampered by differing definitions of the syndrome across studies making cross-study comparisons very difficult. Many studies report the incidence rates of lipodystrophy in general, not lipoatrophy specifically, and the overlapping nature of the components of the lipodystrophy syndrome complicates matters further. At this time, a number of medical, pharmacological, and surgical therapies are used to treat HIV lipodystrophy [14-16]. In addition to exercise and nutritional counseling to prevent the loss of lean body mass, anabolic steroid hormones, testosterone, and human growth hormone have increasingly being used in these patients, with varied success and significant side effects [20-25].

Therefore, ever-increasing numbers of HIV patients receiving HAART are presenting to facial plastic surgeons and requesting reconstructive surgery to ameliorate the unwanted side effects of their treatment protocol such as FW [26].

The pattern of facial wasting seen in HIV patients is pathognomonic for the lipodystrophy syndrome. The distribution of facial musculature, with prominence of the orbicularis oris and oculi, levator labii superioris alaeque nasi, and zygomaticus muscles, frames the defects. In addition to wasting of the buccal fat pad (of Bichat), temporal fat wasting creates a hollow above the zygoma. Actually, the most used classification of facial lipoatrophy is the James Carruthers Lipoatrophy severity scale [27] divided in 4 grades:

- Grade I, mild and localized facial lipoatrophy
- Grade II, deeper and longer atrophy, with the facial muscles beginning to show through
- Grade III, atrophic area is even deeper and wider, with the muscles clearly showing
- Grade IV, lipoatrophy covers a wide area, extending up towards the eye sockets, and the facial skin lies directly on the muscles.

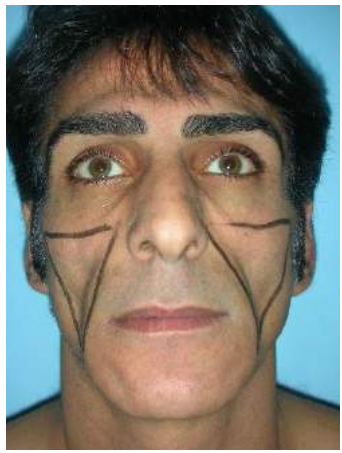


Figure 3. A facial marker related to facial wasting; this is a triangular area of the cheeks, between the malar arch, the naso labial groove and the anterior margin of masseter muscle.

However it has been also described a facial marker related to facial wasting, this is a triangular area of the cheeks, between the malar arch, the naso labial groove and the anterior margin of masseter muscle [66]; studies have shown that patients identify this area as a stigmate of the infection and it is one of the most requested area to fill by the patients [64, 66].

FACIAL LIPOATROPHY REHABILITATION

When lipohypertrophy of the body and lipoatrophy of the face are both present, structural fat grafting for facial wasting rehabilitation seems to be the best option [14, 15].

The use of the peripheral hypertrophied fat, harvested with suction assisted lipectomy (SAL), to restore the hypotrophized areas (lipofilling), such as the face, theoretically seems to be the best option because during the same operation not only can facial wasting be treated, but the body contouring procedures too can be performed [14, 65].

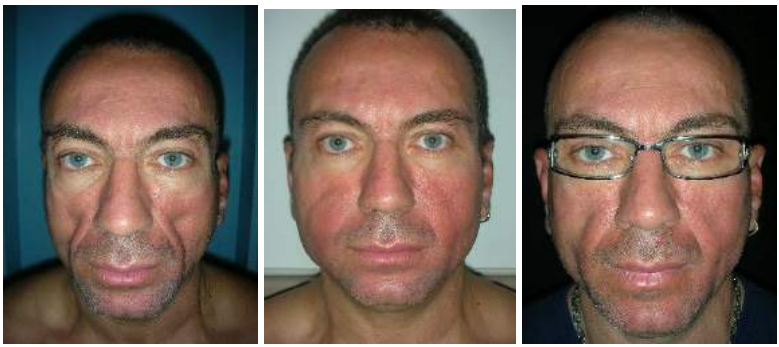
In fact HIV-associated lipodistrophy is characterized by both lipoatrophy in the face and extremities and lipohypertrophy in areas such as the dorso-cervical region (buffalo-hump deformity), lower abdomen, and breast (breast enlargment or gynecomastia) [8].

However different results due to the percentage of fat resorption after transplantation have been reported in literature in facial rehabilitation with lipofilling [14-15]. Autologous fat grafts have been used successfully for structural fat grafting in facial, lip, and hand rejuvenation and body contour improvement [61].

Most investigators believe that fat, as autologous tissue, can be considered the ideal soft-tissue filler because it is abundant, readly available, inexpensive, host compatibile, and can be harvested easily and repeatedly. If fat grafts can truly survive after transplantation, structural fat grafting may present a safe, long-lasting, natural appearing method for soft-tissue augmentation in patients [28-30].

However, one of the main concerns after fat grafting can be the potential high rate of absorption over time in the grafted site, which may reach up to 70 percent of the filled volume [29-30].

However some "enlarged" areas of the body, in patients suffering HIV-related lipodystrophy, have shown to be different from the fat usually present in human body; especially for the dorso cervical fat pad enlargement, the so called "buffalo hump" deformity. The determination of the protein levels of the "proliferating cell nuclear antigen" (PCNA), a marker gene of cell proliferation, showed a high expression of it in fat cell of buffalo hump, this supports the notion of a transformed phenotype in those fat-cells, indicating an intrinsic enhancement in cell proliferation [31].



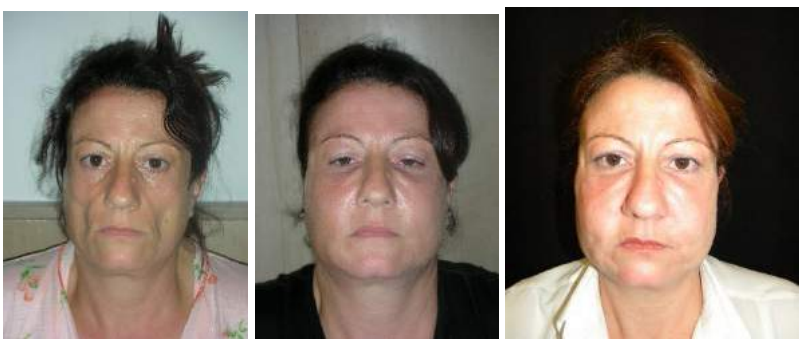
(a) (b) (c)

Figure 4abc. Facial wasting pre operatively (a); clinical control 1 months after structural fat graft harvested from pectoral fat (b); follow up at 1 year, showing resorption of large part of the grafted fat (c).

Auto-transplantation of adipose tissue from "buffalo hump" to facial lipoatrophic areas in reconstructive surgery may result in an enlargement of adipose tissue cheeks, a former lipoatrophic area. This indicates that cells in the buffalo humps may have acquired a high proliferative capacity that remains even when they are placed in a lipoatrophic enviroment. However, in clinical cases where lipoatrophy of the face is the only manifestation of lipodystrophy syndrome, filling materials remain last chance to restore facial features [16]. Facial rehabilitation of these patients is challenging and very expensive for the Economic National Health Balance [32].

Non invasive treatment protocol, such as cessation of the presumed causative antiretroviral agent, have been proposed, but they showed inconsistent improvement; this is why, when body fat is not present, dermal fillers represent the last chance!

When lypodystrophy is characterized by facial lipoatrophy only, fillers: permanent, long lasting and resorbable, are suitable. There are different filling material that can be used in this clinical situations, such as: hyaluronic acid, calcium hydroxyapatite, poly lactic acid, etc. [25]. However, all resorbable and long lasting fillers used for facial wasting rehabilitation are not resolutive because the injections need to be repeated during the time [16]; due to these reasons permanent fillers can be much more suitable to restore patient's facial features.



(a) (b) (c)

Figure 5abc. Facial wasting preoperatively (a); clinical control 1 months after structural fat graft harvested from buffalo hump deformity (b); follow up at 1 year, showing a slight enlargement of grafted fat instead of resorption(c).

PERMANENT SOFT TISSUE FILLERS

The first permanent facial filler developed was paraffin oil in the early 20th century. This was followed by a variety of other synthetic fillers such as beeswax, lanolin, and mineral oils. However, only in the 1970s dermal filler substances consisting of highly viscous fluids or polymer particle suspensions were injected beneath wrinkles and acne scars [24, 43, 46].

These substances are useful for the correction of congenital or traumatic facial, bony, and soft-tissue defects [10] and in patients suffering from scleroderma, Romberg's disease, facial wasting, or facial lipodystrophy following acquired immunodeficiency syndrome treatment. Additional indications are unilateral paralysis of vocal cords [35-37], augmentation of the lip and soft palate in cleft lip patients, anophthalmic orbits [33], and enophthalmos. Other potential applications for fillers are as bulking agents for the lower esophageal sphincter in gastroesophageal reflux patients [38-39] and for the bladder neck or anal sphincter in patients suffering from urinary or fecal incontinence [40-41].

The injection of a permanent filler substance into damaged vertebral disks with an intact annulus might even relieve the pain caused by narrowing of the disks [42].

Animal studies and clinical trials have shown good acceptance and short- and long-term efficacy in accordance with the chemical structure and surface characteristics of polymer microparticles [44, 45]. Resorbable materials such as collagen, hyaluronic acid, hydroxyethyl methacrylate, dextran, and polylactic acid are removed by phagocytosis over a period of 3 to 24 months, depending on the amount and type of bulking agent implanted [45, 52].

Permanent fillers such as paraffin, fluid silicone, Teflon, or silicone particles have an irregular surface that cannot be phagocytized and may eventually form foreign body granulomas [50] because of the memory of macrophages and giant cells (so called frustrated macrophages). Particles and microspheres smaller than 15 micron are generally phagocytized and can be transported to local lymph nodes. Larger microspheres from nonresorbable polymers with a smooth surface are encapsulated with fibrous tissue and escape phagocytosis.

The use of permanent fillers is still debated: proponents of nonpermanent fillers stress the importance of how aging will continually change the overlying structure of a person's face [47-49, 51]. They can fear that as a person ages, the placement of the permanent filler will become out of proportion. However the use of permanent fillers is very debated between

the physicians, but very often patients affected by FW ask for a permanent solution.

POLYACRYLAMIDE BASED FILLER: AQUAMID

Aquamid is manufactured by Ferrosan A/S in Copenhagen, Denmark, and marketed and distributed by Contura International S.A. (Soeberg, Denmark). Aquamid is composed of a homogenous transparent gel composed of 97.5 percent pyrogenic water bound to 2.5 percent cross-linked polyacrylamide, which is obtained by polymerization of acrylamide and N,N = methylenbisacrylamide monomers. The monomer concentration is below 2 ppm.

Each prefilled syringe contains 1.0 ml of product. Aquamid should be stored at room temperature and protected from direct sunlight.

Histologically, the acrylamide gel causes a fine fibrocellular capsule, without evidence of foreign body reaction [61]. At 6 and 9 months, the fibrous capsule is more pronounced around millions of mini droplets of gel. The capsule is surrounded by fibroblasts and macrophages. The water component of the gel is bound to the polyacrylamide polymer and is not absorbed or biologically degraded [62]. The implant is clinically still palpable at 9 months [45].

Reportedly, polyacrylamide has a half life in the human body of longer than 20 years. This may be true for large quantities; however, the injection of 0.1 cc of Aquamid was absorbed in human skin within 9 months.

Aquamid is approved for use in Europe, Australia, South America, and the Middle East; however, it is not U.S. Food and Drug Administration approved. In recent investigations, Aquamid has demonstrated efficacy [55-56], with 93 percent of patients being satisfied or very satisfied with their aesthetic results at 1 year after injection [58-59].

Indications for use include lip augmentation [46]; smoothing of nasolabial folds; filling depressed mouth corners, perioral wrinkles, and glabellar frown lines; and for cheek, chin, nose, and vermilion border contouring [47].

Using a 27-gauge needle in a fine multiline retrograde fashion, Aquamid is injected into the subcutaneous tissue. A 1:1 injection to augmentation effect is expected, as the product is nonabsorbable. Fifteen day intervals are recommended between injection sessions. An adverse event rate of 20.7 percent was reported occurring in 52 of 228 treated patients [54]. Only 30 percent of adverse events were either probably or certainly related to hydrogel injection. Most of these cases were attributed to transient local tissue response that resolved spontaneously. These included hematomas, edema, alteration of skin pigment, itching, tingling, and moderate pain. Ten cases of gel accumulation or lumps occurred, eight of which resolved spontaneously and two of which required gel withdrawal. There were no reports of granulomatous proliferation, observed rigidity of tissues, or other serious adverse events [54]. Granuloma formation does occur, however, with the use of polyacrylamide gel.

POLYACRYLAMIDE INJECTION FOR FACIAL WASTING REHABILITATION

The use of polyacrylamide hydrogel for facial wasting rehabilitation has been widely investigated [55-56, 58], however before considering the facial assessment, it is really important to get a general health assessment of the patient. This patient population is absolutely different form patients seeking for cosmetic improvement of the face, they are HIV infected but, above all, they are patients having HAART regimen. The correlation between highly active anti-retro viral therapy and facial lipoatrophy have been fully explained [4], but we also have to consider the metabolic disturbances induced, represented by insulin resistance, hypercholeste-rolemia, and hypertriglyceridemia [6-7]: this mean that this patient population have also to take drugs to treat the secondary effects of HAART therapy, especially anticoagulants and anti-hypercholesterolemic drugs in order to prevent atherosclerosis.

