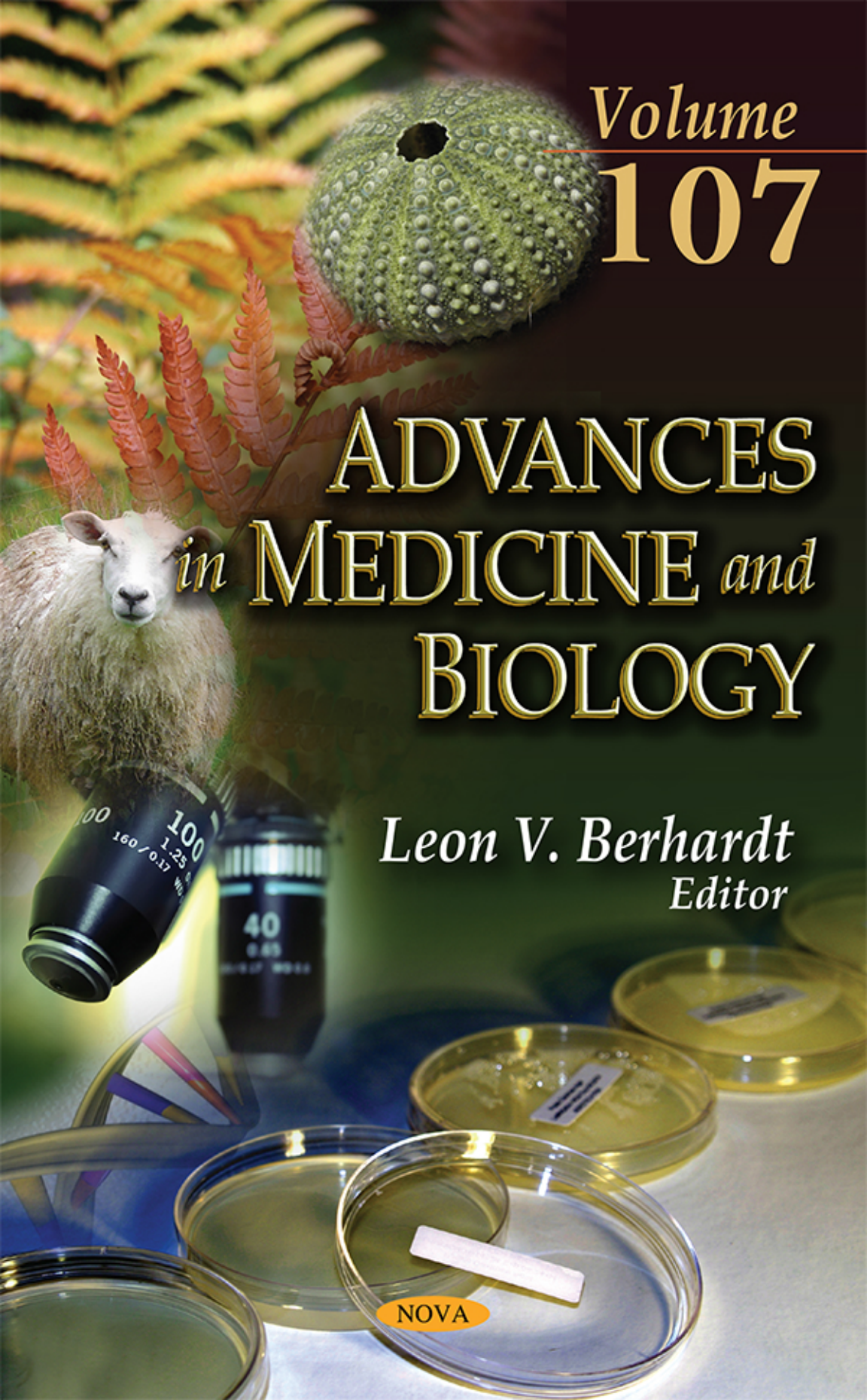
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CONTENTS

PREFACE

The chapters in this volume present the latest developments in medicine and biology. Chapter One reviews the antitumor effecrs of lithium carbonate. Chapter Two analyses the current evidence on the role of epigenetic mechanisms involved in the progression of various types of cancer and their clinical relevance. Chapter Three examines pregnancy and hemodialysis. Chapter Four focuses on the pharmacokinetics, efficacy and safety of levonorgestrel. Chapter Five discusses challenges and management of childhood obesity in the United Kingdom. Chapter six reviews dynamic and integrated synaptic processing in sex-steroid sensitive medial amygdala of female rats. Chapter Seven studies the characterization of anti-coxsackie virus B3 constituents of *Scabiosa arenaria* Forssk by reversed phase-high-performance liquid chromatography. Chapter Eight provides a review of risk factors, diagnosis and management of congenital upper limb anomalies. Chapter Nine summarizes the current knowledge on genetics, environmental factors, clinical characteristics, medical, laser and surgical treatment options of exfoliation syndrome and exfoliative glaucoma.

Chapter 1 - The present chapter is focused to analyze the underlying mechanisms that dictate the antitumor properties of different forms of lithium carbonate. The results of clinical studies on the use of lithium carbonate for cancer treatment are given. Debate exists in literature about contradictory effects of lithium salts are discussed as well.

Chapter 2 - Tumor development is the result of genetic and epigenetic alterations. The epigenetic mechanisms do not imply a change in the DNA sequence but can be as important as the genetic mechanisms for the onset and progression of cancer due to its important role in the regulation of gene expression. Methylation and acetylation of histones induce changes in the

structure of chromatin that can repress or facilitate the expression of genes. Another epigenetic modification studied in recent years is the methylation of DNA. Recently it has been observed that there are abnormal patterns of DNA methylation in many types of cancers. The malignant cells are accompanied by a global hypomethylation that is associated with the activation of genes required for the invasion and metastasis and a local hypermethylation (CpG Islands) associated with the repression of tumor suppressor genes (TSGs). Thus, the methylation of the TSG blocks its expression, contributing to the onset and tumor progression. Classic examples are the genes BRCA1, PRC, TP53 and PRDM in breast, colon, skin and lung cancer, respectively.

The abnormal patterns of DNA methylation, as well as epigenetic modifications on histones, could be used as biomarkers for the diagnosis of cancer and as targets of drugs, in order to correct these alterations and restore the function of the genes. This chapter analyses the current evidence on the role of epigenetic mechanisms involved in the progression of various types of cancer and their clinical relevance.

Chapter 3 - The outcomes of pregnancy in patients on dialysis have greatly improved in the last half-century. Therefore, the current medical opinion regarding pregnancy in women on hemodialysis has recently changed from ‘impossible’ to ‘possible,’ and pregnancy is no longer automatically discouraged, although it is still relatively uncommon and associated with a certain degree of risk. In order to decide whether to become pregnant or not, hemodialysis patients and their family members should be provided with sufficient information regarding the success rate, complications, need of intensive dialysis 6-7 days per week, optimal nutrition, and alternative options, including delaying pregnancy to after renal transplantation. In addition, to avoid delays in the detection of pregnancy, patient education and pregnancy planning is critical. Hence, close collaboration between patients, their family, nephrologists, dialysis staff, obstetricians, and neonatologists is necessary.

Chapter 4 - Levonorgestrel (LNG) is a dextrorotatory isomer of the synthetic progestogen norgestrel. Norgestrel is a racemic mixture. The activity of norgestrel resides in the dextrorotatory isomer, while the levorotatory isomer is biologically inactive. Generally, LNG is rapidly absorbed with a half-life of absorption of less than 1hour. The mean time taken to achieve maximal serum concentration is less than 2 hours with a bioavailability of over 90%. The half-life of elimination is approximately 24 hours. LNG like other progestogens not only binds with progesterone receptors but also binds to other steroid receptors. In conjunction with estradiol, LNG causes anovulation dependent on the follicular stage and acts as an effective contraceptive agent

through several possible mechanisms including endometrium lining thinning, inhibition of the corpus luteum, thickening of the cervical mucus and retardation of sperm motility. LNG is well tolerated and is considered to be safe. No links to pregnancy complications or congenital disorders have been found. Lastly, the safety of breastfeeding is well established with no deleterious effect on the nursing infant.

Chapter 5 - Childhood obesity has been on the increase over the last several years, and has been described as reaching epidemic proportions in the developed world. Current estimates are as high as 30% of children in the United Kingdom are overweight or obese. Obesity in childhood has brought with it the rise in previously rare childhood complications of type 2 diabetes, non-alcoholic fatty liver disease and significant mental health issues relating to obesity. Many public health and health-economic authorities have focused on the significant health and financial burden that could be posed by the possibility of entire generations of children growing up to be overweight or obese adults. The challenge faced by the global community is in managing these children and adolescents with obesity. Drug therapy is limited, and weight-loss outcomes from community based programmes have been limited. More focus has recently been placed on surgical interventions – but these have proved controversial in many sectors of the society.

Chapter 6 - The posterodorsal medial amygdala (MePD) is a sexually dimorphic area that contains one of the highest concentration of gonadal hormone receptors in the rat brain. It modulates the timely display of reproductive behavior in both male and female rats. Dynamic changes affect the local synaptic processing along the estrous cycle. These changes are evident in the number of synapses made directly on dendritic shafts or the complex modulation of the density and the shape of dendritic and somatic spines during the short-term proestrus phase, when the levels of estradiol and progesterone reach a peak in circulation. That is, the MePD of females in proestrus has an increase in the number and diversity of somatic spines. In contrast, there is a marked reduction in the density of dendritic spines during this same phase. Spines are specialized postsynaptic neuronal elements that modulate mainly the excitatory neurotransmission. These findings indicate that the female rat MePD modulates the synaptic input in a complex and dynamic way prior to influencing other interconnected brain areas relevant for the neuroendocrine secretion needed for the ovulation and the display of proceptive behavior. Furthermore, substitutive hormonal therapy to adult ovariectomized females do not resemble the physiological variations in the normally cycling rat. This synaptic organization changes once more when

females have the experience of motherhood. In mothers, the reduced density of dendritic spines is likely involved with less avoidance behavior toward pups and for other adaptive skills developed for the new demands from the environment, for nursing behavior and memory elaboration about this life event. These data provide additional insights about the important role of the rat MePD as a model for the study of the link between sex steroids, the cellular specializations for synaptic processing, and the functional organization of the nervous tissue in females.

Chapter 7 - In the present work, the bioassay-guided fractionation strategy associated with ethnopharmacological information has been used. Thus, various plants belonging to the *Scabiosa* genus (Dipsacaceae) are used to treat skin diseases. In this study, the cytotoxic effect and the antiviral activity of *Scabiosa arenaria* Forssk. extracts were investigated against coxsackievirus B_3 (CVB3) in Vero cells. The cell viability was carried out using 3-(4, 5-dimetylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) assay. The best antiviral activity was shown with the roots ethyl acetate fraction (EtOAc) (IC_{50} = 722.22 ± 0.15 µg/mL and Selectivity index (SI) = 5.53). Its further fractionation yielded 4 subfractions. The most potent anti-coxsackievirus B_3 activity was obtained for SR1 and SR4 subfractions with SI of 6.45 and 5.73, respectively. Chemical analysis of these subfractions revealed the presence of flavonoids and phenolic acid, which could be attributed to this biological activity. The good anti-coxsackievirus B_3 of ethyl acetate roots of *S.arenaria* support the traditional use of many species of *Scabiosa* genus in the treatment of skin diseases of viral origin.

Chapter 8 - Congenital upper limb anomalies (CULA) are a relatively common finding at birth. Recent advancements in developmental biology and clinical genetics have provided new insights into the mechanisms of limb formation and malformation. Depending on the aberration, it may be isolated or affect disparate groups of cells thereby affecting other organ systems. The etiologies of CULA are diverse and disorder-specific, but potential contributors include genetic, mechanical, and a variety of environmental factors. Careful examination, both prenatally and postnatally, is key to early identification and management. Progress has been made recently to improve the classification/diagnosis of CULA using language that is inclusive of both basic and clinical sciences. In this review, the authors incorporate a similar approach in providing a comprehensive overview of CULA combining information regarding the molecular landscape of limb development with the growing database of clinical genetics. The authors will also highlight recent

advances in risk-factor association, diagnosis and management of congenital upper limb anomalies.

Chapter 9 - Exfoliation syndrome (XFS) is a genetically determined systemic condition characterized by the production and accumulation of an abnormal protein, the exfoliative material. Though XFS is a systemic elastosis, the most significant clinical consequences occur in the eye (nuclear cataract formation, Zinn's zonule fragility, increased risk for cataract surgery complications and exfoliative glaucoma [XFG]). XFG is the most common type of secondary open-angle glaucoma, and is characterized by elevated intraocular pressure with high fluctuation, and rapid progression. XFG is typically a disease of the elderly. In many cases XFG starts as a clinically unilateral disease in eyes with XFS. This chapter summarizes the current knowledge on genetics, environmental factors, clinical characteristics, medical, laser and surgical treatment options. It also addresses the significance of systemic vascular diseases associated with XFS and XFG, and the need for consultation between the ophthalmologist and cardiologist or general practitioner during long-term management of the disease.

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Chapter 1

ANTITUMOR EFFECTS OF LITHIUM CARBONATE

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ABSTRACT

The present chapter is focused to analyze the underlying mechanisms that dictate the antitumor properties of different forms of lithium carbonate. The results of clinical studies on the use of lithium carbonate for cancer treatment are given. Debate exists in literature about contradictory effects of lithium salts are discussed as well.

INTRODUCTION

Lithium carbonate has been used clinically since the 19th century to treat psychotic diseases as bipolar disorders, and its safety profile is well documented. For adult patients, lithium carbonate doses of 900 to 1,200 mg/day result in steady-state lithium levels in the range of 0.5 mM to 1.2 mM for effective maintenance of bipolar patients [1]. Lithium carbonate, used orally, accumulates in the colon and does not cause an additional risk for cancer. The study with using all patients diagnosed with incident colorectal

adenocarcinoma during 2000–2012 (n = 36248) in Danish Cancer Registry wasn't found association between long-term (≥ 5 years) lithium use and colorectal cancer risk [2]. Treatment with lithium carbonate is not associated with increased rates of upper urinary tract tumors [3]. This conclusion is made on results from a nationwide population-based study. On the other hand, lithium compounds are regarded as potential agents of target therapy, capable of slowing tumor growth, by inhibiting the enzyme glycogen synthase kinase 3β (GSK-3β) that possesses anti-carcinogenic effects [4]. The tumor cells are heterogeneous morphologically, the degree of differentiation and the ability to proliferate. In addition, there are multidrug resistant tumor cells, which significantly reduce the efficiency of traditional therapy and increase the risk of recurrence and mortality of patients. In this regard, explores new therapeutic approaches that can resolve this problem.