It is mandatory, before to start with facial wasting rehabilitation procedures, a complete blood serum evaluation, considering also the viral load that has to be 0 or next to.

FACIAL ASSESSMENT AND INJECTIONS

The first local contraindication to the use of polyacrylamide hydrogel fillers is represented by other facial cosmetic treatments previously performed; especially permanent filler of different nature form polyacrylamide, previously injected is a full contraindication to the treatment. However also previous resorbable or long lasting fillers injections represent a relative contraindication to the treatment; in case hyaluronic acid fillers have been previously injected, is safe, before to start a treatment based on polyacrylamide injections, to degradate all HA thanks to hyaluronidase injections. Although HA is a natural component of our body, we have to consider that HA fillers may have complications such as tardive swelling, lumps, foreign body reaction and so on, this is why is safe to inject a permanent filler only in a "field not contaminated" by others agents. In case long lasting fillers have been previously injected, it is not recommended to inject polyacrylamide before 24 months, but the physician before to procede with the injections have to exclude the presence of lumps, bumps, foreign body reaction or any other potential complications secondary to filler injections; in case of doubts it can be useful an US examination. Of course, not only fillers can be previously performed on the patient, this is why is mandatory during the anamnesis, to exclude any other cosmetic facial treatment in the anatomical area to treat, such as threads, etc. before to proceed.

Once the patient has been selected for facial wasting rehabilitation with polyacrylamide hydrogel injections it is really important to take pre-treatment pictures (frontal, 3/4 and lateral sides) with the patient in standing position. The same pictures have to be fully evaluated with the patient-self; quite often this patients do not really understand what has changed in their

facial appearance, this is why looking at the patient's pictures with the patient-self is really useful to focus "where to fill".

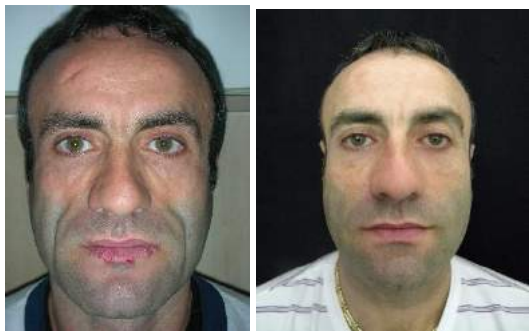
(a) (b)

Figure 6ab. Facial wasting preoperatively (a); clinical control after 14 mL of polyacrylamide hydrogel based filler injections (b).

(a) (b)

Figure 7ab. Facial wasting preoperatively (a); clinical control after 12 mL of polyacrylamide hydrogel based filler injections (b).

Although it is important patient's perception, in literature it is possible to find some areas to fill to restore facial features: Davison et al. [67], described the defects of facial wasting as a series of triangles, the first one is observed above the zygomaticus major, near the temples, the second below it, in the pre-parotid area, and the last is based at the zygomatic arch, extending superiorly; Rauso et al. [66], described another triangular area, in the cheeks, delineated by the zygomatic arch, the naso labial groove and the anterior margin of masseter muscle. Once identified the target areas, the physician has to mark them.



(a) (b)

Figure 8ab. Facial wasting preoperatively (a); clinical control after 14 mL of polyacrylamide hydrogel based filler injections (b).

One of the most fairy complications with the use of permanent fillers is represented by the infection, in order to avoid it is really important to approach to this non surgical treatment as for surgery; before the injection, the facial area to be treated has to be cleaned twice with a solution of chlorhexidine 0.2%, it is also recommendable for the physician to use sterile gloves before to handle the pre-filled syringes that have to be opened by a nurse without contaminating them.

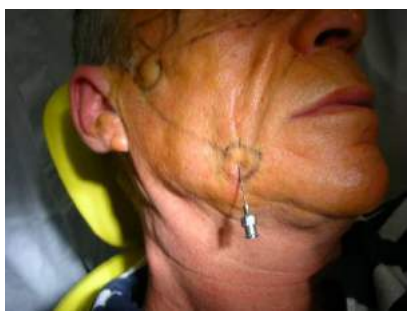


Figure 9. Blunt micro-cannula inserted into the area to fill before the connection with filler's vial.

One hour before the injections the patient has to start oral antibiotic therapy that will be performed for 5 days on average [57]; someone has also advocated intra venous antibiotic therapy during the injections and oral antibiotic therapy once discharged the patient. Polyacrylamide filler

injections can be painful for the patient, this is why is reasonable to perform nerve blocking of the infraorbital and zygomaticofacial nerves before to proceed.

The rehabilitation of facial wasting can be performed in a "one shot" session or can be splitted. When splitted treatments are chosen, it is recommendable to inject 1 or 2 mL per session, every 2-3 weeks, up to full correction. When a "one shot" session, also known as "megafilling" session [57], is chosen, it means that full correction using as much product is required is aimed to be achieved, always avoiding over filling; when megafilling session are performed, the patient can benefit of further touch up of 1-2 mL to optimize the result, however in this cases is useful to wait at least 1 or 2 months before to proceed in order to have an objective evaluation of the result reached.

The injections are usually performed with the needle supplied by the manufacturer and a cross funning injection technique with retrograde release (a "spaghetti like" injection) is recommended; to avoid complications such as nodules it is also recommended to avoid bolus injection and to release a small quantity of product per each injection. The filler has to be always injected in the subcutaneous fat, not intradermally, not intramuscularly [57].

In last years an increasing interest in filler injections with micro-cannula arisen [67]; the main advantages of a blunt micro-cannula over traditional needles are atraumatic injection and the ability to inject a large volume of product using a single puncture site. In addition, it is easier to inject (less plunger pressure) the fillers through this cannula, and there is less injection pain and less oedema and bruising than with needles. Finally, multiple punctures are unnecessary [67]. However, it has be found that the use of cannula instead of needle for polyacrylamide injections is related to a slightly higher percentage of nodules due to product accumulation. Because these cannulas are bigger than needles, and because of the larger hole of the cannula, there is little back pressure on the syringe, requiring less effort and making the injection more fluid and faster than pushing a gel through a needle; this can explain why a slightly high percentage of nodules were recorded with the use of microcannula [67]. Other statistic differences were

not found for complications in term of infections, migrations of the product, ecchymosis development etc.

At the end of the session the patient has to be fully instructed to avoid shaving, apply perfumes or similars for 3 to 5 days at least, and to avoid for ever others facial cosmetic treatments such as thread lifting, use of other types of fillers, radiofrequency, intradermotherapy, etc.

COMPLICATIONS AND THEIR MANAGEMENT

Many surgeons are reluctant to utilize permanent fillers [69], especially because of the potential risks and complications; main ones are represented by migration, foreign body reactions, nodules and infection.

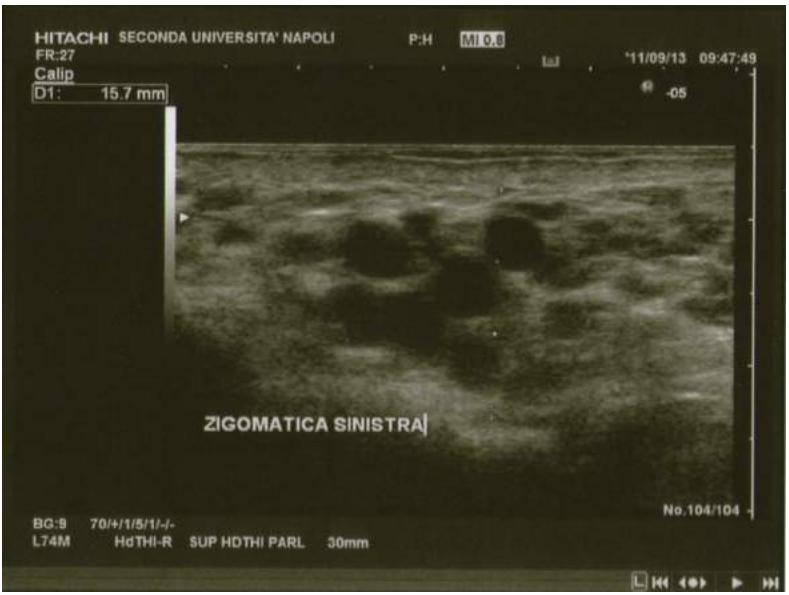


Figure 10. An ultrasonographic examination showing nodules, secondary to polyacrylamide injections, due to product accumulation.

Polyacrylamide hydrogel filler, compared to the other permanent ones, seems not to have an high percentage of migration or foreign body reactions;

this filler consists of 2.5% crosslinked polyacrylamide and 97.5% pyrogenic free water, it is a permanent, hydrophilic filler material that has proven to be stable, nontoxic [61], nonallergenic, nonembryotoxic, and practically nonabsorbable [62].

The plasticity of polyacrylamide hydrogel is similar to that of silicone, but it is hydrophilic and thus has a high capacity to exchange water molecules with the surrounding tissue fluid. This biophysical property of the material elicits minimal tissue response and results in the formation of only a thin capsule, this capsule, at the same time, avoids product migration and let the filler to be well accepted by human body [61].

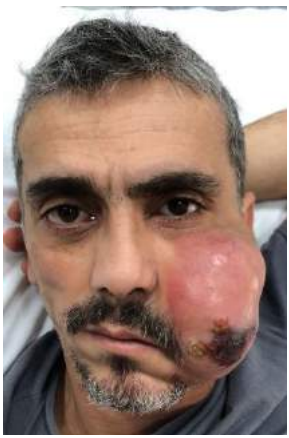


Figure 11. A huge abscess of the left cheek in a patient previously injected (8 years before) with polyacrylamide based fillers for facial wasting rehabilitation.

About nodules, as already stated, this problem is related to product accumulation and is almost due to a bolus injection of the filler or to an excessive release of the product during injections [57], this can be easily avoided injecting in a “spaghetti like” fashion, releasing not more than 0.2 mL during each step of the pass, and avoiding multiple passes in the same area and in the same sub-cutaneous plane. However, in case of nodules secondary to product accumulation the only way to solve it is surgically.

Infection is the most feared complications regarding not only the use of polyacrylamide based fillers but all permanent fillers [67]; infection may occur shortly after injection of permanent fillers, particularly if the treating

clinician is not highly skilled in the technique. The importance of adherence to asepsis rules cannot be overemphasized [67], especially when new injections are performed in areas previously treated with permanent filler (especially when splitted rehabilitation are planned). After administration of polyacrylamide hydrogel, it is essential that patients avoid any procedure that could alter the homeostasis achieved between the filler and surrounding tissues, such as derma-rolling or injecting a different permanent or temporary filler into the previously-treated area. Such procedures may lead to bacterial contamination of the initial filler. A recent study reported the 28% of patients experienced late-onset complications, including infection, after augmentation of facial soft tissue with permanent fillers, although the specific types of filler were not identified [62]. Moreover, the authors noted that abscess formation was significantly more common in patients with HIV. They concluded that the intrinsic characteristics of the injected filler and the immune status of the patient play important roles in the time of onset and the type of adverse events that occurred.

Infection after polyacrylamide injections can onset very lately, even 10 years after their injection, and can be related also to untreated infection present in another anatomical area such as the teeth; in such clinical situation a bacteria can contaminate the thin capsula around the filler. In clinical situation, once recognized the infection, the first approach is represented by the antibiotic therapy, this can rarely solve the problem, more frequently let to compartmentalize the abscess that can be later on drained conservatively; although, sometimes infection can let to disastrous consequences that need surgical repair.

CONCLUSION

Individuals with HIV infection today can expect to live longer than in the past because of new therapeutic options. However, the commonly utilized HAART is associated with various adverse effects, including resorption of subcutaneous facial tissue, which manifests as lipodystrophy, due to rearrangement of body fat.

Hollowing of a triangular area of the face, between the nasolabial fold, the malar arch, and the line that connects these two anatomic landmarks, is a common concern of infected patients and is regarded as a stigma of the illness. This lipoatrophy often prompts patients to consult facial plastic surgeons for facial volume rehabilitation. In this clinical situation polyacrylamide hydrogel filler can be used to restore volume to facial features. Polyacrylamide hydrogel filler consists of 2.5% cross-linked polyacrylamide and 97.5% pyrogen-free water. It attained European Union certication in 2001 and has been in clinical use in Europe for cosmetic purposes since 2000, under the brand name Aquamid (Contura International, Soeborg, Denmark). It is also available in many other countries. This permanent hydrophilic filler has been proven stable, nontoxic, nonallergenic, nonembryotoxic, and virtually nonabsorbable. Although Aquamid has undergone safety and efficacy trials in the United States, it has not yet been approved by the US Food and Drug Administration. Many surgeons are reluctant to utilize permanent fillers, especially because of the potential risks and complications. However, in many cases of HIV-related facial lipoatrophy, the fat loss is so extensive that it cannot be replaced adequately by an absorbable filler; such cases are considered reconstructive rather than cosmetic, and therefore a permanent filler is more appropriate. Favorable results with the use of polyacrylamide hydrogel (Aquamid) for reconstruction of facial lipoatrophy of the face in significantly immunocompromised individuals have been reported in the literature; De Santis et al. [59-60] reported a positive experience at 2 and 5 years in the management of HIV-related facial lipoatrophy in a very-high-risk group in terms of susceptibility to infections.

Although polyacrylamide hydrogel fillers looks to have a low percentage of complications, the patients should be aware of tardive infection; the most important factors to prevent complications are strict adherence to asepsis rules during the injections and instructing patients to avoid any kind of injection to the previously-treated areas; however, even if the complication rate is really low, it is mandatory to discuss with the patient before the treatment and clearly explain how really dramatic complications

related to product infection can appear also after a long term from the implantation.

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Chapter 5

USE OF POLYACRYLAMIDE HYDROGEL FOR VESICOURETERAL REFLUX TREATMENT

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ABSTRACT

Vesicoureteral reflux (VUR) is a common pathology encountered in pediatric urology. It may affect from 1 to 3% of children. Potential complications of VUR are upper urinary tract infections and renal scarring, which can lead to loss of renal function and hypertension. During the last three decades, endoscopic treatment of VUR has evolved to become the first line of intervention in several countries, especially for low grade VUR. This growing interest in subureteral injection has prompted additional research on different types of bulking agents. The ideal bulking agent is described as biocompatible, nontoxic, non-antigenic, non-migratory, causing minimal inflammation and keeping its shape and volume. The perfect agent which meets all of these criteria has yet to be found. Nowadays, the most popular bulking agent is dextranomer hyaluronic acid (Dx/HA, Deflux®), which is the only agent approved by the Food and

Drug Administration for treatment of VUR. Polyacrylamide hydrogel (PAHG, Bulkamid®) is currently approved for the treatment of stress urinary incontinence through urethral injections. Its safety in the urinary tract has been shown in different clinical trials. Recent publications have evaluated the efficacy and safety of PAHG in the endoscopic treatment of VUR in pediatric patients. In this chapter, we propose a review of the use of PAHG endoscopic injection as an alternative bulking agent for the treatment of children with VUR.

Keywords: vesicoureteral reflux, endoscopic treatment, polyacrylamide hydrogel

INTRODUCTION

Vesicoureteral reflux (VUR) is the retrograde flow of urine from the bladder to the ureters and kidneys. This pathology is frequently encountered in pediatric urology practice. Its overall estimated prevalence is between 1 and 3% [1, 2]. Although Galen and da Vinci made the first descriptions of VUR in ancient history, clinical correlation with recurrent pyelonephritis was only described in the 1950s-1960s by Hutch [3]. It is now well established that VUR combined with urinary tract infections (UTI) can lead to renal scarring and loss of renal function. Renal scarring or dysplasia secondary to VUR is also the leading cause of hypertension in children and young adults [4]. Recently, a better understanding of the natural history of VUR has led to many changes in the management of this pathology. The objectives now aim at preventing recurrent febrile UTIs, preventing renal injury and minimizing the morbidity associated with the different treatment options and follow-up [5]. The different alternatives in the management of children with VUR are: active surveillance, continuous antibiotic prophylaxis, endoscopic injections of bulking agents and open, laparoscopic or robotic ureteral reimplantation [5, 6].

During the last three decades, endoscopic injections of bulking agents for the treatment of VUR have gained popularity and have been the subject of several publications [7-10]. In 1984, Puri and O'Donnell reported for the first time the surgical treatment of VUR in piglets by intravesical injection

of polytetrafluoroethylene (PTFE, Teflon®) [11]. Since then, several bulking agents have been investigated, but the perfect agent has yet to be discovered. The ideal bulking agent should be biocompatible, nontoxic, non-antigenic, non-migratory, causing minimal inflammation and keeping its shape and volume [12]. The agents studied include the dextranomer hyaluronic acid (Dx/HA, Deflux®) [13], polyacrylate-polyalcohol copolymer (PPC, Vantris®) [14], polydimethylsiloxane (Macroplastique®) [15], polytetrafluoroethylene (PTFE, Teflon®) [16], bovine collagen [17], calcium hydroxyapathite [18] and autologous materials like chondrocytes [19], fat [20] and blood [10, 21]. More recently, polyacrylamide hydrogel (PAHG, Bulkamid®) has been evaluated for the treatment of VUR by endoscopic injection [22].

Polyacrylamide hydrogel is a nondegradable water-based polymer with high viscoelasticity and is associated with minimal long-term risk of fibrosis or migration [23]. The injected volume tends to remain stable over time as compared to several previously mentioned agents. The bolus appears hypoechoic on ultrasounds, others being more hyperechoic. PAHG has been widely used for injection into the urethra for the treatment of female stress urinary incontinence. A systematic review conducted by Kasi *et al*. concluded that it was an effective and safe treatment option [12]. In this chapter, the use of PAHG as an alternative bulking agent for the treatment of children with VUR will be discussed.

PATHOPHYSIOLOGY OF VESICOURETERAL REFLUX

Vesicoureteral reflux is generally classified as primary or secondary. Primary reflux refers to a deficiency in the antireflux mechanism of the ureterovesical junction, while secondary reflux is the result of an overwhelmed normal ureterovesical orifice by a secondary process generating elevated bladder pressure [6]. The normal ureterovesical junction and the tunnelling of the ureter in the bladder, form a flap-valve mechanism that prevents the retrograde flow of urine while the bladder is filling. This flap-valve phenomenon is caused by the compression of the submucosal

ureter against the underlying detrusor muscle, due to changes in the intravesical pressure [8]. The length-to-diameter ratio of the ureter has been hypothesised to play a major role in this natural flap-valve mechanism. A length-to-diameter ratio of 5:1 was described in normal children [24]; a shorter tunnel ratio of 1.4:1 was observed in children with VUR [25]. Diagnosis of reflux is usually confirmed by a voiding cystourethrogram (VCUG). It is graded from I to V according to the International Classification of Vesicoureteral Reflux [26], grades I and II VUR being low-grade, III being intermediate and IV and V known as high-grade.

ENDOSCOPIC TREATMENT OF VESICOURETERAL REFLUX

When surgical intervention is indicated, endoscopic treatment is a less invasive option than ureteral reimplantation. It is an attractive procedure for surgeons and patients/family, especially because it is mostly done as an outpatient procedure, operative time is short, no surgical incision is needed and patients usually recover quickly (in less than 48 hours). Endoscopic injection of a biocompatible agent is believed to prevent reflux by narrowing the ureterovesical junction without altering the urine flow [8] and also by elongating the intramural ureter, contributing to a higher length-to-diameter ratio, as described earlier. In a randomized clinical trial comparing endoscopic injection of Dx/HA and Cohen's ureteral reimplantation, Garcia-Aparicio et al. concluded that these two surgical options were of similar effectiveness at five years post-surgery [27]. In this study, the Subureteral Teflon injection (STING) technique was used and the authors performed up to two endoscopic injections if indicated (need for second general anesthesia). Many studies comparing the efficacy of ureteral reimplantation vs endoscopic injection in VUR have been published but results remain sparse and the conclusions controversial, especially for low grade VUR [27-29]. In general, ureteral reimplantation offers higher success rates than endoscopic injection but comes with more associated morbidities (longer recovery and more complications).

O'Donnell and Puri described the STING technique in 1984 [16]. They used a 18 gauge needle through a 14 French cystoscope and the needle was inserted 2-3 mm below the ureteral orifice and advanced for 5 mm prior to injection [16]. Kirsch et al. introduced a new technique in 2004 called hydrodistention implantation technique (HIT) where the needle is inserted in the submucosa of the mid to distal ureteral tunnel at 6 o'clock within the ureteral orifice [30]. This technique further evolved to the double-HIT technique which became the most popular technique among urologists [31]. The double-HIT technique is performed by distension of the ureter with irrigation fluid. An injection is first performed in the mid ureteral tunnel to reduce the lumen of the proximal part of the ureteral tunnel. A second injection is then made in the distal tunnel, narrowing the ureteral orifice [32]. After the injection, the mound appearance and the orifice are inspected with an empty and a full bladder. If needed, a third injection using the STING technique can be used to provide more support and a better coaptation of the orifice [32]. Better success rates have been reported with the HIT and double-HIT techniques compared to the conventional STING but they are also associated with an increased volume of injection of the bulking agents [31-35].

Endoscopic injection is usually performed as an outpatient procedure. The technique is carried out with the patient under general anesthesia. Polyacrylamide hydrogel comes in a 1ml syringe and can be injected with a 23 gauge, 35 cm needle through a pediatric cystoscope, similarly to other agents [36]. Due to the fluidic properties of the bulking agent, no special device is needed for the injection as opposed to polydimethylsiloxane, a thick granular agent that requires an injection gun. The Dx/HA needle has been used successfully for the injection of polyacrylamide hydrogel. The choice of the injection technique remains the surgeon's preference. Performing the injection slowly and gently allows the surgeon to assess the location of the implant during the injection.

POLYACRYLAMIDE HYDROGEL AS AN ALTERNATIVE BULKING AGENT FOR VUR

Over the last decades, dextranomer hyaluronic acid (Dx/HA) has become the most widely used bulking agent [7]. Dx/HA obtained FDA approval in 2001 [8]. The injection of Dx/HA has shown a good success rate, ranging between 68% and 92% [7]. Better success rates correlate with lower grades of VUR. A systematic review conducted by Routh in 2010 revealed an overall success rate of 77%, which means that about one quarter of the patients treated will not have their VUR corrected and may need more invasive procedures [37]. Furthermore, a 25% volume reduction of Dx/HA after the injection has been described [38], leading to an increase in the quantity of products that need to be injected (overinjection) and subsequently an increase in the associated cost of the procedure [39]. The search for a better bulking agent is therefore justified. Recent studies showed no difference in the outcomes between Dx/HA and PPC (Vantris®) [40-42], except for a potential increased risk of ureteral obstruction with PPC [14, 43]. Polyacrylamide hydrogel is approved for the treatment of female stress urinary incontinence and is documented as being safe and effective [12, 44]. Moreover, PAHG seems to maintain its shape and size [45]. Treatment of vesicoureteral reflux by endoscopic injection of PAHG obtained approval in Canada in October 2017 [46]. Canada is currently the only country to have given this approval. Three recent studies have evaluated the use of PAHG in endoscopic treatment of VUR as an alternative bulking agent [22, 36, 47].

Cloutier et al. in 2013 was the first group to publish in the English scientific literature. Their trial on the use of polyacrylamide hydrogel to treat VUR by endoscopic injection in pediatric patients was a pilot study [22]. In this series, 40 patients, 30 females and 10 males, underwent endoscopic treatment for VUR. Children with unilateral or bilateral VUR were included, as well as those with primary or secondary reflux, first or second injection, previous surgical procedure and duplicated systems being eligible. Patients with neurogenic bladder and/or untreated voiding dysfunction were excluded. Preoperative evaluation included VCUG, renal bladder ultrasound

(RBUS) and DMSA scan when deemed necessary. A pediatric cystoscope with a 23-gauge needle was used to inject PAHG. A single skilled surgeon performed every procedure using the double hydrodistention implantation technique (double HIT) with patients under general anesthesia. Follow-up included VCUG, RBUS 3 month postoperatively and RBUS 1 year postoperatively. A total of 69 refluxing renal units were treated. When using up to 2 injections, the overall cure rate, defined as grade 0 reflux, was 81.2%. A total of 6 refluxing renal units underwent a second injection. This cure rate differed according to the grade, ranging from 60% for grade V to 100% for grade I. Only one patient suffered from pyelonephritis 3 weeks after surgery. It was interesting to note that no patient had evidence of obstruction after a mean follow-up of 12 months. This was the first study using PAHG in the treatment of VUR. Results were interpreted as comparable to the outcomes of Dx/HA, but follow-up was relatively short and there was no comparative group.

A second trial from the same group was published by Blais et al. in 2015 [47]. The aim of this study was to compare the efficacy of PAHG and Dx/HA in endoscopic treatment of VUR. For this purpose, a single center and single surgeon prospective non-randomized study was designed. Patients under 18 years of age with grade I to IV VUR confirmed by VCUG were included. Exclusion criteria were grade V VUR, previous endoscopic treatment, active infection, untreated dysfunctional elimination syndrome, neurogenic bladder and history of bladder exstrophy. Surgery was performed under general anesthesia, using a pediatric cystoscope and the double HIT technique. The choice of the agent (Dx/HA or PAHG) was based on product availability and alternate use of the two bulking agents was encouraged. A total of 45 patients were included in each group, 78 refluxing renal units in the PAHG group and 71 in the Dx/HA. No significant difference was observed in the two groups. The overall success rate after the first injection was 73.1% with PAHG and 77.5% with Dx/HA ($p = 0.54$) at the 3 month VCUG. One patient in each group suffered from febrile UTI after surgery. In the Dx/HA group, 2 patients developed a symptomatic ureteral obstruction and one needed a temporary double J ureteral stent.