PRECLINICAL DATA ON THE ANTITUMOR EFFECT OF LITHIUM CARBONATE

Currently being studied antitumor influence of officinal forms and nanosized particles of lithium carbonate. It is known that nanosized particles have a high cytotoxicity [5]. Lithium carbonate nanosized particles are available for cells since they have a highly active surface and high sorption capacity. In addition, lithium carbonate nanosized particles able by slow dissolution in the tumor microenvironment to inhibit its growth. Lithium ions can pass through the cell membrane using a selective voltage-dependent sodium channel [6].

It has been shown *in vitro* that lithium carbonate nanosized particles have an inhibitory effect on proliferation of hepatoma-29 cells in lower concentrations compared to officinal salt form. The basis of the antiproliferative effect of lithium salts is the activation of tumor cell apoptosis and delay in phase G_2/M (phase to mitosis preparation) cell cycle [7]. It was previously shown that lithium initiates apoptosis of tumor cells HL60 indirectly via the activation of expression p53 (a transcription factor, which is involved in cell cycle regulation and acts as a suppressor of malignant tumors), Rb (retinoblastoma protein – tumor suppressor protein), Bax (promoter of apoptosis) and inhibition of expression Bcl-2 (intracellular protein factor that inhibits apoptosis) and activation of inflammatory cytokine production IL-2, IL-10 (IL - interleukins) [8]. Anti-apoptotic signaling is regulated by GSK-3

beta which selectively is inhibited by lithium [9]. Lithium increases the phosphorylation of extracellular signal-regulated kinase (ERK), leading to the direct regulation of p53 levels and subsequent G_2/M arrest of glioblastoma cell cycle [10]. Lithium also induces p38 MAPK (mitogen-activated protein kinase p38) phosphorylation, hence, suppresses p53 and cell cycle arrest in G2/M phase.

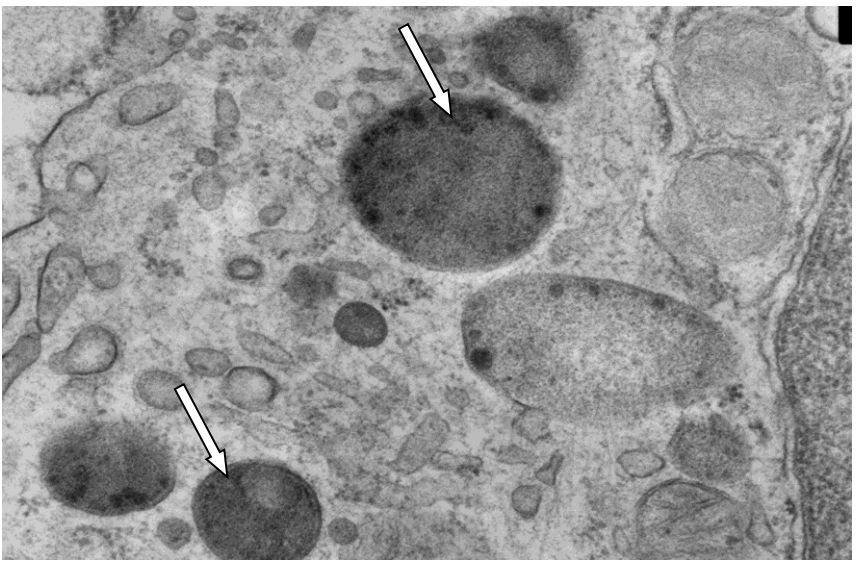

Figure. The ultrastructure of tumor associated macrophage. Macrophage phagosomes loaded lithium carbonate nanosized particles. Their average size of 14 ± 3,12 nm determined using the computer program Image Tool [15].

The effects of nanosized lithium carbonate particles on muscle tissue and development of experimental hepatocarcinoma 29 transplanted into thigh muscle were studied in CBA mice [11]. The injections of lithium carbonate nanosized particles at the periphery of cancer locus caused the injury within the tumor, which was evaluated by electron and light microscopy and biochemical methods. The number of neutrophils and macrophages in the tumor increased, whereas the density of blood vessels and hemoglobin concentration were reduced. Treatment of lithium carbonate nanosized particles protects vital organs including the heart and lungs from the damaging effect of lipid peroxidation secondary products.

Antitumor effect of lithium carbonate nanosized particles may be associated with activation of the NO-synthase way in the tumor. It is known

that arginine promotes tumor growth and enzymatic degradation of this amino acid mediated arginase inhibits proliferation and death of tumor cells [12]. Arginase and NO-synthase pathways compete with each other for arginine. Activation of NO-synthase pathway may contribute to the elimination of tumor cancer cells. It has been established that the combined use of 5-fluorouracil and L-arginine in nude mice bearing hepatocarcinoma leads to rise of expression of inducible NO-synthase, NO (nitrogen oxide) levels in the tumor, thus increasing gene expression of proteins that induce tumor cell apoptosis [13]. Application of NO-synthase inhibitor L-NAME (methyl ester of N-ω-nitro-L-arginine) reduces the effect of anticancer therapy [14]. Effect of lithium carbonate nanosized particles injected after transplantation hepatocarcinoma 29 cells in the thigh muscle, was aimed at increasing the NO levels in tumors and peritoneal macrophages, which could lead to lower the arginine levels required for tumor growth [15].

Treatment of hepatocarcinoma mice with nanosized particles of lithium carbonate maintains of balance between oxidants and antioxidants and may help limit the progression of precancerous condition toward malignancy and tumor growth [16]. Tumor growth after hepatocarcinoma-29 cells injection into muscle right leg changes the levels of lipid peroxidation activity in two-phase manner. The level of 2-thiobarbituric acid-active products is decreased in comparison with the control indicates after invasion of tumor cells, it is raised at excessive tumor growth and diminish at terminal stage. Catalase activity is elevated significantly, but superoxiddismutase activity is reduced in tumor at hepatocarcinoma growth. The repeated injections of lithium carbonate nanosized particles at hepatocarcinoma inhibit lipid peroxidation in tumor tissue, but don’t influence on catalase and superoxiddismutase activities.

Treatment with lithium carbonate officinal forms of two kinds of tumor-bearing mice (hepatoma H22 and sarcoma S180) for 17 or 10 days (advanced and simultaneous administration) significantly inhibit the growth of the two kinds of tumor, and increase the activity of superoxide dismutase and decrease the contents of malonyldialdehyde [17].

Many epithelial cancers, particularly gastrointestinal tract cancers, remain poor prognosis diseases, due to resistance to cytotoxic therapy. An official form of lithium carbonate was shown to induce autophagosomes in esophageal and colorectal cancer cells by western blot analysis of LC3 isoforms, morphology and FACS (fluorescence-activated cell sorting) quantitation of Cyto-ID or mCherry-GFP-LC3 [18]. When combined with the chemotherapeutic agent 5-fluorouracil or oxaliplatin, lithium carbonate

(30mM) showed strong enhancement of non-apoptotic cell death. When either chemotherapeutic agent was combined with lithium carbonate a significant reduction in tumor volume was achieved. In addition, survival was dramatically increased in the combination group ($p < 0.0001$), with >50% of animals achieving long term cure without re-occurrence (> 1 year tumor free).

Metastasis is the main cause of mortality in cancer patients. Angiogenesis and lymphangiogenesis are crucial for cancer progression; in particular, lymphangiogenesis is pivotal for metastasis in cancer. The experimental investigation has demonstrated a novel role of lithium carbonate in the inhibition of colon cancer metastasis by blocking transforming growth factor-β-induced protein (TGFBIp) expression, and thereby TGFBIp-induced lymphangiogenesis, in primary tumors. Lithium reduces the expression of TGFBIp in colon cancer cells by inhibiting Smad3 phosphorylation via GSK3β inactivation [19]. Moreover, lithium carbonate inhibits lymphatic endothelial cell migration, which is increased upon TGFBIp expression in tumor cells. In tumor xenografts model, lithium was found to prevent metastasis to the lungs, liver, and lymph nodes by inhibiting TGFBIp-induced tumor lymphangiogenesis.

RESULTS OF CLINICAL STUDIES ON THE USE OF LITHIUM CARBONATE FOR CANCER TREATMENT

1. An Enhancer Traditional Anticancer Therapy

The addition of lithium carbonate to treatment with 30 mCi ^{131}I in thyroidectomized patients with low-risk differentiated thyroid carcinoma improved the efficacy of thyroid remnant ablation and therefore might be a better alternative than using higher doses of ^{131}I for remnant ablation in these patients [20]. This was a randomized study with endpoint at one year. Sixty one consecutive patients with follicular thyroid carcinoma or papillary thyroid carcinoma were randomized into two groups: group A (n = 32) treated with 30 mCi ^{131}I; group B (n = 29) treated with 30 mCi ^{131}I plus an oral dose of lithium 900 mg/day, for 7 days.

It should be noted that the addition of lithium carbonate (1200mg/day) to treatment with radioactive iodine did not have any beneficial effects on the clinical course in 12 patients with metastatic differentiated thyroid carcinoma [21]. Lithium carbonate enhanced the absorption of radioactive iodine thyroid

tumors, but had no significant effect on the clinical course of the disease, as measured radiographically, and the level of thyroglobulin in serum.

No significant down-trend has been observed in the annual mortality rate for well-differentiated, non-medullary thyroid cancer, a fact reflecting the existence of a "core" population of patients with radioiodine-"resistant" disease [22]. The molecular basis for this phenomenon is believed to be the progressive tumoral de-differentiation over time, with loss of (or marked decrease in) the expression of cellular components responsible for iodine uptake, organification and retention. Adjuvant methods to radioiodine, such as radiosensitizers and lithium carbonate, provide only marginal additional therapeutic effect.

In some cases, the use of lithium carbonate as an additional component of anticancer therapy has a positive effect. The patient 33 years old with ovarian cancer, BRAF-positive follicular variant of papillary thyroid cancer, and abdominal metastases received surgical treatment and radiological therapy [23]. Before re-treatment with radioactive iodine patient received 10 days of 300 mg of lithium 3 times a day. The concentration of lithium in blood serum was 0.9 mEq /l (rate of 0.6-1.2). This treatment resulted in the disappearance of metastases in the abdominal cavity and the absence of symptoms after 6 months of treatment, as well as reduced levels of serum thyroglobulin with 264 ng /l to 18 ng /l.

2. Drug Promotes Restoration of Bone Marrow and Blood Composition after Chemotherapy

a) Normalizes Number of Neutrophils in Blood

Neoplasm therapy is restricted by the hematological side effects of tumor-destructive therapy, requiring expensive supportive care to some extent to overcome and treat leucopenia and its consequences. An effective and very cost-effective alternative for treating neutropenia is to administer lithium carbonate [24]. Lithium leads to a release of hematopoietic growth factors (CSF) and therefore to proliferation of neutrophil granulocytes. Normally, recombinant CSF is only administered when there are indications of severe neutropenia because of the high costs involved, all the more evident in the long-term treatment of persistent leucocytopenia. On the other hand, CSF and leucocytes play an essential role in tumor immunology and with regard to response rates to cytostatic drugs. Lithium salts have shown that they can increase the number of neutrophil granulocytes quite significantly and, to a

lesser extent, the number of eosinophil granulocytes and lymphocytes as well. The average number of erythrocytes does not change significantly.

b) Normalizes Blood Platelet Count

Thrombocytopenia is a major clinical problem caused by chemotherapy and radiotherapy. An effective and very cost-effective option for treating moderate neutropenia is the administration of lithium carbonate [25]. Lithium induces the release of colony-stimulating factors and therefore stimulates proliferation of neutrophil granulocytes. Other cytokines, such as IL-1, IL-6, and tumor-necrosis factor-alpha (TNF-alpha), are also stimulated. Apart from granulocyte-macrophage-CSF (GM-CSF), there are as yet no reports of lithium salts inducing early activating factors for the megakaryocytic lineage, such as IL-3, IL-11, stem cell factor and flt-3 ligand, or maturation factors, such as thrombopoietin. A statistically significant increase in the mean number of platelets for patients with cell counts below 150,000/microL on the commencement of treatment with lithium carbonate could be observed. Patient tolerability of lithium carbonate therapy is very good. Patients with persistent leucopenia and thrombocytopenia following chemotherapy or radiotherapy can be treated with this trace element very cost-effectively.

c) Increases the Content of $CD34^+$ Cells in the Blood

The concomitant administration of lithium carbonate and G-CSF in 33-year-old woman with acute myeloid leukemia following chemotherapy increases the number of circulating hematopoietic progenitor $CD34^+$ cells in peripheral blood [26]. At the time of mobilization, the dose of lithium carbonate was 1200 mg/day and the serum lithium concentration was 0.86 mmol/l. In this case, both percentage and the number of $CD34^+$cells obtained by a single apheresis were higher analogical indicators in patients without lithium carbonate treatment after 2-6 apheresis procedures. This case is consistent with clinical experience Ballin et al. [27] on the mobilization of $CD34^+$ cells successfully in patients with both hematologic malignancies occurring and bipolar disorders, in treatment of lithium carbonate.

d) Increases Cytokine Production

Lithium salt compounds influence hematopoiesis, which is known to be regulated by a number of cytokines, including TNF-alpha, IL-1 and IL-6. Lithium carbonate treatment of neutropenic patients with breast cancer results in increased cytokine production [28]. Results indicate that this therapy produced TNF-alpha and IL-6, but not IL-1 alpha elevations in patients

affected by unmetastasized breast cancer. Conversely, TNF-alpha, but not IL-6, elevations were detected in metastatic patients.

3. Neuroprotective Agent in Cancer Patients in Order to Improve Quality of Life by Maintaining the Cognitive Abilities, Improve Emotional State

Cognitive changes are documented consequence of cancer therapies, including chemotherapy and radiotherapy [29]. Indeed, the ability to inhibit cell division, the key element in cancer therapies, causes reduction in neurogenesis, which is implicated in mood and cognitive disorders. Lithium carbonate is a mood stabilizer with known neuroprotective activity, a characteristic that is thought to underpin its therapeutic efficacy [30]. Drug-induced mental disturbances are frequent in bone marrow transplantation patients who require numerous kinds of drugs [31]. Lithium carbonate was effective for the treatment of depressive disorder in a patient with myeloid leukemia without causing side effects such as renal dysfunction.

COMPLICATIONS AT CLINICAL APPLICATION OF PREPARATIONS OF LITHIUM

Treatment with lithium salts may be associated with the risk of development of numerous adverse effects. Lithium drugs often implicated in hair loss patients. It has been reported that 15 per cent of patients taking lithium developed hair thinning [32].

Long-term treatment with lithium carbonate (8-40 years) may suppress the activation of regenerative processes by reducing the number of very-small embryonic-like stem cells circulating peripheral blood [33].

Glomerular renal dysfunction occurs after an average of 20 years of continuous lithium treatment, and the severity is related to the total lithium load as measured by dose and duration. Recently, several reports have highlighted the relationship between renal microcyst formation and significant reductions in glomerular filtration rate. The proposed mechanism of microcyst formation is related to the antiapoptotic effect of lithium [34].