The third trial was published by Ramsay et al. in 2017 and evaluated the long-term results and safety of PAHG in endoscopic treatment of VUR [36]. This study originated from the same group as the two previously published papers. A prospective single-surgeon study was designed to evaluate PAHG in endoscopic treatment of VUR grade I to V. Patients were included in this trial after exiting previous studies. Children with neurogenic bladder, bladder exstrophy, or untreated voiding dysfunction were excluded. All children had a VCUG and RBUS prior to surgery. DMSA scan was performed only when it was considered necessary. The same double-HIT technique with patients under general anesthesia was performed. Follow-up included VCUG and RBUS at 3 month, followed by RBUS at 12 and 36 month. It was decided by the group not to systematically repeat the VCUG one year after surgery to avoid unnecessary radiation exposure and invasive manipulation in the absence of pyelonephritis after the discontinuation of antibiotic prophylaxis. The definition of treatment success was the absence of both VUR and de novo or worsening hydronephrosis. Authors looked at the complications to assess the safety of the procedure, such as calcification of the injected agent and febrile UTIs. A total of 76 patients, 53 females and 23 males were included. The median age at injection was 45 months. The median volume of PAHG per refluxing renal unit was 1.0 ml, with volumes ranging from 0.6 to 2.5 ml. Overall success rates at 3 month were 70.7% for grades I to V and 72.6% when excluding grade V. Success rates were higher for patients with a duplicated renal system (87.5%), history of renal reimplantation (100%) and history of previous injection with another agent (80%). Only 2 patients experienced a febrile UTI and one had persistent VUR at the 3 month VCUG. At the 36 month RBUS follow-up, no patient had developed de novo or worsening hydronephrosis or calcification of the bulking agent.

The only other trial from a different group, regarding the use of PAHG for the injection treatment of VUR, is a 1999 Russian article for which only the abstract was available [48]. In this study, 11 patients underwent 16 surgeries with Interfall injection, a PAHG variant. The success rate for VUR was 75%, 80% and 50% for grade II to IV, respectively. After discussion

with the senior author, it was confirmed that more patients were also treated, but unfortunately, no data was collected.

All of these studies show an interesting new purpose for PAHG. Its use in VUR treatment appears safe, with few reported febrile UTIs and no evidence of ureteral obstruction following the injection. Also, PAHG being a hydrophilic polymer gel without small particles, it decreases the risk of migration and calcification in the long term [12]. The volume of PAHG has been shown to be stable several years after cosmetic surgeries, which is also promising for long-term results [49]. As previously mentioned, Routh et al. reported in their meta-analysis an overall success rate of 77%, 3 months after one injection of Dx/HA [37]. Blais et al. obtained a success rate of 77.5% with Dx/HA, which was similar to Routh et al.'s results. However, these results were not significantly different from the rates obtained with PAHG. Ramsay et al. reported a median injected volume of 1.0 ml of PAHG per refluxing renal unit, with volumes ranging from 0,6 ml to 2,5 ml. In a recent article, Moore and Bolduc compared polydimethylsiloxane (Macroplastique®) with Dx/HA and they reported a median volume of 1.0 ml for both agents with a range from 0.5 to 2.6 ml and 0.5 to 2.8 ml respectively [50]. Since the mean volume of bulking agent used to inject a refluxing renal unit was very similar for all the bulking agents, the price per ml of each different agent may have a direct impact on the cost of the procedure. In Canada, 1.0 ml of PAHG costs 15-20% less than the same amount of Dx/HA [36]. Considering all these evidences, the use of PAHG as an alternative bulking agent in VUR is an interesting avenue to explore.

CONCLUSION

Vesicoureteral reflux is one of the most discussed topics in pediatric urology and the search for minimally invasive treatments is continuous. The endoscopic subureteral injection of a bulking agent is one of these strategies. Several agents have been studied and dextranomer hyaluronic acid remains the most popular. The use of polyacrylamide hydrogel as an alternative bulking agent has been shown in different trials. The rate of VUR resolution

following PAHG injections was comparable to other agents and no significant difference was found when comparing PAHG with Dx/HA. Ureteral obstruction following endoscopic injections and calcification of the injected bulking agent are two major concerns for pediatric urologists. None of these complications were observed with the PAHG making it an attractive option in VUR treatment. Additional data on the use of PAHG in VUR management from other centers would be valuable information.

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Chapter 6

THE ROLE OF ENDOSCOPIC SINUS SURGERY IN PEDIATRIC PATIENTS

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ABSTRACT

Introduction of pediatric endoscopic sinus surgery (PESS) is relatively recent. Owing to the excellent outcomes of functional endoscopic sinus surgery observed in the adult population, in 1990s the development of suitable pediatric instruments and optics was encouraged. Moreover, improvements in technology and surgical techniques enabled pediatric otolaryngologists to progressively expand the surgical indications of endoscopic nasal approaches. Nowadays, PESS is routinely performed in children affected by sinonasal inflammation pathologies, anatomic malformations, benign expansible lesions, and lacrimal stenosis. Furthermore, this surgical management is favoring PESS over open

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approaches for CSF leak repair and other skull base disorders, including some neoplastic masses. However, specific considerations in PESS must be addressed. The major issues regard reduced nostril aperture and nasal fossae volume, and degree of sinus pneumatization. Herein, the authors describe this surgical technique and its main use in pediatric patients. Finally, the advantages of this less invasive surgery are described compared to traditional surgical treatments.

PEDIATRIC NASAL EMBRYOLOGY AND DEVELOPMENT

Thorough knowledge of nasal and paranasal sinuses anatomy is mandatory to achieve familiarity in endo-nasal endoscopic surgery. A major issue in the pediatric population concerning sinonasal anatomy is the variability according to age, stage of development, and pathology treated [1]. A key point in paranasal sinuses consists in the development of the maxillo- and ethmoido-turbinals by the 10th week of gestation from the lateral wall of the nasal capsule. Each of these ridges contribute to the formation of important anatomical structures and key landmarks in endoscopic surgery. The inferior turbinate develops from the inferior ridge, called maxillo-turbinal; 5 to 6 ridges develop superiorly and form the ethmoido-turbinals. The first ethmoido-turbinal forms the agger-nasi and uncinated process, and the second through fourth participate in the formation of portions of the bulla, middle and superior turbinate. The fifth and sixth ethmoido-turbinals degenerate or contribute to the supreme turbinate [1-3].

Two types of sinus pneumatization are recognized: “intramural” when confined to the nasal capsule (ethmoid primary pneumatization) and “extramural” when extended to the surrounding bones (ethmoid secondary pneumatization and other sinus development) [4]. In fact, whereas the maxillary, frontal, and sphenoid sinuses develop as extensions of the ethmoid (extra-nasal capsule development), ethmoid pneumatization starts within the nasal capsule as intra-capsular diverticular spaces (primary pneumatization) and progressively expanding beyond nasal capsule borders within the contiguous structures. Ethmoidal air cells develop by the 12th week of gestation and continue to grow up to early adolescence (generally around 12 years) [3-4].

The maxillary sinus is the first paranasal sinus to develop (by the 10th week) as a lateral bud from the ethmoidal infundibulum, between the first and second ethmoido-turbinals; at birth the sinus volume is small, but its diameter increases greatly in the first 8 years of life. During childhood and adolescence, the maxillary sinus continues to grow and, reaches an adult size by 16-18 years [5]. Frontal sinus development starts with migration of extramural anterior ethmoidal cells within the inferior aspect of the frontal bone during the 13th week of gestation. Thereafter, the anterior ethmoidal outpouching proceeds further between the frontal bone until about 20 years of age (can generally be seen on X-rays between 2 and 6 years). According to Terracol and Ardouin, the variable drainage pathway of the frontal sinus depends mostly on which ethmoidal cell the sinus developed (nasal, orbital or bullar groups) [4,6]. During the 4th month of gestation, the sphenoid sinus develops as an "extramural" extension of posterior ethmoidal cells from the sphenoethmoidal recess, with a posterior directed gradual extension starting from the antero-inferior wall, proceeding to the floor and then to the planum sphenoidale and anterior sellar wall. A varying degree of sellar floor pneumatization is reached in the majority of patients by age 7; extension beyond the posterior sellar wall, when present, rarely occurs before age 12 [7]. Absence or underdevelopment of the frontal and sphenoid sinuses is typical of Down and Apert's syndromes. The skull base derives almost entirely from the chondrocranium (cartilagineous neurocranium) and starts its development at the 4th week of gestation. The basicranium grows slower than the cranial vault. The anterior skull base is cartilaginous at birth, with a progressive ossification process that becomes evident by 4 months of age. Between the ages of 2 and 4 the anterior cranial fossa reaches an adult conformation, with the definitive closure of the spheno-ethmoidal and ethmoido-frontal sutures by adolescence. Anterior displacement of the anterior skull base, frontal bone, and sinus occur during the first years of life secondary to development of the frontal lobe and nasomaxillary complex [8].

Pediatric ENT surgeons must always keep in mind that any surgical procedure in this anatomical region can potentially affect normal development of the craniofacial complex. Extensive procedures on the skull

base demands a thorough knowledge of sinonasal and skull base growth centers in order to avoid a major impact on their key role.

NASAL ENDOSCOPY AND PEDIATRIC NASAL ENDOSCOPIC ANATOMY

In-office diagnostic nasal endoscopy is generally performed with patients placed in either a sitting position or kept in the arms of a nurse or parent. The application of cottons soaked with decongestants and local anesthetics in the nasal cavity is generally preferable to increase compliance and space of the nasal cavity. When the use of cottons is not tolerated or in patients younger than 36 months, a local anesthetic can be sprayed in the nasal cavity. In neonates, topical drugs are not generally utilized. Before starting the endoscopy, the child should be informed about the procedure in the attempt to get better collaboration. Four-mm rigid endoscopes are generally reserved for compliant patients older than 8 years; 2.7 and 3 mm rigid endoscopes ensure the same visual quality with better performance in small nasal fossae. Flexible endoscopes, despite the lower visual quality, must be used in non-compliant patients because of their nearly negligible traumatic potential. After removal of cottons and application of anti-fog products on the endoscopic lens, the endoscope is delicately inserted into the nasal fossa. Endoscopic access through the nostril in the first 6 years of life can be difficult because of the significantly narrower aperture, and can pose a major issue during surgical procedures that require the simultaneous use of two instruments. Diagnostic endoscopy is a standardized procedure that is generally performed as follows. First, it starts with the inspection of the nasal fossa floor, nasal septum, inferior nasal turbinate, and inferior meatus; it then proceeds along the floor of the nasal cavity to observe the choanal aperture, nasopharynx, Eustachian tube orifices, and torus tubarius. At this point, the endoscope is directed superiorly and proceeding backwards to assess the sphenoethmoidal recess, middle turbinate, and middle meatus. The uncinate process, bulla ethmoidalis, and iatus semilunaris are generally

easily assessable. Finally, anterior to the middle turbinate, the endoscope is rotated superiorly to evaluate the olfactory fossa. Complications are rare, and the most common is nasal bleeding, which is often self-limiting. Parents should be informed to not let their children drink or eat for 20 minutes after the procedure because of partial laryngeal anesthesia from nasal local anesthesia.

Endoscopy plays a major role as a diagnostic tool in the evaluation of the nasal anatomy and functional status by assessing the mucosa, presence of pathologic secretions and their origin, inspection of adenoid hypertrophy, and diagnosis of nasal masses (polyps, congenital malformations, tumors, etc.). In addition, thanks to its high benefit/cost ratio, it is commonly used to evaluate response to medical treatment and for surgical follow-up.

Figure 1. Classic position of the main surgeon (arrow) during pediatric endoscopic sinus surgery. Surgeon operates holding in his hands surgical instrumentations and nasal rigid endoscope connected to camera. The surgeon can check the surgical procedure on the monitor sited in front of him (A). Close up view of nasal endoscope (arrow) and surgical instrumentation (white arrowhead), both inserted into nasal fossa.

PEDIATRIC ENDOSCOPIC SINUS SURGERY

Pediatric endoscopic sinus surgery (PESS) (Figure 1A, B) is performed for several sinonasal disorders ranging from inflammatory diseases to

malignant tumors. Herein, the main sinonasal pathologies treated by nasal endoscopic surgical approaches are presented.

Inferior Turbinate Hypertrophy

Inferior turbinate hypertrophy can be either congenital or acquired. The former is rare, whereas the latter is usually due to septal deviation, allergic rhinitis, or gastroesophageal reflux disease [9-10]. The primary presenting symptom is nasal obstruction occasionally associated with seromucosal rhinorrhea, itching, and sneezing. Moreover, chronic nasal obstruction can modify the normal function of the Eustachian tube causing effusion in the middle ear [11]. Diagnosis is made by nasal endoscopy. Rhinomanometry in basal conditions and after nasal decongestion can be useful in selected cases. Medical therapy is usually effective and consists of regular saline irrigations and the application of topical steroids for at least 3 weeks. Association of topical steroids with an anti-histamine is a valid alternative for patients with allergies. Surgery is reserved for hypertrophy resistant to medical therapy and, in pediatric patients, is generally performed under general anesthesia.

Adenoid Hypertrophy

Adenoid hypertrophy represents the most common cause of nasal obstruction in the pediatric population, and is generally diagnosed in the first 6 years of life. The most commonly observed manifestations are bilateral nasal obstruction, snoring, mouth breathing, rhinorrhea, hyponasal speech, and cough. Clinical history commonly shows recurrent episodes of acute otitis media, persistent effusive otitis and/or chronic or recurrent rhinosinusitis [12]. Adenoid hypertrophy may play a major role in pediatric chronic rhinosinusitis, working as a reservoir for pathological bacteria [13-15]. For this reason, adenoidectomy with or without antral irrigation and balloon sinus dilation can be considered as first-line treatment when

maximal medical therapy fails. Adenoid hypertrophy may also present with episodes of sleep apnea and, albeit rarely, affect the cardiovascular system (cor pulmonale in extreme cases). Nasal endoscopy is the gold standard diagnostic procedure for adenoid hypertrophy, evaluating size, intrachoanal extension, residual choanal lumen, and anatomical relationships with the Eustachian tube orifice. Adenoids (Figure 2) appear as a lobulated, pinkish, pyramid-like shaped aggregations of lymphoid tissue with the apex pointed toward the nasal septum and the base at the level of the superior and posterior nasopharyngeal walls. Many surgical options have been developed for treatment of adenoid hypertrophy. In our opinion, the use of adenotome or microdebrider under endoscopic control with a 70° angle optic rigid scope represents the best surgical option for removal of adenoids.

Sinonasal Polyposis

Except for antrochoanal polys, polyposis is quite rare in the pediatric population [16] and when bilateral should raise concern for a potential diagnosis of cystic fibrosis or fungal allergic rhinosinusitis. Even if some predisposing factors are well known (i.e., hereditary, allergies, asthma and aspirin sensitivity), the etiology of sinonasal polyposis is still debated. Sinonasal polyposis can be classified into 5 categories [17]: 1. antrochoanal polyps; 2. choanal polyps; 3. polyposis associated with chronic rhinosinusitis (non-eosinophilic dominated); 4. polyposis associated with chronic rhinosinusitis (eosinophilic dominated); 5. polyposis associated with specific illnesses (asthma, cystic fibrosis, primary ciliary dyskinesia, etc.).

Children affected by this disorder report hypo/anosmia, nasal obstruction, rhinorrhea, and sometimes headache or facial pressure [16]. Diagnosis is easily confirmed by nasal endoscopy. Polyps (Figure 3) show a characteristic edematous and translucid appearance (closely resembling a grape) and generally originate from the middle meatus or the olfactory region. Computed tomography (CT) scan is the gold standard diagnostic technique, defining the exact extension of disease and allowing for detailed study of sinonasal anatomy in the preoperative setting [16].

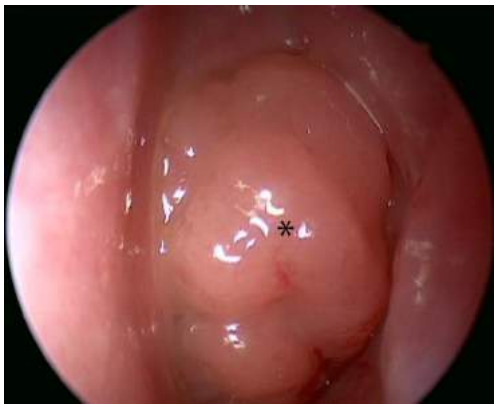

Figure 2. At nasal endoscopy, adenoid hypertrophy (asterisk) obstructing totally the posterior choanae.

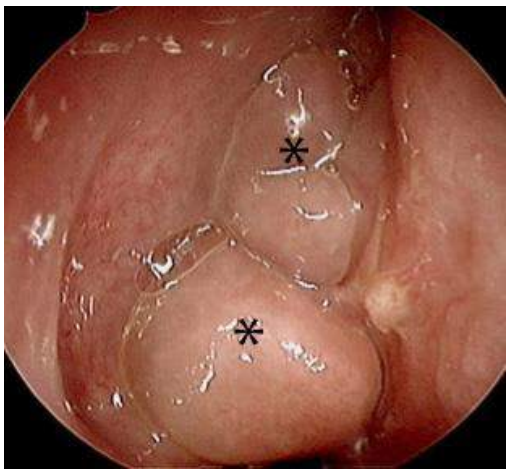

Figure 3. Nasal polyps obstructing completely right nasal fossa.

Medical therapy is usually effective and is managed through saline irrigations and the application of topical steroids for at least 4 weeks. Allergic patients may benefit more from a combination of topical steroids and anti-histamine drugs. When symptoms are not tolerated and polyposis proves to be resistant to medical therapy, surgery is offered to the patient. In the pediatric population, polypectomy is usually performed under general anesthesia. After the application of cottons soaked with decongestants into

the nasal cavity 10 minutes before starting surgery, a solution of local anesthetic and epinephrine is injected at the level of the polyp pedicle, which is then stripped with Weil forceps, or more commonly removed by micro-debrider. When planning a polypectomy, it is recommended to define the surgical strategy according to the necessity to correct anatomic anomalies that have might favored the formation of polyps.

Antrochoanal polyp (ACP) is a peculiar form of sinonasal polypoid formation, more frequently observed in infants, where it is observed in almost one-third of cases of polyposis [18-21]. ACP are usually unilateral and has a male prevalence (M:F = 2.1:1). They originate inside the maxillary sinus, from one of the intramural Tonrwaldt's cysts within sinus walls [22-23]. As it grows, it first occupies the maxillary sinus volume, and then protrudes through the natural or accessory maxillary ostium into the nasal cavity towards the choanal aperture. ACP generally shows a cystic composition in its intra-maxillary portion and a solid composition in the emerging portion within the nasal fossa. Clinical presentation depends on local extension. Patients generally complain of unilateral nasal obstruction, occasional bleeding, rhinorrhea, and foreign body sensation [24-25]. Bilateral symptoms are typical of ACPs with large involvement of the nasopharyngeal lumen or when ACP is bilateral. Dysphagia, dyspnea, and pharyngeal sensation of foreign bodies may be reported by extremely advanced cases. Diagnosis can be easily confirmed by endoscopy. A bright-white mass protruding from the middle meatus and occupies the nasal cavity with a variable posterior extension toward the nasopharynx. CT (Figure 4) offers details of sinonasal anatomy and disease extension. MRI is not routinely performed.

Acute and Chronic Rhinosinusitis

Rhinosinusitis is defined as an inflammatory condition of the sinonasal tract, characterized by the presence of at least two of the following symptoms: nasal obstruction, hypo/anosmia, cough, pain on pressure of tender spots, facial pain and/or headache, rhinorrhea, and postnasal drip

[26]. Diagnosis is generally confirmed by endoscopy, demonstrating diffuse mucosal inflammation, turbinate congestion, and pathologic nasal discharge (more commonly coming from the middle meatus).

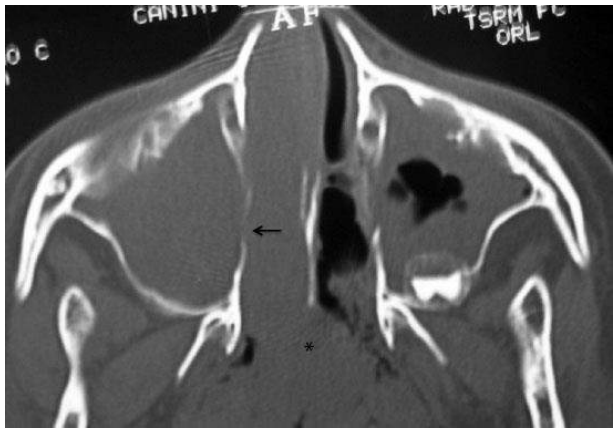

Figure 4. At CT scan, Antrochoanal polyp fills the right maxillary sinus growing the natural ostium (arrow) into the middle meatus to the choana (asterisk).

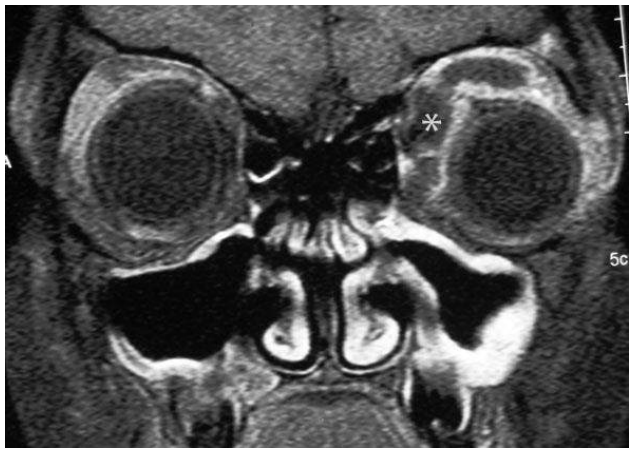

Figure 5. Coronal MRI view of the left extraconal orbital lesion (asterisk) associated with displacement of the orbital globe.

Based on duration of symptoms, rhinosinusitis can be distinguished as:

1. acute rhinosinusitis, when symptoms lasts less than 12 weeks;
2. chronic rhinosinusitis, when symptoms lasts continuously at least 12 weeks; and
3. recurrent chronic rhinosinusitis, when multiple acute episodes occur with complete resolution of each within 12 weeks.

The association of chronic rhinosinusitis with nasal polyposis is far less common in the pediatric age compared to adult population. Recurrent/chronic rhinosinusitis and the concomitant presence of bilateral polyposis should raise concern for immunodeficiency and primary ciliary disorders. CT scan is usually reserved for chronic rhinosinusitis when endoscopy is not sufficient to make differential diagnosis and/or surgical treatment is necessary. Because of the underdevelopment of frontal and sphenoid sinuses in the first years of life, CT scan often shows involvement of maxillary and ethmoid sinuses. The Lund-Mackay score is a reliable and highly predictive model used for diagnosis of chronic rhinosinusitis based on CT findings [27]. MRI is mandatory when clinical presentation raises suspicion for severe forms or orbital (Figure 5) or intracranial complications.

Differential diagnosis should exclude viral or allergic rhinitis and adenoid infection or hypertrophy. Once diagnosis is made, the ENT specialist must always take into account concurrent disorders that may coexist with chronic rhinosinusitis: adenoid hypertrophy, immunodeficiency, asthma, cystic fibrosis, allergy, ciliary dyskinesia, and gastroesophageal reflux disease. Accurate anamnesis and close collaboration with pediatrics, pulmonologists, and immunologists is therefore recommended.

Pathogens most commonly isolated in acute rhinosinusitis are those generally responsible for acute bacterial upper airway infections such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catharralis*. However, *Staphylococcus aureus* and anaerobes can be occasionally found [28]. Origin of purulent discharge can be assessed through endoscopy, and nasal samples for cultures can be easily taken directly from the involved meatus. At first presentation, microbiological cultures are not mandatory since the success rate of empiric antibiotic

therapy is satisfactory. When patients fail to improve despite medical therapy within 72 hours or a second episode recurs within 2 weeks, cultures are necessary to guide antibiotic therapy. Cultures are also mandatory in any case of rhinosinusitis with severe presentation or in immunocompromised patients [29-30].

Medical therapy is the first treatment of choice for chronic rhinosinusitis. The European Position Paper on Rhinosinusitis and Nasal Polyps [26] suggests an algorithm for treatment of pediatric chronic rhinosinusitis based on presentation. Mild symptoms may be managed with nasal saline irrigation and application of nasal topical steroid puff. The use of oral antibiotics is reserved for persistent symptoms or moderate to severe symptoms. Antibiotic therapy is generally empiric (e.g., amoxicillin/ clavulanate) and requires one or two courses. When medical therapy fails to control symptoms of chronic rhinosinusitis and acute infection exacerbates, intranasal samples of nasal discharge should be taken and antibiotic chosen according to the pathogens isolated. When maximal medical therapy fails, surgical therapy must be considered: adenoidectomy with or without antral irrigation and balloon sinus dilation, and PESS are the most commonly performed surgical procedures.

PESS represents the surgical treatment of choice for complicated rhinosinusitis or when associated with systemic conditions (i.e., cystic fibrosis). Because of potential harm to sinonasal and facial growth centers, it is recommended to adopt a conservative approach aimed to restore nasal fossa patency by polypectomy, correction of turbinate hypertrophy or concha bullosa, anterior ethmoidectomy, and maxillary antrostomy. PESS is always performed under general anesthesia and requires the use of rigid endoscopes of different angles (0°, 30°, 45°, 70°) and size (4 mm, 3 mm, 2.7 mm diameter). Generally, the application of cottons soaked with decongestants in the nasal cavity 10 minutes before starting surgery increases nasal fossae volume. A solution of 1% mepivacaine chlorohydrate and 1:200,000 epinephrine is then injected in the submucosa at the roof of the middle turbinate and uncinated process. With a small seeker, the posterior aspect of uncinated process and main ostium of maxillary sinus are probed before proceeding with uncinectomy. In order to be as conservative

as possible, the uncinectomy is partial and the upper third is conserved. Natural ostium of maxillary sinus is then widened both inferiorly and posteriorly. Once the uncinate process has been removed, the anterior wall of the bulla ethmoidalis is visible and is opened using a debrider or a Weil forceps. Following this, the opening of basal lamella medially and inferiorly is performed to give access for posterior ethmoidectomy. The aperture of the sphenoid sinus is achieved at an infero-medial angle because of the close relationship on the lateral side with optic nerve and internal carotid artery. Just below the sphenoid sinus aperture runs the posterior septal branch of sphenopalatine artery, which should be preserved if a Hadad-Bassagasteguy flap [31] for skull base reconstruction is planned. Sphenoid sinusotomy can also be effectively performed through a trans-nasal or a trans-septal corridor. Frontal sinusotomy is rarely required as the frontal sinuses are usually underdeveloped until late childhood. When necessary, this procedure should be performed by pediatric ENT surgeons with expertise in sinonasal and skull base surgery, even if it is generally sufficient to remove agger nasi cells after anterior ethmoidectomy in order to expose the frontal recess, where a sinusotomy can be easily performed using angle circular-biting forceps.