Lithium carbonate induced endocrine complications include: thyroid dysfunction, nephrogenic diabetes insipidus and hyperparathyroidism. It is described patient who had undergone removal of a parathyroid adenoma and

later developed lithium-induced hyperparathyroidism, but cessation of lithium treatment normalized parathyroid function. The pathogenic mechanism for parathyroid dysfunction in lithium-treated patients is still unclear [35].

CONCLUSION

Development of novel and efficient treatment strategies for epithelial cancers, particularly gastrointestinal tract cancers and hepatocarcinoma has vital importance considering their treatment refractoriness and very high mortality. The understanding from different forms of lithium carbonate interactions with the tumor cells, tumor environment cells and cellular targets that have been identified may provide leads for the rise of effectiveness antitumor therapy.

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Chapter 2

EPIGENETIC MODIFICATIONS IN TUMOR SUPPRESSOR GENES (TSG): ITS CONTRIBUTION TO TUMOR DEVELOPMENT AND ITS CLINICAL RELEVANCE

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ABSTRACT

Tumor development is the result of genetic and epigenetic alterations. The epigenetic mechanisms do not imply a change in the DNA sequence but can be as important as the genetic mechanisms for the

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onset and progression of cancer due to its important role in the regulation of gene expression. Methylation and acetylation of histones induce changes in the structure of chromatin that can repress or facilitate the expression of genes. Another epigenetic modification studied in recent years is the methylation of DNA. Recently it has been observed that there are abnormal patterns of DNA methylation in many types of cancers. The malignant cells are accompanied by a global hypomethylation that is associated with the activation of genes required for the invasion and metastasis and a local hypermethylation (CpG Islands) associated with the repression of tumor suppressor genes (TSGs). Thus, the methylation of the TSG blocks its expression, contributing to the onset and tumor progression. Classic examples are the genes BRCA1, PRC, TP53 and PRDM in breast, colon, skin and lung cancer, respectively.

The abnormal patterns of DNA methylation, as well as epigenetic modifications on histones, could be used as biomarkers for the diagnosis of cancer and as targets of drugs, in order to correct these alterations and restore the function of the genes. This chapter analyses the current evidence on the role of epigenetic mechanisms involved in the progression of various types of cancer and their clinical relevance.

Keywords: Epigenetic, DNA-methyltransferases, histone deacetylases, tumor suppressor genes, tumor progression

INTRODUCTION

Epigenetic and genetic alterations are two independent mechanisms that have an important role in the modification of gene expression that are involved in the onset and progression of cancer. Epigenetic changes can be as important as the genetic mechanisms in the regulation of gene expression.

Cell proliferation is a process strongly regulated by various enzymes such as cyclins dependent-kinases (CdKs) and proteins encoded by tumor suppressor genes (TSG). In normal cells, the variations in the levels of expression of CdKs regulate entry to the different phases of the cell cycle while the proteins encoded by the TSG stop the cell cycle progression in response to DNA damage or signals of the extracellular medium.

The loss of TSG function by deletions, mutations or epigenetic modifications prevents the cell response to cell cycle checkpoints or cell death by apoptosis if the DNA damage is high. This leads to an increase in mutations and uncontrolled cell division.

Epigenetic mechanisms are reversible changes in DNA that modulate the expression of various genes, without altering the DNA sequence. They occur during development, differentiation, tumorigenesis, and aging as well as in many other biological processes. They include changes in DNA and its associated proteins such as the histones [1].

The amino-terminal groups of histones have residues of amino acids that can be modified by methylation, acetylation or phosphorylation inducing changes in the structure of the nucleosomes. These changes regulate the binding and activities of the transcription factors and other proteins that interact with the promoter regions of specific genes, regulating the expression of these genes. In this regard, numerous studies show a correlation between increased acetylation of histones and increased transcriptional activity while the deacetylation and methylation of histones is correlated with gene silencing [2].

Despite this generality, some exceptions have been described such as methylation of lysine 4 of histone H3 in active transcription regions that promotes its acetylation [2]. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are enzymes that mediate the acetylation and deacetilacion process of histones, respectively.

In mammals, DNA methylation is an epigenetic mechanism that involves the enzymatic addition of a methyl group to the carbon 5 of cytosine in DNA. These epigenetic modifications occur in the dinucleotides CpG distributed in the human genome with a rate five times higher in regions called "CpG islands". In general, CpG islands are located in the promoter regions of genes and the methylation process regulates the transcription of these genes. DNA methylation inhibits gene expression through the recruitment of repressive proteins such as Methyl-CpG-binding proteins (MeCP) and proteins with a domain of binding to methyl groups (MBD). Hypermethylation of promoter regions is associated with transcriptional repression while hypomethylation is generally correlated with gene activity.

DNA-methyltransferases (DNMTs) are the enzymes that carry out DNA methylation. At least three functional DNMTs have been identified in eukaryotic cells. DNMT1 preferentially methylates hemimethylated DNA and participates in the maintenance of methylation patterns with each cell division [3]. DNMT3a and DNMT3b contribute to *de novo* methylation during embryogenesis [4]. DNMT2 has no relevance in the DNA methylation activity.

In normal cells, DNA methylation maintains gene silencing and participates in development, genomic imprinting and X chromosome

inactivation. Conversely, aberrant methylation patterns participate in the promotion of some diseases, including cancer.

In cancer cells, epigenetic alterations that have been found most often involve hypomethylation of DNA sequences normally methylated and the aberrant hypermethylation of the usually non-methylated sequences. Hypomethylation and hypermethylation occur at specific sites in the genome, but these are different depending on the type of tumor cell. While hypomethylation of usually methylated regions is associated with the activation of genes required for the invasion and metastasis, the hypermethylation in promotor regions of genes suchs as cell cycle regulatory proteins and TSG is correlated with transcriptional inactivation and carcinogenesis in many types of cancers. In this regard, regulators of the cell cycle, such as p16, p21, p27 and p53, have been found silenced by methylation mechanisms in many cancers. Aberrant hypermethylation inactivate genes related to the control of the cell cycle, apoptosis and DNA repair. Epigenetic silencing of TSG by promoter hypermethylation is associated with increased tumor severity and poor survival [5].

In general it has been observed a low degree of DNA methylation in human tumors, favoring the transcription of genes that normally are not expressed.

In opposition, an aberrant hypermethylation that inhibits the expression of TSG, such as BRCA1, PRC, TP53 and PRDM has been observed in breast, colon, skin and lung cancer, respectively. This knowledge has various applications, changes in the patterns of methylation or modifications in histones can be used as biomarkers for the diagnosis of several types of cancer. So, for example, the RARβ gene methylation is useful in the detection of prostate cancer and the PRDM gene methylation is used as a predictor of the response to chemotherapy drugs.

The miRNA are small non-coding RNA molecules that interact with its mRNA target, inhibiting its transcription. Evidence shows that the hypermethylation of the miRNA can downregulate its function and contribute to the generation of cancer. This is another important epigenetic mechanism that is deregulated in cancer.

The potentially reversible state of the epigenetic alterations generates targets for therapeutic strategies to restore the normal epigenetic patterns. Also, enzymes that mediate the epigenetic processes such as the histone acetyltransferases (HATs), histone deacetylases (HDACs) and DNA methyltransferases (DNMTs) are being analized as potential targets of anticancer drugs.

In this chapter, we analyze the current evidence on the role of epigenetic mechanisms involved in the progression of various types of cancer and their clinical relevance.

EPIGENETIC ALTERATIONS IN DIFFERENT TYPES OF CANCER ASSOCIATED TO TSG

The BRCA1 (breast cancer 1) gene is a tumor suppressor gene that encodes a protein involved in the cell cycle regulation and DNA damage repair. Its action prevents the cell uncontrolled proliferation. Several evidence show, that different mutations of this gene are involved in some types of cancer, especially breast cancer. Also, it has been observed that hypermethylation at the BRCA1 gene promoter inhibits the transcription and promotes the genesis of cancer [6]. Evidence of hypermethylation in other TGS associated with breast cancer has been found. In this sense, it has been observed the silencing of RASSF1A (Ras association domain family 1A), SLIT2 (Slit Guidance Ligand 2) and WIF1 (WNT Inhibitory Factor 1) by promoter hypermethylation in hereditary breast cancer [7]. The silencing of the RASSF1A by promoter methylation is also involved in kidney tumorigenesis [8]. In accordance with these evidences, Papadopoulou et.al [9], showed the RASSF1A promoter hypermethylation in plasma samples obtained from patiens with breast cancer. This study shows that free-circulating DNA can be detected in cancer patients and suggests a noninvasive approach for early detection of cancer.

The loss of expression of RASSF through promoter methylation is associated with many types of cancers such as leukemia, melanoma, breast, prostate, neck, lung, brain, colorectal and kidney cancers [5].

The inactivation of TP53 and retinoblastoma TSG by promoter methylation has been demonstrated in Squamous Cell Carcinomas (SCC), the most aggressive type of skin cancer [10].

PRDM (PRDI-BF1 and RIZ) family proteins are factors of transcription that act as suppressors of tumors in humans and have an important role in cell differentiation and malignant transformation. It has been shown the inactivation of PRDM by promoter hypermethylation in squamous cell carcinoma of lung and adenocarcinomas [11]. Also, it has been demonstrated the involvement of PRDM11 in B-cell lymphomagenesis. Fog et. al. [12] showed in animal models that PRDM11 inactivation by hypermethylation

accelerates the MYC oncogene-mediated lymphomagenesis. Moreover, patients with diffuse large B-cell lymphomas (DLBCLs) and PRDM11 deficients have a lower survival. Also, PRDM11 is a novel tumor suppressor that associates with transcriptional start sites of oncogenes such as FOS and JUN, downregulating their expression.

Colorectal cancer (CRC) remains to be one of the most common causes of cancer-related death [13]. Aberrant methylation of DNA is an alteration epigenomic prevalent in the CRC, in combination with the BRAF mutation [13]. PRC proteins mediate trimethylation of the lysines 9 and 27 of histone H3 (H3K9me3 y H3K27me3) silencing a TSG specific group in human cancers. It has been found in the CRC that folate deficiency induces the hypermethylation of specific genes and global hypomethylation of genome [14].

Prostata cancer (CaP) is one of the most common human cancers generated by genetic and epigenetic mechanisms. Retinoic Acid Receptor β (RARβ) is a TSG frequently silenced in CaP by promoter methylation. Also, DNA methylation levels are positively correlated with trimethylation of the lysine 27 of histone H3 mediated by PRC proteins [15]. In addition, deregulation of miRNA expression can have a critical role in prostate carcinogenesis. It has been shown in prostate cancer that miRNA-205 transcription is commonly repressed by hypermethylation. The downregulation of the expression of miRNA 205 increases the MED1 expression contributing to tumor progression. So, miRNA-205 is an epigenetically regulated tumor suppressor that could be a potential biomarker in prostate cancer [16].

EPIGENETIC THERAPY

Several epigenetic alterations can produce the silencing of TSG or inhibit the function of the miRNAs. These epigenetic changes can be reversed by treatment with DNMT and HDAC inhibitors. So, DNMT or HDAC inhibitors can produce the hypomethylation at the promoters of genes, inducing the expression of TSG, cell differentiation or death by apoptosis.

Many drugs commonly used for the treatment of cancer have high toxicity, undesirable side effects and often generate resistance, an example of this is the use of doxorubicin or daunorubicin for the treatment of breast cancer. Also, there is evidence that epigenetic alterations might be responsible

for resistance to drugs in tumor cells. Then, the alternate use of conventional anticancer drugs with epigenetic drugs has been proposed.

The effectiveness of DNMT and HDAC inhibitors in the treatment of various human cancers is being analyzed in several clinical trials. So, it has been demonstrated the effectiveness of the 5-aza-2'-deoxycytidine (5-aza-2-dC; decitabina), a DNMT inhibitor that causes the DNA demethylation or hemi-demethylation, in the treatment of myelodysplastic syndromes and leukemias [17, 18]. Several preclinical and clinical studies have demonstrated the efficacy of epigenetic treatment for the lymphoma non-Hodgkin (NHL) and some specific inhibitors of DNMT (5-aza-cytidine and 5-aza-deoxicitidine) and HDAC (vorinostat and romidepsin) have been approved for clinical use. *In vitro*, it has been shown that 5-aza-2-dC reduces the methylation of PRDM2, PRDM5 PRDM16 genes in adenocarcinoma and lung squamous cell carcinoma, decreasing tumor proliferation [11]. Another DNMT1inhibitor, 3,6-dihydroxyflavone (DHF), inhibits carcinogens in human breast epithelial cells [19].

Several HDAC inhibitors have been developed up to the present. The inhibition of these enzymes leads to the acetylation of histones and transcription of genes, such as regulators of the cell cycle. The suberoylanilide *hydroxamic acid* also known as *Vorinostat*, is a HDAC inhibitor used for the treatment of patients with cutaneous T cell lymphoma that do not respond or are refractory to conventional chemotherapy [18]. *Vorinostat* is also being analyzed for the treatment of other types of cancer.

Given the close interrelationship between DNA methylation and histone modifications in TSG transcription inhibition, the combined use of HDAC and DNMT inhibitors that could have a synergistic effect in the treatment of cancer is being analyzed. So, it was demonstrated that the combination of hypomethylating agents and histone deacetylase inhibitors produce marked synergy in preclinical models of T-cell lymphoma [20].

CONCLUSIONS

Epigenetic modifications may have implications of clinical relevance, in the diagnosis, prevention, and treatment of cancer. The knowledge of the epigenetic patterns in different regions of the human genome will allow us to establish an association with the development of different tumors. Also, due to the reversible character of epigenetic changes, the design of therapeutic

strategies to correct the epigenetic alterations in the DNA and the histones, particularly those affecting the cell cycle regulatory genes seems to be one of the paths for the prognosis and treatment of cancer.