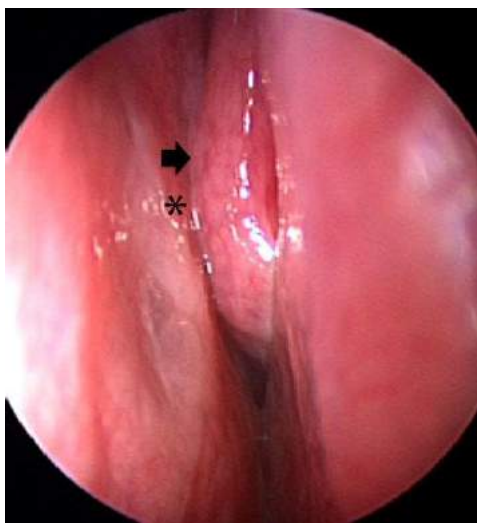

Figure 6. Endoscopic view of the anterior dislocation of the uncinate process (asterisk) and medialization of the middle turbinate (arrow).

Adenoidectomy with intranasal irrigation and balloon sinuplasty represent a surgical alternative to PESS, especially for children without complicated rhinosinusitis [32-33]. The role of adenoids in chronic rhinosinusitis in children is under debate. The long-term benefit some patients with chronic rhinosinusitis gain from adenoidectomy may suggest a major role of adenoids in maintaining sinonasal chronic infection [13], [14-15] although a definitive conclusion cannot be established with current evidence. In chronic rhinosinusitis, adenoidectomy is generally performed along with maxillary antral irrigation. Balloon sinuplasty may be performed to increase the definitive outcome. However, its real impact on sinonasal restoration is yet to be determined.

Complications and Quality of Life

PESS is considered a safe and effective treatment for pediatric chronic rhinosinusitis. The success rate, defined as increased quality of life, reduced symptoms and/or willingness to suggest PESS to other patients, varies from 76% to 89% [27,34-35]. Patients affected by chronic rhinosinusitis with or without cystic fibrosis, eligible for PESS, reported an impaired general quality of life prior to surgery. Dedicated questionnaires were submitted to analyze specific quality of life after PESS for these patients, showing a net improvement after 1-3 months [36].

Reported complications from PESS range between 0.6% - 1.4% [27,34,37] Although rare, PESS may result in major complications, including orbital damage, skull base lesion with subsequent CSF leak, and meningitis, requiring prompt intervention. Minor complications (i.e., minor bleeding, synechiae, local infection) are usually treated conservatively. The low overall risk of readmission, reoperation and complication rates were also confirmed by a recent study, with reported rates of 4.5%, 2.6%, and 3.0%, respectively. In this study, independent factors associated with an increased risk of postoperative adverse events were urgent/emergent procedures and presence of bleeding disorders. Age < 3 years was an independent risk factor only for bleeding requiring transfusion [38].

Mucocele

Mucocele is a benign, cyst-like, locally expansive paranasal sinus mass consisting of a collection of aseptic mucus, and products of mucosal debris, lined by respiratory mucosa, that develops within a paranasal sinus [39,40]. A mucocele grows slowly and expands by eroding the surrounding bony walls as a consequence of ostium blockage. The obstruction can result from congenital anomalies, adherences from chronic rhinosinusitis, previous radiotherapy and/or surgical treatment, trauma, and sinonasal neoplasms [41]. Recognized predisposing factors are congenital illnesses such as cystic fibrosis and primary ciliary dyskinesia. Mucoceles occur rarely in the pediatric age, and most cases described were associated with the abovementioned congenital conditions [42-44]. The most common site of origin is the frontal sinus, where it occurs in 60% of cases, followed by the ethmoid labyrinth and maxillary sinus with 30% and less than 10% of cases, respectively. Few cases are localized in the sphenoid sinus [45]. The higher incidence of mucoceles in the frontal sinus seems to be related to anatomical complexity of the frontal recessal [46]. Mucoceles are almost always monolateral, and no gender prevalence has been observed. Clinical presentation is dependent on localization, but is generally characterized by nasal obstruction, headache or facial pain, rhinorrhea, check swelling, allodynia or dental pain, and local hypo/anesthesia. When a frontal mucocele grows enough to involve the orbital content, periorbital pain, edema, proptosis, diplopia, blurred vision, and sudden vision loss can occur [40,47]. Whenever erosion of the anterior or posterior wall of the frontal sinus is present, a Pott's puffy tumor or neurological symptoms may be evident. Diagnosis is based on signs and symptoms, nasal endoscopic evaluation, and imaging. Nasal endoscopy is generally silent during the intra-sinusal phase, whereas, during subsequent expansion of the mucocele, alterations of the bony walls of the paranasal sinus may be evident. Advanced maxillary mucocele may cause medialization of the middle turbinate, anterior dislocation of the uncinate process (Figure 6), and bulging of the agger nasi cells. Submucosal remodeling or bulging of the sphenoethmoid recess or posterior ethmoid, on the other hand, is more typical of high-volume

sphenoidal mucoceles. In frontal mucoceles, endoscopic examination is usually negative, since the lesion expands inferiorly to involve the agger nasi [48]. CT scan shows an homogenous lesion that completely occupies the involved sinus with smooth clear-cut margins of bone erosion of its walls, giving details on the origin, extension of disease, entity of bony erosion, and hyperostotic changes (Figure 7). MRI is usually reserved for mucoceles secondary to sinonasal expansive tumors.

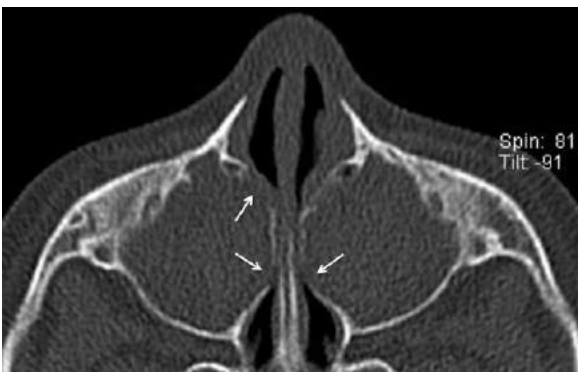

Figure 7. At CT scan, lesion involves completely the maxillary sinus bilaterally and erosion of the medial bone walls (arrows) is evident.

Naso-Lacrimal Duct Congenital Disorders

Naso-lacrimal duct obstruction (NLDO) in the newborn and infants is usually congenital, secondary to a defect of its canalization during embryogenesis. This defect typically presents at the level of Hasner valve. Causes of this disorder include persistence of a membranous diaphragm, bony stricture, or ab-extrinseco closure from edematous nasal mucosa. At birth, the persistence of a membrane at the Hasner valve can be found in 20 -30% of newborns. However, in many cases canalization occurs spontaneously hereafter [53]. Children affected by NLDO show persistent epiphora and an increased risk of lacrimal system infection. Crusting of the ipsilateral medial canthus is commonly observed. Diagnostic work-up generally includes nasal endoscopy and ophthalmologic evaluation.

Dacryocystography (Figure 8) is commonly performed to confirm diagnosis and localize the site of obstruction [54]. When dacryocystorhinostomy (DCR) is planned, presurgical CT scan of the sinus is mandatory to evaluate sinus anatomy.

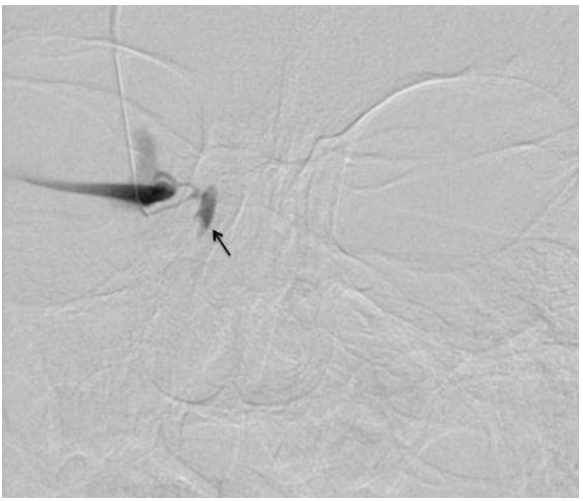

Figure 8. Dacryocystography shows obstruction of the lacrimal system at inferior third of the lacrimal sac (arrow).

Treatment of this disorder includes conservative and non-conservative procedures. When conservative approaches such as lacrimal massage, probing and irrigation of lacrimal system, balloon dacryoplasty, and nasolacrimal duct intubation fail, DCR is mandatory. Despite the optimal success rate and diffusion of external approaches, endoscopic-DCR is encouraged because of the far lower incidence of complications and recovery time and because it is less invasive than external surgerry. Use of a nasolacrimal stent is debated, since it might elicit local inflammatory response and granulation, increasing the risk of re-stenosis [57,58]. Known risk factors for surgical failure are pre-saccal obstruction and presence of craniofacial anomalies. Major complications following endoscopic DCR are exceedingly rare [52,54-56]. Minor complications include bleeding, synechiae, and eye itchiness. A new promising and more conservative endoscopic-assisted procedure has been reported. After inspection of the

inferior meatus, the valve of Hasner is incised and the nasolacrimal duct is irrigated [59].

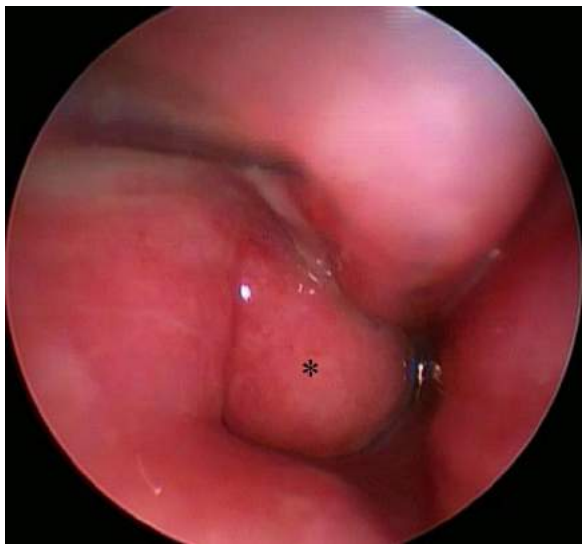

Figure 9. At endoscopic evaluation, a naso-lacrimal duct cyst (asterisk) is observed.

Naso-lacrimal duct cysts (NLDC) (Figure 9) are congenital cystic dilation of the distal portion of the nasolacrimal duct secondary to failure of the Hasner membrane to canalize. It is a rare condition, most commonly symptomatic at birth or within few weeks of age [60]. It is more commonly observed in females. Clinical presentation depends on extension and laterality. Monolateral NLDCs typically present as ipsilateral persistent epiphora, recurrent dacryocystitis, and nasal obstruction, whereas bilateral NLDCs may cause acute respiratory distress at birth because of bilateral nasal obstruction. Susceptibility to aspiration and feeding difficulties may accompany bilateral NLDCs [61]. The evidence of a grayish-blue cystic mass in the inferior meatus on nasal endoscopy confirms diagnosis. CT scan can further confirm diagnosis showing a cystic formation at the level of Hasner's valve in the lateral wall of the inferior meatus, with progressive dilation of nasolacrimal duct and lacrimal sac. Surgical intervention is indicated for symptomatic patients, since spontaneous canalization occurs in

most patients within 9 months after birth. Under general anesthesia, the cyst is endoscopically incised, drained, and marsupialized into the inferior meatus [62-63].

Congenital Choanal Atresia

Congenital choanal atresia (CCA) is defined as the congenital pathological closure of the posterior choanae, either mono- or bilateral, by a diaphragm of membranous, bony, or mixed tissue. CCA is the most common congenital malformation in the sinonasal tract, with an incidence of 1 in 5000-9000 live births, with a prevalence for the female gender and the right side (both with a ratio of 2:1) [64,65]. The pathogenesis of CCA is still debated. Persistence of the bucco-pharyngeal membrane, naso-buccal membrane of Hochstetter, misdirection of neural crest cell migration, and abnormal location of mesoderm are the most accepted theories [66,67]. CCA is an isolated finding in 50% of cases, while in the remaining is due to syndromic conditions. CHARGE (Coloboma, Heart disease, Atresia choanae, Retarded growth, Genital anomalies, Ear anomalies), Di George, Treacher Collins syndromes and Blackfan-Diamond anemia are the most important in this regard [67]. Choanal narrowing is caused by the presence of a pathologic diaphragm, extended from the floor of the sphenoid bone to the horizontal process of the palatine bone, and from the vomer to the medial pterygoid plate. Since the condition is mainly secondary to bony malformation of vomer and sphenoid bone, in almost all cases the diaphragm has a mixed (70%) or bony (30%) structure, while a pure membranous occlusion is extremely rare [68-70].

CCA is unilateral in two-thirds of cases and bilateral in the remaining. Bilateral CCA usually presents at birth as an airway emergency, since newborns show an obligate nasal ventilation. Severe respiratory distress, paradoxical cyanosis and stridor are the most commonly observed symptoms [64]. Differential diagnosis includes other sinonasal congenital malformations (midnasal or pyriform aperture stenosis), bilateral nasolacrimal duct cysts, septal deviation, nasopharyngeal wall collapse and

reflux, and congenital sinonasal lesions (e.g., teratoma) [71]. In these cases, prompt intervention to secure airway is mandatory, and pediatric ENT surgeons must be prepared for tracheostomy in selected cases. Unilateral CCA usually shows a delayed presentation (5-24 months or even after), with symptoms of unilateral nasal obstruction and ipsilateral otitis media on occasion. Differential diagnosis includes septal deviation, nasal foreign bodies, inferior turbinate hypertrophy, antrochoanal polyp, and expansive sinonasal benign of malignant lesions [64].

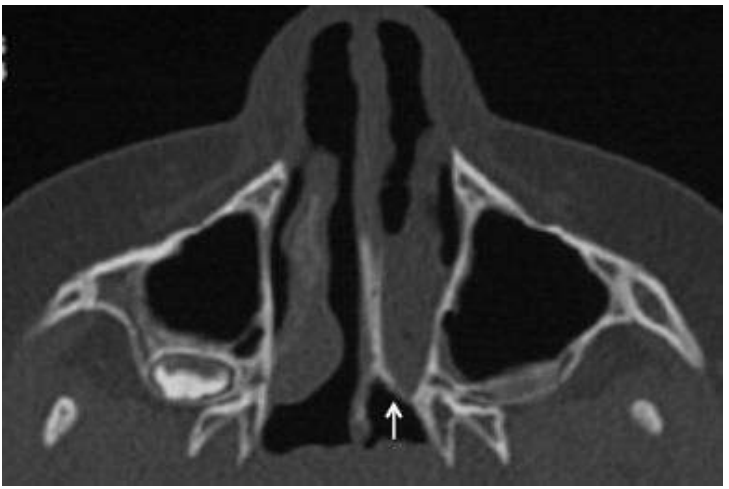

Figure 10. CT-scan shows left choanal atresia (white arrow).

Diagnosis can be easily confirmed by observing fogging on a cold metal speculum positioned at the nasal pyriform aperture [64]. For differential diagnosis with pyriform aperture stenosis, a catheter is inserted into the nasal cavity: if it passes through the nasal cavity, but is blocked more posteriorly, CCA is confirmed. Nasal endoscopy and CT (Figure 10) represent the gold standard for definitive diagnosis. Nasal endoscopy shows a complete uni- or bilateral choanal occlusion, usually associated with abundant mucous secretions. CT allows evaluation of the bony details of the CCA and other craniofacial malformations that can accompany choanal atresia in syndromic cases. In order to exclude CCA as part of a syndromic condition, thorough

diagnostic work-up, including evaluation of craniofacial malformations, muscle tone, and airway assessment, is usually recommended [64].

Therapeutic strategies are planned based on type, grade of stenosis, and associated conditions. While bilateral CCA requires prompt intervention aimed to secure the airway and stabilize ventilation, surgical planning for unilateral atresia might be postponed after the first years of life in order to increase the rate of success. The first surgical approaches introduced were transnasal puncture and transpalatal repair, but were progressively abandoned in favor of transnasal endoscopic choanal plasty [72,73]. In order to maximize the chance of success and reduce the rate of restenosis, medial choanal enlargement over lateral extensive drilling, and protection of exposed bone with local mucosal flap are encouraged [74].

A metanalysis published in 2008 reported that endoscopic choanal plasty had a success rate of 85.3%, although criteria for definition of failure varied widely among the individual studies considered [72]. Risk of restenosis is mainly associated with bilateral CCA, CHARGE syndrome, and age and weight at the time of surgery [75]. Recent data show a higher risk of failure with the employment of choanal stents, secondary to granulation and inflammatory reactions that may locally cause [76,77]. For this reason, their use is discouraged. Application of topical steroids and accurate endoscopic toilette of the nasal cavities have been reported as fundamental precautions in reducing the risk of re-intervention.

Nasal Dermoids

Nasal congenital midline lesions, consisting of nasal dermoids, gliomas, and encephaloceles, are rare developmental malformations with and estimated incidence of 1:3000 - 1:40,000 births [78,79]. Nasal dermoids (ND) are defined as frontonasal inclusion cystic masses, sometimes associated with sinus opening. Males are slightly more affected. NDs may associate with other congenital malformations (5 - 41%), such as external ear anomalies, cleft lip and palate, hypertelorism [80]. Foramen caecum plays a key role in the pathogenesis of these congenital anomalies. It is a

transient skull base corridor, anterior to the crista galli, that forms from the third week of gestation. Through the foramen caecum, the dura protrudes towards the prenasal space, located posteriorly to the nasal bone, where the ectoderm is apposed. As the foramen caecum regresses spontaneously during the eighth week of gestation, the dura retracts back into the intracranial space. Nasal dermoids typically form from ectodermal and mesodermal tissues entrapped along the dural descent pathway towards the prenasal space [81].

Histologic examination confirms the presence of ectodermal and mesodermal elements (i.e., hair, keratin, sweat, and sebaceous glands). Lack of the endodermal component differentiates them from teratoma. Unlike encephaloceles and gliomas, NDs do not present glial elements. Generally, clinical presentation is peculiar. A dimple, fistula or cystic lesion can present anywhere along the embryologic midline line, from the nasal tip into the skull base, with the rhinion as the most common location. Although a rare finding, hair protruding through the midline lesion is pathognomonic for nasal dermoid. When a midline external lesion presents protruding hair, this is pathognomonic for nasal dermoids. A widened nasal bridge, intermittent sebaceous discharge, and recurrent infections are commonly observed. Unlike encephaloceles, NDs do not transilluminate and do not expand with crying. NDs may have an intracranial extension in 4% 45% of cases, with a potential for recurrent meningitis (caused by skin flora bacteria) [80].

Diagnostic work-up includes nasal endoscopy and radiological imaging, and should be aimed at identifying the location and size of ND, indirect or direct signs of intracranial extension, nasal anatomy, and associated malformations. Indirect signs of intracranial extension are well-established with CT and include bifid crista galli, enlarged foramen caecum, and cribriform plate anomalies [82]. If intracranial tract is suspected, contrast-enhanced MRI is recommended.

Surgical treatment is mandatory since progressive enlargement of NDs can cause craniofacial deformities, recurrent infections, meningitis, and brain abscess. The surgical strategy should obtain radical removal, allow proper skull base reconstruction, with optimal cosmetic outcomes [83].

Conservative treatment, such as curettage, drainage, and aspiration are not recommended, because of the high risk of recurrence [83]. Different surgical strategies have been proposed according to the type of ND and its extension, with external rhinoplasty incision as the most widely adopted. When NDs show intracranial extension, coronal incision and frontal craniotomy are generally required. Although external approaches are considered safe and effective [84-87], efforts have been made to introduce endoscopic-assisted transnasal excision for intracranial NDs [88-89].

Encephalocele

Encephaloceles are sac-like herniations of intracranial content caused by a skull base defect [90]. They are called meningoceles when formed only by meningeal linings and CSF, and meningo-encephaloceles when brain tissue is also included. Encephaloceles form secondary to failure of proper neural tube closure. No gender predominance has been reported. The incidence is higher in Asia, with a reported incidence of 1 in 6000 births [91]. Low levels of maternal folic acid during first weeks of development may be a major risk factor. Encephaloceles typically present as smooth, white-bluish, pulsatile lesions, increasing in volume with crying or on jugular vein compression (Furstenberg sign) [90]. If left untreated, encephaloceles can cause recurrent meningitis and epilepsy. Depending on their localization, encephaloceles are classified into 3 major classes: sincipital, basal, occipital.

Sincipital encephaloceles are further classified as naso-frontal, naso-ethmoidal, and naso-orbital. *Nasofrontal* encephaloceles develop through a defect between nasal and frontal bones, presenting as a protruding mass in the glabellar region and causing inferior displacement of nasal pyramid and lateral displacement of the eyes (telecanthus). *Nasoethmoidal* encephaloceles protrude through a patent foramen caecum towards the prenasal space, where junction of nasal cartilages and bones is located (rhinion). Typical presentation is a mass on the nasal dorsum causing a superior displacement of nasal bones and inferior displacement of alar cartilages. If an encephalocele protrudes through a patent foramen caecum

and laterally extends through a defect in the medial orbital wall, then a *naso-orbital* encephalocele forms, causing ipsilateral proptosis, visual impairment, epiphora, and recurrent dacryocystitis. *Basal* encephaloceles originate more posteriorly, between the cribriform plate and the middle skull base. They are further classified as *transethmoidal*, *sphenoethmoidal*, *transsphenoidal*, and *sphenorobital*. *Transethmoidal* and *sphenoethmoidal* encephaloceles present as an endo-nasal protruding mass, causing nasal obstruction and orbital displacement (hypertelorism). They occur secondary to a defect in the cribriform plate or at sphenoethmoidal junction, respectively. *Trans-sphenoidal* encephaloceles develop secondary to a persistent craniopharyngeal canal, protruding into nasopharynx, causing nasal obstruction and affecting swallowing. Trans-sphenoidal encephaloceles are typically associated with cleft palate defects. *Spheno-orbital* encephaloceles expand toward the spheno-palatine fossa, passing through the superior and inferior orbital fissures. Ipsilateral proptosis and visual impairment with no visible masses is the typical presentation. Finally, *occipital* encephaloceles occur secondary to a defect in the posterior cranial fossa.

The neural tissue in meningo-encephaloceles was initially considered dysplastic and non-functioning, but since functioning brain has been found in some occipital and trans-sphenoidal encephaloceles, this concept has been recently revised [90]. The presence of ependymal tissue on histologic examination is pathognomonic [92]. Diagnosis of an endo-nasal protruding encephalocele is made by nasal endoscopy and CT and/or MRI. Contrast-enhanced MR allows to identify the composition of the encephalocele (meninges with or without brain tissue), and also provides details on skull base defects and possible associated malformations. In order to obtain the best outcomes, prompt surgical intervention within the first months of life is advisable. Traditionally, small lesions could be managed endoscopically, while external craniotomic approaches were reserved for larger lesions.

Gliomas

Nasal gliomas (NG) are a rare benign developmental disorder, composed by heterotopic glial tissue, sequestered outside the cranium secondary to premature and abnormal skull base closure (e.g., foramen caecum) [94-96]. For this reason, gliomas lack a patent communication with the brain (in contrast to encephalocele), and therefore do not transilluminate and do not show a change in size with straining, crying, or jugular vein compression (negative Furstenberg sign) [90]. However, a fibrous attachment to the dura can be identified in a minority of cases. NGs are classified into *extranasal* (60% of cases), *intranasal* (30% of cases), and *mixed* (10% of cases). *Extranasal* NGs present as a firm, smooth mass, external to the nasal bones and cavities (more commonly at the glabella). *Intranasal* NGs lie within the nasal cavity (more commonly from nasal lateral wall close to the middle turbinate) and can protrude through the ipsilateral nostril, causing nasal obstruction. Further extension to adjacent sinuses is not common. *Mixed* NGs show a typical dumbbell shape with a connecting band between the extra- and intra-nasal components. NGs are present at birth, although clinical presentation occurs typically between 5 and 10 years of age and may include: nasal obstruction, epistaxis, CSF rhinorrhea, broadening of the nasal bridge, hypertelorism, and epiphora secondary to nasolacrimal duct stenosis [96-98]. The intranasal components present as a non-pulsatile polypoid mass with a gray-reddish-purple color on nasal endoscopy. CT and contrast-enhanced MR are mandatory for diagnosis, in order to study nasal anatomy, bony structures, skull base attachment, and possible associated malformations. Extranasal gliomas generally require external surgical access that allows possible exploration of the skull base, with external rhinoplasty as the most commonly adopted approach. Intranasal gliomas are generally successfully treated with an endoscopic approach. In patients with fibrous attachment to dural layer, it is recommended to follow its entire course, in order to be radical and exclude intracranial extension. If required, endoscopic skull base reconstruction can be performed with synthetic, allogeneic, autologous tissue and/or local flaps [93].

Cerebrospinal Fluid Leak

Cerebrospinal fluid (CSF) leak occurs when there is an abnormal communication between the subarachnoid space and the sinonasal tract and/or the ear cavity, through a breach of the skull base and underlying meninges [90]. CSF leaks are classified as *non-traumatic* (with high or normal CSF pressure) and *traumatic* (accidental and iatrogenic) [99]. Traumatic skull base lesions are the most common cause of CSK leak: 80% secondary to craniofacial trauma and 16% secondary to sinonasal or skull base surgery [100]. Spontaneous CSF leak may be caused by congenital defects (potentially associated with encephaloceles, large arachnoid granulation cysts), skull base erosion by malignant tumors, or indirectly by increasing CSF pressure (i.e., aqueductal stenosis). The most common locations for spontaneous CSF fistula are the cribriform plate, sphenoid sinus, and pterygoid recess. Unilateral or bilateral watery rhinorrhea (rhinoliquorrea), which is exacerbated when the patient bends forward, is the most typical presentation. Clinical manifestation of CSF leak may also include salty or sweet taste, recurrent meningitis, and signs of CSF hypertension such as headache, vomit, and papilla edema [90]. The "reservoir sign" is suggestive for sphenoid CSF fistula. In a recumbent position, CSF accumulates within the sphenoid sinus, until the patient resumes an erect position and sudden abundant watery rhinorrhea occurs [101].