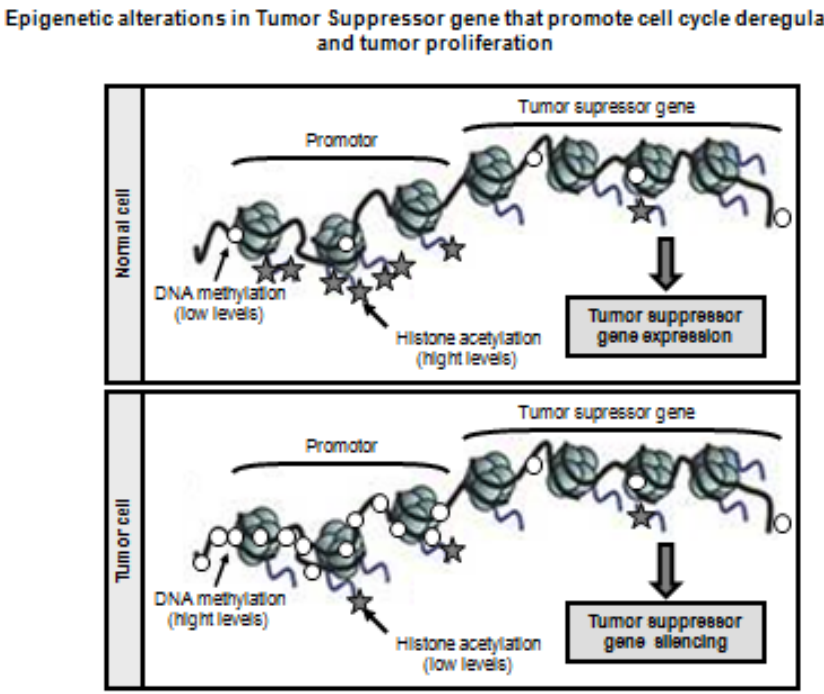

Figure 1. Epigenetic alterations such as the methylation of DNA or modifications in histones silence transcription of the GST and promote tumorigenesis.

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Chapter 3

PREGNANCY AND HEMODIALYSIS

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ABSTRACT

The outcomes of pregnancy in patients on dialysis have greatly improved in the last half-century. Therefore, the current medical opinion regarding pregnancy in women on hemodialysis has recently changed from 'impossible' to 'possible,' and pregnancy is no longer automatically discouraged, although it is still relatively uncommon and associated with a certain degree of risk. In order to decide whether to become pregnant or not, hemodialysis patients and their family members should be provided with sufficient information regarding the success rate, complications, need of intensive dialysis 6-7 days per week, optimal nutrition, and alternative options, including delaying pregnancy to after renal transplantation. In addition, to avoid delays in the detection of pregnancy, patient education and pregnancy planning is critical. Hence, close collaboration between patients, their family, nephrologists, dialysis staff, obstetricians, and neonatologists is necessary.

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INTRODUCTION

For almost 45 years, there have been case reports on successful outcomes of pregnancies in women on maintenance hemodialysis (HD) [1]. Initially, this success was considered to be exceptional, and given the high rate of poor fetal outcomes, medical opinion was to discourage pregnancy. However, the frequency of pregnancy in women on HD has increased worldwide. Accumulating reports concerning the challenged experiences of pregnancy in women on HD have provided some key information regarding ways to improve fetal outcome and reduce maternal complications. The most effective management contributing to successful outcomes has been proven to be increased dialysis dose [2]. Although still uncommon, and carrying a certain degree of risk to pregnancy, the current medical opinion regarding pregnancy in women on HD has recently changed from ‘impossible’ to ‘possible’ and pregnancy should not be automatically discouraged [3]. Therefore, it is imperative to provide sufficient informed consent to patients and their family members before pregnancy. Herein, we present a review, based on published reports, of the major key points regarding the history, change in outcomes, current management recommendations, and the necessary information that should be provided to women on HD, and their families.

PREGNANCY OUTCOMES ASSOCIATED WITH INTENSIVE DIALYSIS TREATMENT

The initial report of a successful pregnancy and delivery in a patient undergoing chronic HD was published in 1971 [1]. This case was considered to be an extremely exceptional occurrence. In 1980, the registration committee of the European Dialysis Transplant Association reported 16 successful pregnancies occurring in women on dialysis, all of whom had some residual renal function, as evidenced by a mean urine volume of 800 ml/day (range: 200-1600 ml/day) [4]. Four of the 16 babies born to these women on dialysis were conceived before dialysis was commenced; the periods of dialysis before delivery were 4, 10, 10, and 19 weeks. The remaining 12 pregnancies were completed after a mean of 2.2 years of dialysis. During the pregnancies, the mean dialysis time was 18 hours per week (range: 7 to 27 hours) and mean frequency of dialysis was 3 times per week (range: 2 to 6). In the total 16 newborns, the mean birth weight was 1.9 kg (range: 0.8-2.5 kg) and the mean

gestational age was 33.2 weeks (range: 25-42 weeks). Two patients suffered from severe anemia during pregnancy. At that time, anemia was not properly managed as erythropoietin analogues were not yet developed. Overall, these results indicated relatively favorable outcomes in some of the women; however, these patients all had residual renal function, a condition known to be associated with better fetal outcomes compared with those in patients with anuria and oliguria.

In 1994, Hou reported on 60 pregnancies from 1,281 women of childbearing age who were undergoing treatment in dialysis units in the United States [5]. This report included some pregnancies in patients on continuous ambulatory peritoneal dialysis. The study showed that 37% of pregnancies resulted in surviving infants, while 44%, 8%, 3%, and 5% resulted in spontaneous abortion, elective abortion, stillbirth, and neonatal death, respectively, while the outcome of pregnancy was not known in 3% of the pregnancies. This report also showed that only 21% of the pregnancies resulted in live infants prior to 1990, in contrast to 52% after 1990. During both time periods, spontaneous abortion was the major cause of pregnancy loss, accounting for 55% and 35% of terminated pregnancies before and after 1990, respectively. In 1998, Bagon et al. reported successful outcome in 50% of pregnancies occurring in HD patients [6] and Okundaye et al. reported an infant survival rate of 40.2% in 184 pregnancies in women who conceived after starting dialysis (HD or peritoneal dialysis) [7]. In line with these data, the outcome of successful pregnancy in women on HD between 1990 and 2002 was 30-60% [5-9]. However, while the outcome of pregnancy improved during this time period, the above studies found that most deliveries were premature, at around 29-32 weeks of gestational age, with an average newborn weight of $\leq$1600 g, and these results were not satisfactory to ensure healthy offspring. The neonatal outcomes are significantly worse before 32 weeks of gestational age; hence, reaching a delivery at over 32 gestational weeks is important [10].

An effective approach to address these problems involves an increase in dialysis dose in terms of the frequency per week and duration per session [11]. With the growing popularity of intensive dialysis treatment, the probability of a healthy offspring was reported in 2010 to range from 50 to 100% as determined by evaluating 90 pregnancies observed in 78 patients [3]. Moreover, in 2008, Barua et al. [12] reported extremely favorable outcomes of pregnancies in women on HD with super-high dose dialysis at a mean of 48 ± 5 hours per week during pregnancy, which resulted in a mean pre-dialysis blood urea level within the normal range. The mean gestational age of the

cohort was 36.2 ± 3 weeks and the mean birth weight was 2417.5 ± 657g. Hladunewich et al. reported, in a Canadian and American cohort in 2014, that there was a significant dose-response relationship between the HD intensity and live birth rate, which improved from 48% in women receiving <20 hours HD, to 75% in women receiving between 21 and 36 hours, and to 85% in women receiving ≥37 hours of HD each week [13].

Recently, in 2015, an Italian group described that women on dialysis should be advised that there is at least a 75% chance of success by intensive dialysis treatment and that they should be kept under a strict clinical course; the authors strongly recommended that a dialysis schedule of 6-7 days (with an ideal target of at least 36 hours) per week should be offered during pregnancy to, at least, patients without residual renal clearance [14, 15]. The dialysis schedule should be adjusted according to the renal function and uremic waste product levels in the blood. However, intensive dialysis still faces some issues. First, a nocturnal dialysis program (at home or in a center) cannot be offered everywhere due to the required facilities and staff not being readily available, and intensive dialysis is not always covered by health insurance. Second, overworking the mother may result in reduced motivation during the progression of the pregnancy.

UREA TOXINS AND PREGNANCY

The blood in patients with end-stage renal disease (ESRD) contains many accumulating uremic waste products. Among the different dialysis modalities, hemodiafiltration is superior to HD for the removal of middle-molecule substances. Adequate verification of the status of all of uremic waste products in terms of transplacental transfer and potential harmful effects on the fetus has not been performed, and future studies are thus required. Accordingly, there are currently limited available data regarding the most appropriate dialysis modality, HD or hemodiafiltration, during pregnancy. However, at the present time, HD is the standard extracorporeal treatment in pregnancy, suggesting at least equal benefits of the two modalities [14]. Our group has previously shown that the maternal urea nitrogen (UN) levels were significantly and negatively associated with gestational age and birth weight [16] and, in line with these data, another study revealed that a high level of maternal serum UN was associated with poor fetal outcomes [17]. Therefore, among the various uremic toxins, the maternal serum UN level is currently used as an important marker associated with gestational age, birth weight and

fetal outcomes, and the intensive dialysis dose must be adjusted according to the maternal serum UN levels.

COMPLICATIONS

Here, major complications are described based on the previously published reports on the topic. Overall, maternal hypertension is the most commonly reported complication in patients on dialysis, occurring in 40-80% of cases [7, 9, 11]. Preeclampsia is a severe condition associated with severe hypertension. A report of available data from 67 patients on HD or peritoneal dialysis in 1998 showed that 79% (n = 53) of women had some degree of hypertension, while 48% (n = 32) had blood pressure higher than 170/110 mmHg, and 7% (n = 5) of patients required admission to the intensive care unit for hypertensive crisis [7]. The rates of preeclampsia were 29% [18] and 67% [19] in studies reported in 2004 and 2005, respectively. In addition, a study conducted from 1966 to 2008 in Australia and New Zealand showed that, out of a total of 49 pregnancies and 30 live births, 19.4% of women developed preeclampsia [20]. Intensive dialysis treatment is likely to result in a lower risk of maternal hypertension, because a daily HD schedule helps avoid fluid overload between dialysis sessions, leading to better control of hypertension. The results of patients treated with intensive dialysis at least 6 times per week, with a mean dialysis time of 28.6 ± 6.3 hours per week, showed that the rate of severe hypertension was 40%, [11] and the mean blood pressure remained within the normal range, although 33% of pregnancies required antihypertensive medications [12].

Preterm delivery is another frequent complication. One previous report [14] described that every effort should be made to prolong pregnancy as much as possible in cases in which viability was possible but the risk of long-term problems was very high (“extremely preterm period;” 24-28 weeks) [21, 22], while delivery after the 34th completed gestational week is especially desirable, on account of the important reduction in major fetal risks after this term (the “late preterm period,” defined as 34-37 gestational weeks) [21-24]. As described above, the neonatal outcomes are significantly worse before 32 weeks of gestational age, and to reach a delivery at over 32 gestational weeks is hence important [10]. In the 1990s and early 2000s, most outcomes of women who went into labor at an average gestational age of less than 34 weeks were not satisfactory, even though live births were generally obtained [6-9, 11, 17-19, 25]. In the second half of the 2000s and beyond, along with

the popularization of intensive dialysis treatment, the proportion of pregnant women on HD with deliveries in the "late preterm period" increased, indicating that most infants were born healthy [12, 13].

Lastly, polyhydramnios is a common complication, with a rate of 30-70% in pregnant women on HD [9, 11, 16, 17, 19]. On the other hand, the risk for maternal death is considered very low [20], although a few cases were reported in 1998 [7]. In addition, the rate of malformations in infants born from patients on HD is considered similar to the risk in the overall population [3].

ANEMIA, NUTRITION AND MINERAL ADJUSTMENT

Treatment of anemia during pregnancy is critical. Previous studies, including a meta-analysis, have shown that the rate of preterm delivery increased in women with moderate to severe anemia [26, 27]. In patients on HD, our group also showed that the average hemoglobin level was significantly higher in women with successful pregnancies compared with that in women with unsuccessful pregnancies [16]. In line with these data, the third-trimester hematocrit has been observed to be positively related to birth weight and to be associated with a lower risk of adverse fetal outcomes [17]. Management of nutrition and mineral adjustment are also important. Nutrition management during pregnancy is important, because patients on HD are likely to have deficits of nutrients such as folate and iron [14]. Administration of vitamin D and calcium is recommended [14]. Furthermore, as intensive dialysis with regular dialysate is likely to cause hypokalemia, attention to serum potassium level is required.

INFORMED CONSENT AND COUNSELING BEFORE PREGNANCY

Prior to pregnancy, medical doctors and staff must provide certain information to women on HD and their family members. First, the chances of conception and pregnancy outcomes, including the rate of live births, long-term infant outcomes, and risk to both the mother and fetus, are known to be significantly better in patients after a successful renal transplantation compared with those in patients on HD. For example, one report stated that

women on HD were estimated to have a 10-fold lower probability of delivering a live-born baby compared to those who had undergone renal transplantation [28]. In addition, information regarding renal transplantation must also be provided to women on HD and their families, including its risks, locating a potential donor, the use of immunosuppressive medication, and the requirements in terms of the condition of the mother for the permission of pregnancy after transplantation, such as the levels of renal function and urine protein. This information may influence the decision regarding whether to postpone pregnancy until after a kidney transplantation or not.

Second, pregnancy planning and avoiding delays in the detection of pregnancy are critical because of the necessary changes in the medical care and treatment strategy due to pregnancy. In patients on HD, starting an intensive dialysis treatment as early as possible in pregnancy results in a better outcome.

However, early detection of pregnancy is likely difficult as patients with ESRD usually have abnormal hormone function of the ovarian axis, resulting in a tendency toward irregular menstrual cycles, anovulation, and decreases in fertility [29-34]. Moreover, a lack of a regular menstrual cycle makes it difficult to become aware of pregnancy through the cessation of menstrual periods, and pregnancy tests using serum levels of beta-human chorionic gonadotropin may not be accurate, as women with ESRD have consistently increased levels [35]. Therefore, to avoid delays in pregnancy detection in women on HD, the patient must proactively plan to see an obstetrician/gynecologist who can diagnose pregnancy using ultrasound.

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Chapter 4

LEVONORGESTREL, PHARMACOKINETICS, EFFICACY AND SAFETY

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ABSTRACT

Levonorgestrel (LNG) is a dextrorotatory isomer of the synthetic progestogen norgestrel. Norgestrel is a racemic mixture. The activity of norgestrel resides in the dextrorotatory isomer, while the levorotatory isomer is biologically inactive. Generally, LNG is rapidly absorbed with a half-life of absorption of less than 1hour. The mean time taken to achieve maximal serum concentration is less than 2 hours with a bioavailability of over 90%. The half-life of elimination is approximately 24 hours. LNG like other progestogens not only binds with progesterone receptors but also binds to other steroid receptors. In conjunction with estradiol, LNG causes anovulation dependent on the follicular stage and acts as an effective contraceptive agent through several possible mechanisms including endometrium lining thinning, inhibition of the corpus luteum, thickening of the cervical mucus and retardation of sperm motility. LNG is well tolerated and is considered to be safe. No links to pregnancy complications or congenital disorders have been found. Lastly, the safety of breastfeeding is well established with no deleterious effect on the nursing infant.