Laboratory test on watery rhinorrhea can confirm the CSF leak by demonstrating the presence of beta-2 transferrin or beta-trace protein, polypeptides exclusively produced by the CNS. Fluorescein test consists in the intra-techal administration of fluorescent dye solution, allowing the surgeon to localize the specific site of the leak. Fluorescein test can be used for pre-operative diagnosis, have a pre-operative diagnostic role, or an intra-operative role in guiding surgery. Contrast-enhanced CT and MR are recommended to study skull base anatomy, extension of the CSF leak, and possible associated disorders [90,102-103].

Reconstruction of Pediatric Skull Base Defects

Endoscopic skull base reconstruction can be performed with synthetic, allogeneic, autologous tissue and/or vascularized pedicled flaps. For large skull base defects, especially with moderate-high CSF pressure (as in the middle cranial fossa), vascularized pedicled flaps are generally preferred. Vascularized pedicled flaps are divided into sinonasal and scalp flaps [1,106].

The most commonly used vascularized sinonasal vascularized flaps are all based on branches of the sphenopalatine artery: *nasoseptal flap* [31] (Hadad-Bassagasteguy flap), *inferior turbinate flap*, and *middle turbinate flap*. Recently, some authors [104-105] have advocated the *naso-septal flap* as a safe and effective reconstruction for anterior skull base defects in the pediatric population, showing a success rate similar to open reconstructions. It is a robust mucoperiosteal-mucoperichondrial flap, potentially eligible for most skull base defects. The Hadad-Bassagasteguy flap is pedicled on the posterior septal artery, a branch of the sphenopalatine artery that runs along the inferior surface of the sphenoid ostium bilaterally. Therefore, if skull base reconstruction is planned, this artery must be preserved during sphenoidotomy. After the age of 13 years, the width and length of this flap have the same dimension of adults, whereas between 24 months and 10 years the length may be insufficient for repair of larger defects, thus requiring accurate pre-operatory surgical planning. Under 24 months of age the nasoseptal flap is not recommended because of its limited overall dimensions [105]. Traditional scalp pedicled flaps are the *pericranial flap*, based on the supraorbital and supratrochlear arteries, the *temporoparietal fascial flap*, based on the superficial temporal artery, and the *temporalis muscle flap*, based on deep temporal arteries. For reconstruction of smaller anterior skull base defects, free grafts of iliotibial tract fascia and fat is a safe option, with high rate of success [1,106-107].

Juvenile Nasopharyngel Angiofibroma

Juvenile nasopharyngeal angiofibroma (JNA) is a rare, benign, highly vascularized expansive lesion that typically affects male adolescents [108]. JNA has a controversial etio-pathogenesis, sharing features of both vascular malformations (origin from incomplete regression of the first branchial artery along its course) and tumors (chromosomal alterations, relentless growth) [109-112]. These lesions typically increase their growth rate following hormonal changes during puberty and can potentially regress when sexual hormonal levels decrease [113]. JNAs originate at the level of the sphenopalatine foramen, from where it extends typically to adjacent structures, widening skull base foramina and displacing fat, muscles, nerves, and vessels. This disorder has a peculiar propensity to expand infiltrating the cancellous bone of the sphenoid, especially at the level of the vidian canal, pterygoid process, and middle cranial fossa floor [114].

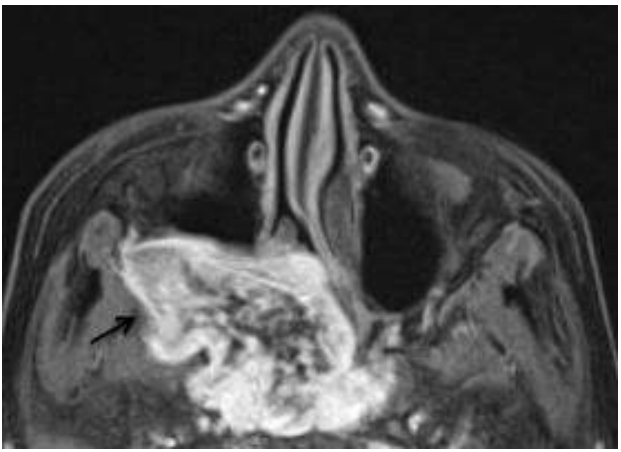

Figure 11. At axial MRI, juvenile nasopharyngeal angiofibroma involves infratemporal fossa (arrow).

Patients complain of recurrent spontaneous epistaxis with persistent monolateral nasal obstruction usually occurring in a young male adolescent. Less frequently, advanced JNAs can present with visual and ocular motility impairment, headache, conductive hearing loss, or facial pain/swelling and

remodeling [114-115]. Nasal endoscopy shows a lobulated, rubbery, red-pink to gray mass covered by vascular structures, occupying the nasopharynx and nasal cavity. When JNA is suspected on nasal endoscopy, biopsy is strongly discouraged because of the high risk of high-flow bleeding [108,114]. MRI (Figure 11) is necessary to confirm diagnosis and for surgical planning. MRI is considered the gold standard diagnostic procedure, thanks to its optimal evaluation of sphenoid cancellous bone infiltration, involvement of cavernous sinus, orbital apex, and potential close relationship with the dura [108,114,116]. JNA is traditionally classified according to Andrews classification, as reported in Table 1 [117].

Transarterial embolization of the lesion, followed by its endoscopic transnasal resection is considered the mainstay of treatment [108,114].

Table 1. Classification of Juvenile Nasopharyngeal Angiofibroma according to Andrews [117]

Type I	Tumor limited to the nasopharynx and nasal cavity. No bone destruction or limited to the sphenopalatine foramen.
Type II	Tumor invading the pterygopalatine fossa or the maxillary, ethmoid or sphenoid sinus with bone destruction
Type III	Tumor invading the infratemporal fossa or orbital region: • IIIa: without intracranial extension • IIIb: with intracranial (parasellar) extradural involvement
Type IV	Intracranial intradural tumor: • IVa: without infiltration of the cavernous sinus, pituitary fossa or optic chiasm • IVb: with infiltration of the cavernous sinus, pituitary fossa or optic chiasm.

When present, postsurgical JNA residuals generally show an indolent course and can be effectively managed with a "wait-and-see" strategy, especially if located in critical areas. Revision surgery is usually reserved for symptomatic growing residuals [118].

Infantile Intranasal Hemangioma

Infantile hemangiomas are benign, painless, easily-bleeding, solitary lesions most commonly present in the skin and in the mucosa of the upper aerodigestive tract [119]. They are typically not observable at birth, but

rapidly grow in the first months of life. Head and neck hemangiomas localize in the sinonasal tract in 7% - 29% of cases, typically involving the anterior portion of the nasal septum or head of the turbinates [48]. Sinonasal hemangiomas present with recurrent epistaxis and nasal obstruction. Local pain, headache, and hyposmia are rarely observed. Nasal endoscopy shows a reddish-purple, hypervascularized, easy-to-bleed, polypoid mass. MRI is generally required, especially when the actual extension of larger hemangiomas is not assessable with endoscopy [44,48]. Medical treatment of hemangiomas include propranolol, which reduces their size significantly [120-121]. Surgical intervention is generally reserved for symptomatic lesions with critical relationships with the orbit or skull base and/or non-response to medical therapy [93]. Wide endoscopic excision is generally sufficient. Pre-operative angio-embolization has been reported for large hemangiomas as an efficient strategy to reduce intra-operative bleeding [122].

Ameloblastoma

Ameloblastoma is a benign epithelial tumor that is derived from the dental lamina remnants or from the developing enamel organ. It is the most common odontogenic tumor, generally arising in the mandible or less frequently in the maxilla [123]. Primary sinonasal ameloblastoma is a rare condition [124]. No gender predilection has been reported. The peak of incidence is between the third and fifth decade, although ameloblastoma can occur in the pediatric population. Primary sinonasal and maxillary ameloblastoma with secondary extension in the sinonasal tract can be both asymptomatic and present as unilateral nasal obstruction, chronic/recurrent rhinosinusitis, bleeding, facial swelling, headache, and dizziness [124]. Radiographically, sinonasal ameloblastoma present as more commonly opacification of the nasal fossae and/or paranasal sinuses [125]. Imaging (CT and/or MRI) (Figure 12) is mandatory to identify the origin and extension of the lesion, and to plan its surgical removal Children are more commonly affected by the unicystic subtype, with a lower rate of recurrence

and better response to conservative treatment [123]. Trans-nasal endoscopic-assisted resection may be considered in primary sinonasal ameloblastoma and in combination with more traditional, open approaches for lesions arising in the maxilla and extending within the sinonasal tract [125,126].

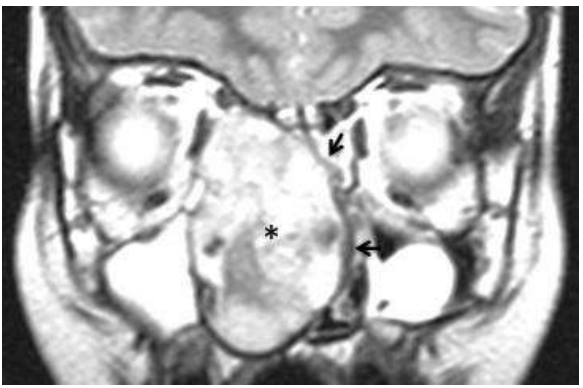

Figure 12. Giant lesion (asterisk) occupies totally the right nasal fossa with displacement of the nasal septum (arrows).

Teratoma

Teratomas are rare neoplasms, with a reported incidence of 1 in 20,000-40,000 live births; however, they represent the most common type of germ cell tumor in the pediatric age [127]. They are formed by a heterogeneous mixture of tissues from each of the 3 embryonic germ layers (ectodermal, mesodermal, endodermal), such as hair follicles, teeth, bony, and muscular structures. They are rarely malignant. Up to 5% of pediatric teratomas occur in the head and neck region (nasopharynx, oropharynx, nasal cavities, orbit, cervical region, etc.). Larger teratomas are generally detected on prenatal ultrasound, allowing for prompt management at birth to secure the airway. Smaller lesions may present later as respiratory difficulties, dysmorphic features, or dysphagia, depending on their location [128]. Sinonasal and nasopharyngeal teratomas can be successfully treated endoscopically,

avoiding palate splitting or resection with transoral approaches. Abnormal levels of serum alpha-fetoprotein during follow-up raise suspicion for recurrence.

Sinonasal Malignancies

Pediatric sinonasal malignancies represent an extremely rare form of cancer, with a reported incidence of 0.036 per 100,000 individuals [129]. Although more commonly associated with male gender, a clear gender predominance has not been demonstrated in the pediatric population [129], [130]. These diseases commonly present with non-specific symptoms, such as monolateral nasal obstruction, epistaxis, facial pain/swelling, headache, ipsilateral visual acuity or ocular motility impairment, thus frequently delaying diagnosis to an advanced stage [129]. The most commonly affected sites are the nasal cavity, followed by the maxillary sinus, ethmoidal complex, and sphenoid sinus [131]. Diagnosis is based on biopsy of the lesion performed by a transnasal endoscopic approach and imaging (Figure 13), which allows to identify the origin and extension of the mass, involvement of nearby structures, and to hypothesize a histological diagnosis. Rhabdomyosarcoma is the most frequently observed histology, followed by NK-T cell lymphoma, olfactory neuroblastoma, and sarcoma. Mesenchymal tumors are more common in the first years of life, while the incidence of olfactory neuroblastoma increases during adolescence. When indicated, naso-ethmoidal malignancies can be treated through an endoscopic assisted wide removal with or without trans-nasal craniectomy and anterior skull base reconstruction. Prognosis is generally extremely poor and mostly depends on high grading and advanced staging at presentation. Both distant and local recurrences and failures are frequent [129-132]. Non-lymphoma sinonasal malignancies show a 5-year overall survival of 37.2% - 60.2% and a 20-year overall survival of 20.6% [129-130].

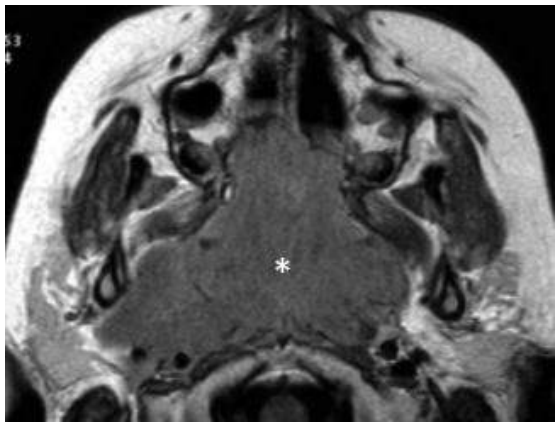

Figure 13. Large lesion (white asterisk) involves rinopharynx and it expands anteriorly and laterally.

CONCLUSION

The use of transnasal endoscopic approach for the treatment of several sinonasal disorders in the pediatric age has radically modified long-term results. Endonasal optic fibers permit to identify and assess sinonasal sites that are not easily evaluated by traditional surgical procedures and to better evaluate relationships with adjacent anatomical structures. These advantages make PESS more precise and resolute. PESS is usually performed with good results for sinonasal inflammatory diseases, anatomical malformations, benign expansible lesions, benign tumors, and CFS-leak. The role of this surgical technique for malignant neoplasms is above all diagnostic. The therapeutic utility of PESS for these latter pathologies is limited to highly selected cases and to treatment of tumor residues.

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