INTRODUCTION

Progestogens

The progestins are derived synthetically from either testosterone or progesterone. The pharmacological properties of progestins depend on the original molecule from which they are derived. Very small changes in the structure of the original molecule may significantly modify the effects of progestins. Norgestrel was synthesized in the 1950s as a racemic mixture. Norgestrel was found to be a more potent progestogen with progestational and ovulation inhibitory activity that was higher than existing progestogens. Norgestrel has a dextrorotatory isomer and the levorotatory isomer. The dextrorotary isomer of norgestrel is active while the levorotary form is biologically inactive. The active dextrorotatory form of norgestrel was later named Levonorgestrel (LNG). The two isomers differ significantly in their pharmacokinetics. Due to its potent biological activity and high efficacy in small doses, LNG became the progestogen of choice for contraception. Initially, LNG was incorporated in oral contraceptive pills (OCPs) along with estrogen. Later it was used alone for emergency contraception, in implants and in intrauterine devices (IUDs).

Synthetic progestogens are metabolized and inactivated much more slowly than natural progesterone. Two types of compounds have been synthesized, compounds with or without the angular methyl group in the C-19 position of the steroid molecule (the 17-acetoxy compounds, such as medroxyprogesterone acetate, and the 19-nor compounds, such as norethindrone). Only the 19-nor compounds are used in OCP or IUDs. The 17-acetoxy compounds are used mainly in injectable contraceptives.

The level of progestogens in the blood depends on several factors such as rate of absorption, metabolism, and binding to plasma proteins. Synthetic progestogens are metabolized and inactivated much more slowly than natural progesterone. Since LNG is a synthetic hormone, the pharmacokinetics of LNG is different compared to natural progesterone. The binding of several progestogens to sex hormone binding globulin (SHBG) also differs significantly. LNG has approximately half the SHBG binding affinity of testosterone. The degree of binding to plasma proteins affects the clearance rate of the drug and the concentration of free steroid in blood. The concentration of free or unbound drug is that which is available to diffuse from the bloodstream into the target organs to bind to receptors in the

hypothalamus, pituitary, mammary gland, and uterus. The activity of the progestogens also depends on the time-course of the serum concentration of the progestogen after the use. This depends on several factors such as the absorption, metabolism, liver physiology (first-pass effect), distribution and storage in fat and other tissues, binding to serum proteins, inactivation and conjugation. Depending on the route of administration, oral or parenteral (vaginal, intramuscular, transdermal), progestins can manifest different effects due to differences in metabolism. This has been demonstrated experimentally for progesterone [1].

The pharmacokinetics of the 19-nor progestogens are relatively similar. The individual properties of progestogens are more related to the specificity of binding to the receptors for progesterone, androgens, and glucocorticoids. The progestogen activity may be measured as the affinity for the progesterone receptor. It may also be measured as the ability to suppress luteinizing hormone (LH) secretion or to stimulate endometrial development in rabbits. There appear to be several different forms of progesterone receptors, usually called PR-A and PR-B, the difference being the particular sequence of amino acids in the two forms. Biologically, both forms have different specifications [2]. Biological effects at the cellular level are mediated by intracellular steroid receptors. The ability of any progestin to bind to the progesterone receptor differs between the compounds, and this influences the biological effect of the progestin. This is also true in relation to binding to other intracellular steroid receptors.

All progestins have in common the progestogenic effect i.e., the induction of a characteristic change in the estrogen-primed endometrium. The total progestogenic or progestational activity of any substance also depends on the route and timing of the administration. It varies widely and is often expressed by the difference in the dose required for the endometrial transformation in a woman, called the transformation dosage. It is crucial for the compound to be able to bind to the progesterone receptor. Suppression of gonadotropin secretion by progestogen appears to be chiefly limited to the preovulatory surge at midcycle. Tonic levels of gonadotropins at other times during the menstrual cycle have not been found to be greatly suppressed [3]. Progestogenic effectivity on the level of the endometrium and antigonadotropic effects (dose for ovulation inhibition) of the different progestins varies.

Androgenic activity of progestogens varies among different progestogens and it is responsible for some of the undesirable side-effects. In addition, the 19-nor steroids are also capable of exerting some estrogenic activity since they

have been shown to stimulate proliferation of mammary tumor cells only in cells that contain the estrogen receptor. In vitro studies with COS7 cells on the ERα and ERβ have shown some differences between progestins. The most significant up-regulation of ERα-activity was found with norethisterone, norethinodrel and desogestrel. In contrast, ERβ was markedly activated only by norgestrel and to a lesser extent by norethisterone, norethinodrel and LNG.

LNG is used as a steroidal contraceptive in different formulations and administered in a number of different ways. Most of the information regarding the pharmacokinetics of LNG is available from studies with oral contraceptives. As the other delivery methods of this compound were introduced more knowledge has been attained regarding pharmacokinetics in other delivery methods.

PHARMACOKINETICS OF LEVONORGESTREL

After oral administration, synthetic progestins are in general rapidly absorbed and reach a maximum serum concentration within 2–5 hours, they have a longer half-life than progesterone and display stable plasma levels for long-term use. Many of them are metabolized in the liver and are excreted via the urine [4, 5, 6 and 7]. When taken orally, LNG is rapidly absorbed and is not subject to first-pass effect in the liver. It is approximately 90% bioavailable after oral intake and the circulating half- life is around 15 hours [8]. The peak-plasma-level is obtained between 1 and 3 hours after administration. There is insufficient information regarding the AUC (Area under the serum concentration -time curve) and the dose. Since LNG is a synthetic hormone, the pharmacokinetics of LNG is different compared to natural progesterone. The binding of different progestogens to sex hormone binding globulin in the plasma also varies. In circulation, 47.5% of LNG is bound to SHBG, 50% to albumin, while 2.5% is unbound [9]. LNG causes a 50% decrease of SHBG [5, 6, 10]. When LNG binds to progesterone receptors in the hypothalamus, it results in slowing of gonadotropin-releasing hormone (GnRH) pulse release, which extinguishes the preovulatory luteinizing hormone (LH) surge. Eventually, this phenomenon results in inhibition of ovulation. [11].

LNG not only it binds to progesterone receptors, but like other progesterone agonists, it also binds to several other steroid receptors. In vitro, relative binding affinity of LNG to steroid receptors has been demonstrated as follows: for the progesterone receptor, 323% that of progesterone; for the

androgen receptor, 58% that of testosterone; for the mineralocorticoid receptor, 17% that of aldosterone; for the glucocorticoid receptor, 7.5% that of cortisol; for the estrogen receptor, <0.02% that of estradiol [12].

LNG has about a threefold greater affinity than progesterone for the progesterone receptor. The effect of different progestogens in the body depends on the absorption of the drug, the duration of the presence of the drug in the body and the activity of the drug at the target cell. Since LNG and norgestimate have similar absorption characteristics and half-lives, the biological activities are proportional to their affinities for the progesterone receptor. The doses of the two progestogens for the LH and endometrial responses correspond to their binding affinities. However, natural progesterone is poorly absorbed and has a much shorter half-life, approximately 20 min, and, therefore, the dose for an equivalent effect is much higher.

Similar to synthetic steroids, the specificity of the progestogens is less than that of the natural hormones. LNG, a derivative of testosterone, has relatively high affinity for the androgen receptor and stimulates an increase in the rat ventral prostate weight. LNG has a higher affinity for androgen receptors compared to other progestogens. Relative to dihydrotestosterone, the affinities of progesterone, norgestimate, 3-ketodesogestrel, gestodene, and LNG for the androgen receptor are 0.003, 0.005, 0.118, 0.154, and 0.220, respectively [13].

Since 19-nortestosterone derivatives, such as gestodene and desogestrel do not have any affinity for the estrogen receptor, it is supposed that the estrogen activity of norethisterone, norgestrel and LNG is at least in part a consequence of their metabolism into the 3(α,β) 5α-reduced derivatives by 5β-reductase activity in breast cancer cells [14].

EFFICACY OF LEVONORGESTREL

All progestins have one common effect, and that is the progestogenic effect on the estrogen-primed endometrium of rabbits. But many other biological effects present large differences between progestins. In practice, clinically used synthetic progestins have been selected based on activity after oral administration, favorable bio-availability or inhibition of ovulation, but not on pregnancy-maintaining capacity, a very important biological role for progesterone.

LNG is the most widely utilized contraceptive progestin, alone or in combination with EE. Marketed for the first time in the 1960s, LNG can be

found today in combined OCP, progestin-only pills, long-acting contraceptive implants, IUD and in the most widely utilized emergency contraceptive (EC) forms. Over time, the dosage in OCP containing LNG and ethinyl estradiol decreased. This is mainly to minimize adverse reactions due to either the progestin or the estrogen component of the pill. There are multiple reasons why LNG has been selected as a progestin of choice. A long successful clinical experience with LNG has documented its safety, randomized studies have shown that LNG containing products have better bleeding patterns than those containing norethisterone[15-17] and newer progestins have experienced a controversy surrounding an increased risk of venous thromboembolism.

Mechanism of Contraceptive Action of Levonorgestrel

All contraceptive progestins have a double mechanism of action. They act at the central and peripheral level, but the relative importance of a particular mechanism of contraceptive protection depends on the route of administration [18]. LNG exerts contraceptive action by inhibiting ovulation, making the endometrium inactive and by altering cervical mucus.

When combined with 30-35 µg of Ethinyl estradiol in OCP, taking into account the synergistic action of estrogen, LNG can block fertility by inhibiting ovulation at the daily oral dose of 60 µg (equivalent to peak plasma levels of approximately 1ng/ml). This inhibitory action takes place at the hypothalamus where physiologically progesterone decreases the number of LH pulses [11, 19].

When taken orally, LNG produces an inactive or atrophic endometrium; however, after discontinuation of hormonal exposure, there is a rapid return to normal endometrial cycling [20]. A different endometrial effect is seen when LNG is delivered directly to the uterine cavity; in this case, there is extensive decidualization of endometrial stromal cells, atrophy of the glandular and surface epithelium and changes in the uterine vasculature. There is also modulation of local mediators that regulate endometrial function [21]. In addition, local delivery of LNG seems to counteract the priming effect of endogenous estradiol (E2). In fact, circulating levels of estradiol are within the same range, irrespective of whether women are menstruating or amenorrheic, suggesting a completely local effect of LNG that cannot be influenced by E2 [22].

Another mechanism of contraceptive action of LNG is an alteration of the cervical mucus. This is obvious even when LNG is administered at doses that

do not constantly inhibit ovulation. LNG in such low doses can still remain effective as a contraceptive by acting at the level of the cervical mucus, by significantly decreasing the amount, ferning and spinnbarkeit of cervical mucus, while at the same time increasing the viscosity. Under the effect of progestins, cervical mucus scores do not exhibit the normal mid-cycle peak, cervical mucus receptivity to sperm is lowered, sperm penetration in cervical secretion is inhibited and the ovulatory peak of the karyopyknotic index of vaginal cytology is suppressed. After insertion of subcutaneous LNG-delivering implants (Norplant), cervical mucus scores decrease within one week, indicating the profound effect of LNG on the cervical mucus, even in the event of possible ovulation [23].

Levonorgestrel Combined Oral Contraceptive

LNG efficacy has been determined in various formulations in the form of OCP, patches, implant, and IUD. In OCP the efficacy is determined along with estrogen. The combined oral contraceptive pill is an effective contraceptive method which can also offer other benefits. Pills containing LNG or norethisterone in combination with 35 mg or less ethinyloestradiol are considered first-line. They are effective if taken correctly and have a relatively low risk of venous thromboembolism [24].

LNG containing OCP are being used in different formulations. Newer extended regimens of LNG containing OCP have been highly effective at decreasing the frequency of menstrual bleeding. Many women would prefer to eliminate or reduce the frequency of scheduled bleeding if given the choice [25, 26]. Loudon et al. were the first to show that reducing withdrawal bleeding to four times a year (using an oral contraceptive containing EE 50 μg plus lynestrenol 2.5 mg) was both acceptable and effective: 82% of the 196 women experienced fewer menses and a reduction in menstrual and premenstrual symptoms [27]. This regimen was not introduced into clinical practice until the development of combined OCP with minimum feasible estrogen daily dose and the introduction of a series of new progestins.

In last several years new options have been considered to decrease the frequency of free intervals or to eliminate them altogether [28]. In addition, extended pill use (with or without interruption) has also been advocated as a maintenance treatment for endometriosis-associated pelvic pain in women not wishing to become pregnant [29, 30] and to avoid menstruation-associated symptoms such as migraine headaches[31] and premenstrual syndrome[32].

The first approval in the USA of an extended regimen combined OCP was based on a large randomized controlled trial that demonstrated the efficacy of 84 days of LNG (LNG) 150 μg/EE 30 μg followed by seven placebo days. Pearl Indices, based on method failure rates, were 0.60 and 1.78 for the 84/7 and the 21/7 regimen, respectively [33]. Compared with the 21/7 regimen, the 84/7 regimen was associated with significantly fewer total days of scheduled bleeding/spotting; however, an increased incidence of unscheduled breakthrough bleeding/spotting was reported [33] thus revealing an important limitation of extended regimen combined oral contraceptive (COC)s compared with traditional 21/7 regimens [33].

Two recent extensive systematic reviews of extended and continuous regimen COCs concluded that the risk of pregnancy did not differ between cyclical and extended regimens [34, 35]. Observational data, however, suggest that regimens with shorter or fewer hormone-free intervals may be associated with reduced pregnancy rates [36, 37]. One analysis of a retrospective claims database revealed lower contraceptive failure rates with 84/7 regimens compared with 21/7 and 24/4 regimens. At 1 year, rates of pregnancy were significantly lower with 84/7 regimens vs. 21/7 regimens (4.4% vs. 7.3%; $p < 0.0001$) and with 84/7 regimens vs. 24/4 regimens (4.4% vs. 6.9%; $p < 0.0001$ [37].

The most important goal of extended regimen COCs is to reduce the incidence of scheduled withdrawal bleeds as well as overall bleeding; most studies evaluating the effectiveness of extended regimens have shown improved overall bleeding patterns [33, 38-40]. A recent Cochrane review by Edelman and colleagues [34] concluded that most trials found no difference or less bleeding and/or spotting with extended/continuous vs. cyclical regimens; although most users of extended regimen combined OCP will experience occasional unscheduled (breakthrough) bleeding or spotting. Although an increased incidence of unscheduled bleeding with extended regimens during early cycles has been reported [34, 35], the frequency and intensity of such bleeding decreases over time [35]. By the fourth extended cycle, the incidence of unscheduled bleeding is generally comparable to that seen among users of conventional cyclical regimens [33, 41].

Levonorgestrel Emergency Contraception

The most commonly-available emergency contraceptive option is LNG 1.5 mg, sold in the United States as Plan B One-Step (Teva Pharmaceuticals,

North Wales, PA) with generic forms including Take Action (Teva Pharmaceuticals, North Wales, PA).

The failure rate of LNG is lower when it is used continuously as a regular contraceptive than when it is used as a single dose for emergency contraception (EC). When LNG-EC is used during the follicular phase, it can inhibit or delay ovulation, and this interference depends on how much the follicular phase is advanced [42]. The efficacy of LNG-EC decreases when the interval between unprotected intercourse and treatment increases, and this suggests that it does not interfere with implantation. Several studies have looked at the actions of LNG-EC on endometrial histology and physiology. Deleterious effects of LNG impairing endometrial receptivity to subsequent implantation have not been found either in vivo [43-45], or in vitro [46, 47].

Defining the efficacy of LNG-EC as the ratio of observed/expected pregnancies, the efficacy of LNG-EC when used before ovulation is 100% [48, 49]. When used after ovulation has occurred, the number of observed and expected pregnancies is not statistically different, indicating that LNG-EC is ineffective in interfering with any reproductive process subsequent to ovulation. This finding is incompatible with the inhibition of implantation by LNG-EC and is consistent with the mechanism of action of EC reported by Gemzell-Danielsson [50]. LNG-EC is highly effective for preventing unintended pregnancy when it is used prior to ovulation, but it is ineffective in preventing pregnancy when used after ovulation. This is because it has no effect on subsequent reproductive processes, including implantation of the embryo [51].

Levonorgestrel Implant

LNG containing implants are a long-acting contraceptive implant for birth control and was sold under the brand name Norplant. A continuous low dose of LNG diffuses through the wall of each capsule. It prevents pregnancy by multiple mechanisms including inhibition of ovulation, thickening of the cervical mucus and thinning of the endometrium. It is 100% bioavailable with plasma levels around 0.3 ng/ml over 5 years but levels are highly variable depending on individual metabolism and body weight. Norplant is a highly effective contraceptive method. The most common side effect experienced by levonorgestrel implant users is menstrual problems, but serious bleeding problems are not more frequent than in controls [52]. Norplant compared to other implants took a longer time to remove [52, 53]. Norplant was

discontinued due to multiple reported side effects and procedural difficulty in removing the implant.

Levonorgestrel Intrauterine Device

Mechanism of Action of the LNG Releasing IUD

The LNG releasing IUD (LNG-IUD) has been available since 1990 in Europe and is available in the United States since 2000. It is marketed under the name Mirena® (Bayer Schering Pharma, Berlin, Germany). Another two LNG-IUD introduced later in the market are Skyla and Liletta. The LNG-IUD Mirena provides highly effective contraception for up to five years, while Skyla is approved for 3 years.

The mechanisms of action for LNG-IUD are similar to that of LNG implants or LNG-containing mini-pills. It achieves these effects with much lower peak serum levels than other progestin-containing contraceptives (0.1–0.4 ng/ml vs 1.7–15.2 ng/ml with combined and progestin-only oral contraceptives, respectively, and 5.4 ng/ml for combined vaginal preparations)[54]. The LNG-IUD is a T-shaped device containing 52 mg of LNG. Initially, 20 μg of LNG is released every 24 hours, decreasing to 11 μg every 24 hours by the end of five years, with an average release rate of 14 μg per day over the life of the IUD. LNG causes thickening of the cervical mucus and also suppresses endometrial proliferation (preventing decidualization of the stroma). The thickening of the cervical mucus creates an unfavorable environment for the survival of the sperm, inhibiting motility and capacitation and thus it prevents fertilization [54].

The LNG-IUD also produces endometrial thinning with fragile superficial vessels which, in the unlikely event of fertilization, may prevent implantation. The low serum levels of absorbed progestin are below the threshold for inhibition of ovulation, therefore with the LNG-IUD ovulation is not inhibited [54].

Contraception

LNG-IUD is a highly effective, long-acting, and reversible contraceptive agent. Due to various contraceptive mechanisms of action, the efficacy rate of the LNG-IUD is high, with only 0.1% of women experiencing an unintended pregnancy within the first year of typical use. In four clinical studies which represented more than 10,000 woman-years of use, the average Pearl Index (a statistical estimation of the number of unintended pregnancies in 100 woman-

years of exposure to a contraceptive method) was 0.1[55]. The LNG-IUD, in particular, is the most effective IUD available as reported in a number of studies with a global cumulative pregnancy rate of <0.5% [56].

The LNG-IUD is also an effective contraceptive option for women to select immediately post-abortion. One study followed 305 women who received the Mirena® IUS immediately post-abortion for up to five years. There was a total of two pregnancies for a pregnancy rate of 0.8% at five years. There was a discontinuation rate due to expulsion of 7.1% at one year and 10.5% at five years [57] which is higher than the interval expulsion rate of 2%–3% per year [58]. An LNG-IUD is therefore safe to insert immediately after either spontaneous or induced abortions. An LNG IUD can also be easily inserted after childbirth, but the expulsion rate is higher compared with interval insertion.

The LNG-releasing IUD has a significant role in the medical management of gynecological conditions. The Mirena® LNG-IUD can be effective in treating a variety of gynecological disorders including menorrhagia, dysmenorrhea, pain associated with endometriosis and endometrial hyperplasia. It can also be used as an alternative to hysterectomy for women with bleeding problems as well as an adjunct to estrogen replacement therapy.

Heavy Menstrual Bleeding

The LNG-releasing IUD (LNG - IUD) has been found to be more effective than oral hormonal treatment for heavy menstrual bleeding. LNG-IUD has been reported to result in a reduction in menstrual blood flow of 86%–97% [59]. One study comparing the LNG-IUD and norethisterone showed that the LNG-IUD reduced menstrual blood flow by 94%, while with norethisterone the reduction in menstrual blood loss was 87% [60]. After three cycles of treatment, 76% of the LNG-IUD group wished to continue the treatment, compared with only 22% of the medical therapy group [60]. In a Cochrane review which included 21 RCT studies with LNG-IUD, there was a greater reduction in menstrual blood loss, improvement in the quality of life and an overall acceptance of the IUD for longer time durations. The only drawback in comparison to oral therapy was that it was associated with more minor adverse effects than oral therapy [61]. In comparison to hysterectomy, adverse effects such as bleeding and spotting are more likely to occur with LNG-IUD, but LNG-IUD provides a better alternative to surgery in most cases. There is no conclusive evidence of a difference in satisfaction rates between surgery and LNG-IUD [62].

LNG-IUD has been evaluated in numerous studies to compare outcomes with hysterectomy for the treatment of menorrhagia. A randomized controlled trial of 236 women assigned either to LNG-IUD or hysterectomy showed that after one year the two treatments were associated with equal improvements in health status, quality of life, and psychosocial well-being, but the IUD was more cost-effective. However, 20% of the women in the LNG-IUD group went on to receive hysterectomies due to continued bleeding [63]. Another study which looked specifically at the cost-effectiveness of OCP versus LNG-IUD versus surgical management (including both ablation and hysterectomy) for dysfunctional uterine bleeding (DUB) showed the LNG-IUD to be the most cost-effective of all three treatments [64]. A Cochrane review concluded that use of the LNG-IUD results in a significant decrease from baseline in the amount of menstrual bleeding, and that it is more cost-effective as a treatment for menorrhagia than hysterectomy both at one and five years [65]. The LNG-IUD is therefore a satisfactory, effective and economical alternative to medical and surgical treatment of menorrhagia.

Endometriosis

Endometriosis affects 5-10% of women in the United States and is associated with chronic pelvic pain, dyspareunia, infertility, and significant effects to a patient's quality of life [66]. Various treatments have been used alone or in combination, which include nonsteroidal anti-inflammatory medications (NSAIDs), progestational medications such as depot medroxyprogesterone acetate (DMPA) that function as anti-estrogens, ovulation suppression with OCP, androgenic medications such as danazol, gonadotropin-releasing hormone (GnRH) analogues to induce temporary pseudo-menopause and surgical ablation. However, the side effects associated with many of these medical treatments, mainly the hypoestrogenic effect and the invasive nature of the surgical treatment are a limiting factor for patient compliance and long-term continuation of the treatment. LNG-IUD has been introduced in the treatment of endometriosis as it is associated with fewer side effects and is more acceptable for the long term.

In a randomized clinical trial of 82 women with chronic pelvic pain from endometriosis, 39 women were treated with the LNG-IUD and 43 women were treated with a GnRH analogue. Both groups showed significant improvement in pain scores throughout the six months of treatment. The fastest improvement was noted in women with stage III or IV endometriosis. There was no difference in the quality of life in two groups. The LNG-IUD group had the benefits of fewer hypoestrogenic side effects and required one

intervention every five years [67]. Fifty-nine percent of the women in the LNG-IUD group were still using the IUD after 36 months. The VAS pain score in this group was 0–3 (indicating excellent pain control) in 82.6%, and ≥7 in only 8.6%. In the former users of the GnRH analogue, 28% were using some form of hormonal contraceptives to control pain, and 47.5% continued to have excellent pain control, while only 2.5% had a pain score ≥7. While both treatments are effective in pain control for up to 3 years, the LNG-IUD has an additional benefit that it provides contraceptive protection along with good pain control [68]. Lockhart et al. reported significant pain improvement with LNG- IUD in women with minimal to moderate endometriosis. 34 women were treated with the LNG-IUD for up to 36 months. There was a decrease in the visual analog pain scale (VAS) from an initial score of 7.7/10 to 2.7 at 36 months. There was also a decrease in the verbal rating scale (VRS) of both dysmenorrhea and noncyclic pelvic pain from an initial 25/96 to 8.4 after 36 months as well as a decrease in total days of pain per 28-day period from 15.0 to 6.0 after 12 months [69].

LNG-IUD is also effective in preventing recurrence of endometriosis after conservative surgical treatment for moderate to severe endometriosis. In a randomized-controlled clinical trial of 40 women, 20 women were treated postoperatively with LNG-IUD while 20 women who were managed expectantly following conservative surgical treatment for moderate to severe endometriosis. After 12 months, women who received the LNG-IUD had a recurrence rate of 10% of moderate to severe endometriosis, compared with a recurrence rate of 45% in the expectantly managed group. There was also an absolute risk reduction in recurrence of dysmenorrhea in the LNG-IUD group of 35%, as well as a decrease in the recurrence rates of dyspareunia [70].

The LNG-IUD is an effective treatment for the relief of pain associated with endometriosis, requires fewer interventions, has fewer hypoestrogenic side effects and results in increased patient satisfaction compared to other alternative treatments.

Adenomyosis

Adenomyosis is a relatively common disorder affecting women, usually in their 40s and 50s. The symptoms include menorrhagia (40%–50%), dysmenorrhea (15%–30%), and metrorrhagia (10%–12%) [71]. Traditionally hysterectomy has been the definitive treatment but over last several years less invasive treatments have been tried. The less invasive treatment options include endometrial ablation, danazol and GnRH agonists. Endometrial

ablation is not effective for this condition as it does not penetrate deeply enough into the myometrium. Danazol and GnRH agonist's side effects and the inability to use these agents in the long term make treatment with these agents less acceptable. Use of LNG-IUD for the treatment of adenomyosis is rapidly increasing. LNG-IUD causes decidualization and atrophy of the endometrium and in addition it down-regulates estrogen receptors in glandular and stromal endometrial tissues which causes adenomyosis foci within the myometrium to atrophy. This leads to decreased blood loss during menses and may also reduce the size of the uterus [72].

Vercellini et al. evaluated the effectiveness of the LNG-IUD in 25 women with menorrhagia associated with adenomyosis over a period of 12 months. The average blood loss decreased by approximately 75% (211 ± 61 to 44 ± 18 mL), resulting in a significant increase in hemoglobin, serum iron and ferritin levels. In addition, there was a significant decrease in both uterine volume and endometrial thickness [70].

In another study, 47 women with adenomyosis were treated with LNG-IUD and followed for up to 36 months. At the end of 36 months, 32 women were still enrolled in the study. In these women, serum hemoglobin and ferritin levels significantly increased and CA-125 values significantly decreased. The uterine volume decreased at 12 months. However there was a slight increase in uterine volume but this was not significantly different from the initial pretreatment volume. Pain scores decreased significantly by 6 months and remained there at 24 months. Menstrual blood loss was also markedly reduced, with a reduction of >90%, and remained similar at 24 months. Even though some symptoms recurred after 3 years, they were still significantly less than the initial presentation. The exact cause of this occurrence is not known, but it is probably due to low levels of daily hormone released by the LNG-IUD over time [73]. LNG-IUD is an effective treatment for adenomyosis, showing an improvement in pain and bleeding scores over two years. Replacing the IUD in less than 5 years may be beneficial in producing long term symptom relief.

Endometrial hyperplasia and Endometrial intraepithelial neoplasia

Management depends on whether the underlying pathology is primarily hormonal (benign endometrial hyperplasia) or essentially a premalignant disease (endometrial intraepithelial neoplasia). Endometrial hyperplasia belongs to the functional category of estrogen effects and is usually treated with hormonal treatment. Endometrial intraepithelial neoplasia is precancerous and the management is hormonal or surgical treatment. Both conditions

respond fairly well to oral medroxyprogesterone acetate or micronized progesterone, but treatment with oral progesterone sometimes is associated with intolerable side effects and patients may not be able to continue oral progesterone treatment. During the last 10 years, LNG-IUD has been introduced in the treatment of endometrial hyperplasia. Several studies have shown a complete response (reversal of endometrial hyperplasia to a progestational-type endometrium) in 25 to 90% of cases. In general, endometrial hyperplasia responds better (90–100% response) than endometrial intraepithelial neoplasia (67–88% response) to intrauterine LNG [74-76]. In addition to the dominant progestational effect of Mirena on the endometrium, adverse events (side effects) that are commonly experienced by patients on oral progestational therapy are considerably reduced. This is because the systemic absorption of LNG is considerably reduced compared to oral progestational therapy [74-77].

In a small study of 20 women, which included 12 women with endometrial hyperplasia without atypia and eight women with atypia, LNG-IUD was found to be very effective over a period of 36 months. At the start of the study, all women with atypical hyperplasia showed progesterone receptor expression in the epithelial cells. There was a significant decline in progesterone receptor expression over the course of the study, suggesting the strong antiproliferative effect of the LNG-IUD [78].

In a prospective randomized study, Dolapcioglu et al. compared the efficacy of LNG-IUD to oral progestin for the treatment of endometrial hyperplasia. A higher success rate was noted with LNG-IUD compared to oral MPA. The success rates of LNG-IUD treatment and oral medroxyprogesterone for three months therapy were 84% and 50%, respectively. In the six-month treatment group the regression rate was even higher, 100% with LNG-IUD and 64% with oral medroxyprogesterone. LNG-IUD was found to have a significantly higher success rate [79].

Another study compared the efficacy and safety of the LNG-IUD with dydrogesterone in 138 patients with endometrial hyperplasia without atypia. LNG-releasing IUD has been shown to be highly effective in the treatment of endometrial hyperplasia. After 6 months of treatment, endometrial hyperplasia regressed in 96% of women in the LNG-IUD group versus 80% of women in the oral group. Minimal differences were noted regarding adverse effects between the two groups. Intermenstrual vaginal spotting and amenorrhea were more common in the LNG-IUD group. Patient satisfaction was significantly higher in the LNG-IUD group. Hysterectomy rates were lower in the LNG-IUD group than in the oral treatment group Recurrence rate was 0% in the

LNG-IUD group compared to 12.5% in the oral group. LNG –IUD is highly effective for the treatment of endometrial hyperplasia and it decreases the hysterectomy rate [80].

In a prospective multicenter study, 75 patients with endometrial hyperplasia were treated with LNG –IUD. One hundred percent of the patients achieved complete regression of hyperplasia within 3 months after LNG-IUD insertion. At 12 months complete regression rate overall was 94.7%: 100% of patients with atypical endometrial and 93.7% with endometrial hyperplasia without atypia [81].

As Adjunct to Estrogen replacement therapy

Hormone replacement therapy for menopausal symptoms requires combined estrogen and progesterone treatment in the presence of the uterus. Estrogen treatment is prescribed to relieve menopausal symptoms, but progesterone is needed in order to offset the proliferative effect of estrogen on the endometrium. The standard treatment is oral progestin in combination with the estrogen. The side effects of oral regimen such as vaginal bleeding and androgenic effects may make it unacceptable for women to continue the treatment. In addition, studies have shown possible links with systemic progesterone and increased cardiovascular risks in addition to increased breast cancer risks [82, 83]. The LNG-IUD has been evaluated as an alternative progestin method to provide endometrial protection with minimum adverse systemic effects, due to its significantly low serum concentration. In one study of 40 perimenopausal women with menopausal symptoms, authors evaluated two treatment regimens: three-week cyclic treatments with 2 mg oral estradiol (E2) valerate combined with 250 μg of oral LNG for the last 10 days of each cycle versus 2 mg of E2 valerate continuously with the LNG-IUD. After one year the subjective symptoms were improved in both groups, and none of the women had endometrial proliferation. However, in the LNG-IUD group 15/18 women were amenorrheic, versus all women in the oral LNG group continued with cyclic bleeding [84].

In a meta-analysis of 19 studies with a total of 826 subjects regarding LNG-IUD in combination with estrogen, and with a duration of six months to five years, none of the women developed endometrial hyperplasia throughout the course of the study [85].

Hampton et al. evaluated 82 perimenopausal women treated with oral estrogen and the LNG-IUD for long term protection of the endometrium. There were no cases of hyperplasia throughout the entire 60 month period.

Amenorrhea was present in 54.4% of the women after 12 months, and 92.7% after 60 months. The treatment was well tolerated, with 79.8% of the women still continuing with the estrogen and LNG-IUD after the full 60 months of the study and 92.4% of the participants rated the treatment as highly effective [86].

The LNG-IUD is well tolerated with a good safety profile. Women using the LNG-IUD plus estrogen replacement therapy report improvements in quality of life. Adherence and continuation rates are also high [87].

Endometrial cancer

Conservative hormonal treatment to spare fertility in younger women with early-stage endometrial cancer has been described by several groups [88-90]. There is no consensus regarding the ideal progestin agent, dose, or duration of treatment. The two most common regimens are medroxyprogesterone acetate (MPA) at 500–600 mg daily and megestrol acetate (MA) at 160 mg daily [91, 92]. Although early stage endometrial cancer in young patients responds well to progestins, treatment with high doses of progestins is associated with side effects and complications, resulting in a higher likelihood of noncompliance [91, 92]. An alternative to systemic progestins that may reduce these risks is the LNG-IUD. The LND-IUD has not been as well studied as oral progestins, but complete response rates from 40% to 100% have been reported in premenopausal women with well-differentiated early stage EC [93-95]. None of these studies reported any specific difficulties with follow-up in the presence of an IUD. Maintenance treatment with low-dose cyclic progestin or an LNG-IUD has been shown to lower the risk of recurrence after complete response among young women with endometrial cancer undergoing fertility-sparing treatment [96-98]. The LNG-IUD can be an alternative to oral progestins for treatment of early stage endometrial cancer. However, more data on long-term follow up is needed before it can be widely implemented in clinical practice.

SAFETY OF LEVONORGESTREL

LNG is a safe and effective progestogen and this has led to its incorporation into many forms of contraceptive treatments, and its use has been extended to the management of certain gynecological conditions. However, LNG is not without its side effects and in order to minimize the side

effects, the lowest effective dose in OCP has been introduced. Use of LNG in IUD has also resulted in a reduction of some of the systemic side effects of LNG due to low-level systemic levels.

The side effects depend on whether the progestin is combined with estrogen or is used alone. Some side effects may be due to blockage of follicular maturation, resulting in a decrease in endogenous estrogen production. In addition, the side effects of progestin may be associated to its estrogenic effects or its androgenic or glucocorticoid properties [6]. The side effects are usually related to the combined effects of estrogen and progesterone in combined products, or are related to higher doses of medroxyprogesterone, LNG or norethindrone acetate.

The usual side effects reported with progestins are bleeding problems, headaches, bloating, mastalgia, weight gain, mood changes and acne [99, 100]. Not all progestins cause the same side effects and to the same degree. OCP-containing levonorgestrel is quite effective in regulating the menstrual cycle but with levonorgestrel-containing implants (Norplant) and LNG-IUD, menstrual irregularity is one of the common side effects. With Norplant approximately 20 and 30% of users experience irregular, unpredictable uterine bleeding [101-106]. Irregular bleeding is the main side effect associated with the use of Norplant. The extent of uterine bleeding, even though it can be persistent and prolonged, is usually not clinically significant and does not cause anemia or a significant drop in hemoglobin concentrations. Rather, a rise in the hemoglobin concentrations has been reported in Norplant users [107]. Unpredictable uterine bleeding or spotting, which occurs often and for >5-6 days, interferes with personal life rather than being a problem of any medical significance.

Menstrual irregularity is also the most common side effect of LNG-IUD. Women may experience irregular bleeding in the beginning followed by oligomenorrhea or amenorrhea. Practically 25% of women discontinue using the LNG-IUD because of amenorrhea [54]. Approximately 20% of LNG-IUD users will be amenorrheic by the end of 12 months [108], and 70% of users will be oligomenorrheic or amenorrheic by 24 months [109]. Overall the LNG-IUD is well tolerated.

Baldaszti et al. evaluated the long-term acceptability of the LNG-IUD in 165 women over 3 years. The study showed a three-year continuation rate of 90.3%. The number of women who expressed that they were very satisfied with the LNG-IUD increased steadily with the duration of the treatment, 29% after two weeks, 56% after two months, 69% after six months and 77% after 36 months [110].

In another study, patient satisfaction was evaluated in 78 women who had the LNG-IUD inserted for menstrual disorders. 12% of the women had the IUD prematurely removed, with the major reason being pain and heavy bleeding. However, the majority of women were satisfied with the IUD. 78% of the women reported improvement in their periods, and 84% reported improvement in menstrual discomfort. 72% of the women reported they would use the LNG-IUD again, 73% would recommend it to their peers and the overall satisfaction rate was 76% [111].

Although the systemic absorption of LNG in IUD form is much less compared to oral LNG containing contraceptives, it can occasionally be associated with some undesirable side effects such as ovarian cysts, acne, weight gain, depression and decreased libido. Three meta-analyses have reported systemic side effects of the LNG-IUD. These identified a significant increase in headaches and breast tenderness and a fivefold (but not significant) increase in acne [112-114].

The LNG-releasing intrauterine system is a safe, effective and acceptable form of contraception and has a multitude of non-contraceptive benefits. Most of the side effects are minor and tolerable.

Other side effects encountered with different progestins varies depending on their androgenic or mineralocorticoid activity. Undesirable side effects due to androgenic activity include acne and seborrhea, weight gain, blood pressure increase, and an increase in the low-density lipoprotein (LDL) to high-density lipoprotein (HDL) ratio. Acne is one of the side effects observed when an androgenic molecule such as LNG is used in higher doses.

A headache is observed due to hyperestrogenism in combination pills where estrogen is predominant. Alopecia may be another effect of systemic absorption of LNG in women using this device, although the exact mechanism is unknown. Alopecia has been cited as an adverse effect of progestin OC [115] and has also been reported with progestin implants with incidence rates of 1.1% [116] to 11.8% [117]. The LNG-IUD product information includes hair loss as an “uncommon” side effect. The incidence is quoted at 0.1–1%. The analysis of IMMP cohort suggests that the incidence is likely to be between 0.18% and 0.33% [118]. The etiology of hair loss with LNG-IUD and implants has not been explained but may result from a lower circulating estrogen level in some women [119]. Although alopecia is rare (0.1–<1.0%), it definitely is psychologically upsetting for women [120].

Cardiovascular effect: Most of the OCP have progestins derived from testosterone, and their main side effects are due to their androgenic or corticosteroid properties. Natural progesterone and some of its derivatives,

such as the 19-norprogesterones, do not exert any androgenic effect and therefore have no negative effect on the lipids. 19-nortestosterone derivatives and even some 17-hydroxyprogesterones have a partial androgenic effect, and hence exert a negative effect on surrogate markers of cardiovascular risk. Estrogen has an antiatherogenic effect and this beneficial effect of estrogen is not reversed by the progesterone. Estrogen also preserves the normal endothelium-mediated dilation of coronary arteries, and Progesterone does not reverse this cardioprotective mechanism. Not all progestins have a similar effect on the cardiovascular system. Progestins, such as medroxyprogesterone or norethisterone acetate, exert a partial negative effect on the beneficial actions of estrogens with regard to lipid changes, atheroma development or vasomotion. In contrast, progesterone itself does not have this inhibitory effect on lipid changes and vascular reactivity. Nonandrogenic molecules of progesterone itself and of derivatives such as 19-norprogesterones have a neutral effect on the vessels 121].

Effect on Lipoproteins: Studies in pre-menopausal women using OCPs have shown a dose-related response in the lipid profile. Women using a 20-µg Ethinyl estradiol (EE)/100-µg LNG OCP demonstrated reductions in high-density lipoprotein cholesterol (HDL-C) and small increases in low-density lipoprotein cholesterol (LDL-C) and triglycerides (TG), in contrast to a 30-µg EE/150-µg LNG OCP [122]. The amount of lipid alteration also depends on the delivery route, where transdermal contraceptive hormone delivery is relatively less potent compared with oral [123]. Barkfeldt et al. [124] conducted a randomized, double-blind study that evaluated the effects of lipid metabolism on 98 women who received 2 different types of progestin-only pills, desogestrel 75 µg/day or LNG 30 µg/day. There were minimal changes seen in the lipid profile except for decreasing trends with levels of HDL-C, its subfractions, and the apolipoproteins apolipoprotein-I and -II. No differences were observed between the 2 formulations despite the higher progestin dose found in desogestrel, including no changes in LDL-C or apolipoprotein-B [124].

Blood pressure: Most studies have shown an increase in blood pressure in normotensive women with OCP use [125]. A review of 2 studies found an increase in systolic blood pressure by 7 to 8 mm Hg on average compared with systolic blood pressure in those not using OCP [126, 127]. Different progesterones have a different effect on blood pressure. In a study of 120 women randomized to drospirenone/EE or LNG/EE, the drospirenone group demonstrated a mean decrease in the systolic blood pressure (from 107.4 to 103.5 mm Hg) and had a statistically significant lower mean blood pressure

compared with the LNG group [128]. Another study of 80 healthy women randomized into groups of 3 mg of drospirenone combined with a 30-, 20-, or 15-μg dose of EE found that systolic blood pressure at 6 months fell by a range of 1 to 4 mm Hg across the groups, compared with an elevation of blood pressure of 4 mm Hg in the control group of LNG/ [129]. Additionally, there was a weight loss of 0.8 to 1.7 kg in the groups receiving the drospirenone compared with an increase in weight in the LNG/EE group by 0.7 kg.

Glucose Tolerance and Diabetes Mellitus: Contraceptive hormones can also impact glucose tolerance and diabetes mellitus. Oelkers et al. [129] studied glucose levels in 80 healthy women assigned to 4 equal groups who received 3 mg of drospirenone combined with 30-, 20-, and 15-μg doses of EE or LNG/30-μg EE. Each woman performed oral glucose tolerance tests at the pre-treatment and at the end of the 6-month OCP cycle. On treatment, fasting glucose was unchanged for all groups, but the area under the curve for the glucose tolerance increased for all formulations. Although not statistically significant between groups, the drospirenone/30-μg EE group had a 19% worsening of glucose tolerance [129]. No worsening of diabetes has been demonstrated with the earlier generation OCPs [130, 131].

Contraceptive use in the past in younger and perimenopausal women has not shown any increased risk of cardiovascular disease. The Nurses' Health Study, an 8-year self-report prospective study did not find increased risk of cardiovascular diseases among past users of OCPs compared with those who had never used OCPs [132]. Among current OCP users, however, there was a 2.5 relative increased risk of adverse cardiovascular events [132]. The increase in cardiovascular deaths and nonfatal MI and stroke in current users but not with past use was believed to be associated with the prothrombotic effects. Stopping OCPs was associated with a declined risk of adverse cardiovascular events, with an RR of 0.95 (95% CI: 0.81 to 1.11) among past users, suggestive of a reversal of the OCP prothrombotic effects with cessation of use. Two separate case-control studies evaluated the association between OCP use and MI, based on the second- and third-generation preparations with differing progestins and reached varying conclusions [133, 134].

In a meta-analysis which included 24 studies oral contraceptive pill users were not found to be at increased at increased risk of myocardial infarction or ischemic stroke compared with non-users (OR 1.0, 95% CI 0.9 to 1.0). The risk did not vary according to the generation of progestagen or type of the progestagen type. The risk of myocardial infarction or ischemic stroke appeared to increase with the usage of higher doses of estrogen. The risk of myocardial infarction or ischemic stroke was only increased in women taking

OCP with ≥ 50 μg of estrogen. Prescribing OCP with < 50 μg of estrogen is safe in regards to myocardial infarction or ischemic stroke [135]. Women who used OCP with EE at a dose of 30 to 40 μg had a risk of arterial thrombosis that was 1.3 to 2.3 times as high as the risk among nonusers, and women who used pills with EE at a dose of 20 μg had a risk that was 0.9 to 1.7 times as high, with only small differences according to progestin type [136].

There is insufficient data with regard to longer-term past OCP use and subsequent cardiovascular disease in the post-menopausal period. Stampfer et al. [132] reported a lower RR for adverse coronary disease events of 0.8 (95% CI: 0.6 to 1.0) among the past OCP users compared with non-prior users in 119,061 women followed for 8 years. A quantitative meta-analysis of 13 studies reported an estimated RR associated with past OCP use of 1.01 (95% CI: 0.91 to 1.13), suggesting that past OCP use had little or no impact on subsequent cardiovascular disease [137].

Terauchi et al. in their study evaluated the effects of oral estradiol and LNG on cardiovascular risk markers in postmenopausal women. They assessed the changes in cardiovascular risk markers induced by low (1.0 mg) and ultra-low (0.5 mg) doses of oral estradiol combined with LNG. In conclusion hormone therapy using 1.0 or 0.5 mg of EE and LNG lowers the serum levels of Total cholesterol, HDL-C, LDL-C, and TG without significantly affecting coagulation/fibrinolysis parameters [138].

Several studies have reported on the cardiovascular disease risk markers and metabolic effects in LNG-IUD users [139-143]. LNG-IUD use was not associated with adverse effects on blood pressure, lipid profile,CRP levels or insulin sensitivity, compared with users of nonhormonal contraception. Regarding glucose tolerance, one study described slightly increased fasting blood glucose in premenopausal women, however, impaired glucose tolerance was not diagnosed [139]. Ferreira et al. in randomized controlled trial evaluated the cardiovascular risk markers associated with endometriosis and the influence of the LNG intrauterine device (LNG-IUD) compared with the GnRH analogue (GnRHa) leuprolide acetate on these risk markers after 6 months of treatment. This study showed that some cardiovascular risk markers are influenced by both GnRHa and the LNG-IUD, but the latter had a greater positive impact on the lipid profile, which could lead to a favorable effect during long-term treatment. Both treatments had no effect on blood pressure. LNG-IUD users had lower total cholesterol and triglyceride values [140].

Progestins and Venous Thromboembolism

The risk of thromboembolism is very rare in the younger women using contraceptives but this adverse effect has received considerable attention due to morbidity and mortality associated with this condition. The increased risk of venous thromboembolism with use of OCP is well known. The risk of venous thromboembolism with OCP is mainly associated with the dose of EE. Estrogen has known pro-thrombotic effects and elevates cardiovascular venous thrombo-embolism (VTE) risk by increasing prothrombin and decreasing antithrombin III. The VTE risk for use of an OCP with a third-generation progestin was found to be about twice that for the use of an OCP with second-generation progestins. Jick et al. in a case-control study assessed the risk of nonfatal VTE with the use of low-dose estrogen < 35 mcg plus second generation (levonorgestrel) or third generation (desogestrel or gestodene) progestins and found that there was a higher risk of nonfatal VTE with the third generation progestins compared to second generation progestins [141]. In a meta-analysis, a relative risk of VTE of 1.7 was reported for use of OCs with third- generation progestins versus second generation progestins [142]. The difference of effects observed between second and third generation OC may be related for some of the variables to the difference in the androgenic properties of the progestin. Third-generation progestins are less androgenic than second-generation compounds, and this difference is reflected on the changes in SHBG and HDL [143].

Although there is no marker for venous thromboembolism risk, epidemiological studies document that second-generation OCP carry lower risks than do third generation OCP. Sex hormone binding globulin(SHBG) has been proposed as a possible marker of the venous risk [143]. OCP containing EE increase SHBG synthesis, but this effect is modified by the progestogen in the pill [143]. An OCP containing the androgenic progestogen LNG is not associated with a high increase in SHBG, while other third generation progestagens,which are less androgenic or antiandrogenic, are associated with a higher SHBG increase as they do not oppose the estrogenic effect. The dose of EE influences the rise in SHBG, but the progestogen constituent in the pill tempers the dose response. The effect of progestins on coagulation factors depends on type and dose of the association with that of an estrogen, the route of administration and the duration of its use. When used with ethinyl-estradiol (EE), several combinations of estrogens and progestins lead to an acceleration of coagulation and fibrinolysis [144]. This is primarily induced by the hepatic impact of EE. Wiegratz et al. in their study showed that LNG has an

antagonistic effect on the EE-induced rise on some coagulation and inflammation markers such as factor VII activity and fragment 1 + 2 and also it reduced the EE-dependent total and free protein S. This effect was not seen with dienogest, which is an antiandrogenic progestin [145]. Therefore estrogen-induced effect on coagulation is counteracted by the progestogens with androgenic properties while nonandrogenic progestins will not have an effect [145-147].

Based on a collective review of studies on the risk of venous thrombosis in women who use combined oral contraceptives, it appears that the OCP containing LNG and 30 μg of estrogen is the safest oral form of hormonal contraception [132].

Progestogen-only pills cause only minor effects on coagulation and fibrinolysis [144]. A systematic review that included 26 published articles did not suggest an increase in odds for venous or arterial events with the use of most progestin-only contraceptives [148].

Progestins and Risk of Breast Cancer

Progestins' effect on the breast cells differs according to the progestin type, dose and duration of use and the resulting balance between cell proliferation and apoptosis [149, 150]. However, in hormonal contraceptives progestins are usually combined with EE and the effect on breast tissue depend on a balance of both hormones. Different progestins have variable effect on proliferation of breast cells. In a study conducted in surgically postmenopausal cynomolgus monkeys conjugated equine estrogen (CEE) plus medroxyprogesterone acetate induced a diffuse epithelial proliferation in the mammary glands but the combination of Ethinyl estradiol and norethindrone acetate did not cause the proliferation of the cells [151]. In another study Conner et al. obtained fine needle biopsy specimens from mammary glands of women using estradiol (2 mg) or estradiol valerate 2 mg plus norethindrone acetate (1 mg) or dinogestrel (2 mg). In the study Ki67, used as a proliferation marker, did not differ significantly between norethindrone or dinogestrel groups, although the dinogestrel group showed a tendency for a weaker proliferation [152].

The Million women study published in Lancet Journal in 2003 reported that hormone replacement therapy in postmenopausal women is associated with an increased risk of incident and fatal breast cancer. The incidence was significantly increased in women taking estrogen-progestagen combinations

than for other types of hormone therapy. For each type of hormone treatment, the risk of breast cancer increased with increasing total duration of use [153].

The risk of breast cancer with hormone therapy varies with the type of progesterone. Fournier et al. reported that the risk of breast cancer was significantly greater with hormone replacement therapy containing synthetic progestins than with hormone replacement therapy containing micronized progesterone, the RRs being 1.4 [1.2–1.7] and 0.9 [0.7–1.2] respectively [154].

Whether the use of hormonal contraceptives increases the risk of breast cancer later in life, when the incidence of breast cancer is increased has been principally studied. Previous epidemiological studies suggested that women who currently use OCP or who have used them in the previous 10 years have a slightly increased risk of breast cancer, whereas women who have used OCP less recently do not have an increased risk [155, 156]. Later a widely conducted population-based, case–control study reported that current or former oral contraceptive use was not associated with a significantly increased risk of breast cancer. Their data showed strong evidence that former oral-contraceptive use does not increase this risk later in life, when the incidence of breast cancer is higher [157].

In a systematic review which included 6 studies on the use of progestin and breast cancer risk. Five of the six studies reported no association between progestin-only formulations (including norethindrone oral contraceptives, depot medroxyprogesterone acetate, injectable, LNG system users, implantable and IUDs) and breast cancer risk [158]. While Soini et al. reported that LNG-IUD use was associated with a higher than expected incidence of breast cancer. They examined the association between premenopausal use of the LNG-IUD and cancer incidence in Finland. The standardized incidence ratio for breast cancer among all LNG-IUD users was 1.19 (95% CI 1.13-1.25; 1,542 observed compared with 1,292 expected cases) [159]. In a later study, the same group reported that the levonorgestrel IUD users had an increased risk for both ductal breast cancer and for lobular breast cancer as compared with the general female population [160].

Safety of LNG Emergency Contraception

LNG has a significantly lower incidence of side effects compared to combination-method ECs [161]. In a randomized, double-blind clinical trial with full results for 1956 women at 21 sites worldwide, LNG (specifically Plan B) demonstrated a superior safety profile relative to the Yuzpe regimen

(i.e., nausea was reduced from 50.5% with Yuzpe to 23.1% with Plan B; vomiting was reduced from 18.8% to 5.6%). There are only three contraindications to the use of LNG EC: existing pregnancy, undiagnosed vaginal bleeding or a known allergy to any ingredient in the product. There is only one report in the literature of a potential drug interaction with LNG-only EC; this was an apparent interaction with warfarin [162].

There were some concerns initially regarding the increase in ectopic pregnancy rate when emergency contraception fails. In a systemic review that included 136 studies, there was no increase in the rate of ectopic pregnancies when the treatment failed. In the LNG studies, 3 of 307 (1%) were ectopic while the incidence of ectopic pregnancy in the general population is around 2%. Because emergency contraceptive pills are effective in lowering the risk of pregnancy, their use will reduce the chance that an act of intercourse will result in ectopic pregnancy [163].

Safety in Pregnancy

There is no evidence that LNG would harm a pregnant woman or would harm a developing fetus if the product is taken accidentally during early pregnancy. Use in pregnant women is contraindicated because the product would be ineffective, not because it has been shown to be unsafe [164–166]. No studies have examined potential teratogenic effects of LNG-EC. However, there have also been no reports of adverse birth outcomes in safety and efficacy studies of the drug covering over 6000 women. Only one adverse birth outcome has been reported to Women's Capital Corporation (the distributors of Plan B) since Plan B has been on the market, and this was following the use of another brand of LNG-only emergency contraception in the UK.

Jatloui et al. reported on the safety of emergency contraceptives. This was based on a PubMed and Cochrane database search for articles published from the date of inception until May 2015 pertaining to the safety of LNG, ulipristal acetate or Yuzpe ECP use. Four studies examined LNG or Yuzpe use among pregnant or breastfeeding women, and one reported the risk of ectopic pregnancy among women repeatedly using LNG ECPs. Poor pregnancy outcomes were rare among pregnant women who used LNG or Yuzpe ECPs during the conception cycle or early pregnancy. Breastfeeding outcomes did not differ between women exposed to LNG ECP and those unexposed, and there was no increased risk of ectopic pregnancy versus intrauterine pregnancy after repeated use of ECPs compared with nonuse. One study demonstrated that LNG passes into breastmilk but in minimal quantities. Studies suggest that

serious adverse events are rare among women taking any of these ECP formulations [167].

Levonorgestrel is a highly effective and safe synthetic progestogen used in different forms, for contraception and treatment of certain gynecological conditions.

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Chapter 5

CHILDHOOD OBESITY IN THE UNITED KINGDOM: CHALLENGES IN MANAGEMENT

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ABSTRACT

Childhood obesity has been on the increase over the last several years, and has been described as reaching epidemic proportions in the developed world. Current estimates are as high as 30% of children in the United Kingdom are overweight or obese. Obesity in childhood has brought with it the rise in previously rare childhood complications of type 2 diabetes, non-alcoholic fatty liver disease and significant mental health issues relating to obesity. Many public health and health-economic authorities have focused on the significant health and financial burden that could be posed by the possibility of entire generations of children growing up to be overweight or obese adults. The challenge faced by the global community is in managing these children and adolescents with obesity. Drug therapy is limited, and weight-loss outcomes from community based programmes have been limited. More focus has recently been placed on surgical interventions – but these have proved controversial in many sectors of the society.

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INTRODUCTION

Childhood obesity represents an evolving pandemic, with 30% of English children noted to be overweight or obese. This is comparable to rates in America where 32% of children are classed as obese or overweight [1, 2]. Globally, an estimated 43 million preschool children (aged less than 5) were overweight or obese in 2010, representing a 60 percent increase since 1990 [3]. The World Health Organization, [4, 5] United States (US) Centers for Disease Control and Prevention, [6, 7] and the International Obesity Task Force [8] have some variation in the definitions of overweight and obesity in children. Therefore at different ages, this can result in slightly different estimations of the prevalence of overweight and obesity [9]. United Kingdom (UK) definitions of overweight are based on comparisons of body mass index (BMI) for age and sex on the UK 1990 growth reference (Table 1) [10].

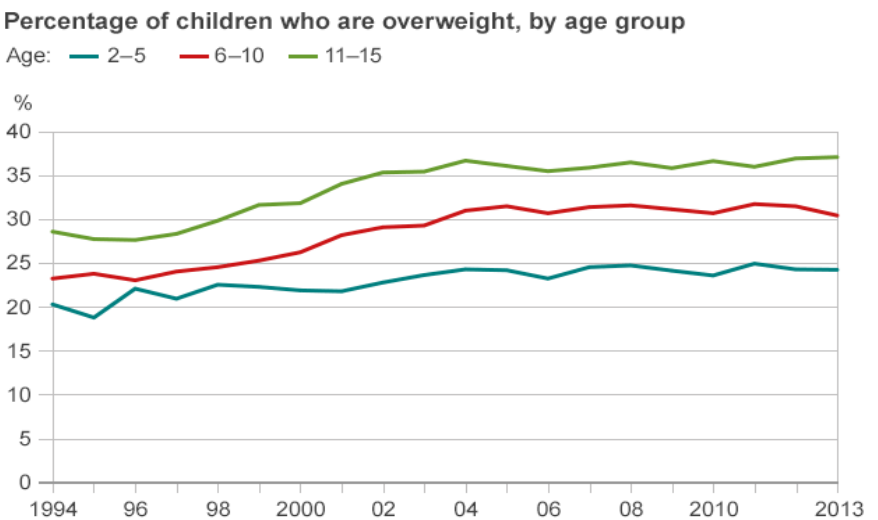
Table 1. Levels of overweight/obesity

Overweight	>91st centile
Obese	>98th centile (>2S.D)
Severely obese	>99.6th centile (>2.67 S.D)
Very severely obese	>3.5 S.D
Extremely obese	>4 S.D

In the U.S. the definition is based on the standard growth charts developed by the Centers for Disease Control and Prevention. In children and young people aged 2 to 20 years old, a BMI in the 85th to 94th percentiles for age and gender is considered overweight; a BMI in the 95th percentile or higher is considered obese. Studies over the last three decades (up to around 2010) have shown the frequency of childhood overweight and obesity to be on the increase – some figures suggesting a tripling of numbers over the last few decades [11]. The prevalence of child obesity in the U.S. was stable through the 1960s and 1970s, but then began to rise in the 1980s. There were no national surveys of child obesity prior to 1963. Authors von Hippel and Nahhas used data from a local study near Dayton, Ohio, to extend the history of child obesity back to the 1930s. Although girls' BMIs were already increasing in the 1930s, obesity among both boys and girls was very rare until obesity prevalence started increasing after birth year 1970. The results are in keeping with the theory that the obesity epidemic is primarily a recent phenomenon [12]. The problem of obesity is not confined only to the

developed world – indeed obesity has replaced malnutrition as the leading nutritional problem in certain parts of Africa, in particular among the poorer, less educated families residing in the urban cities [13].

More recent data however (2010-2015) suggests that the exponential increase in childhood obesity prevalence is abating. This could be in part due to the effect of healthy-living campaigns, but it could also be a reflection of how data is collected and analysed [14-17]. Experts from the University College of London (UCL) suggest that childhood obesity may now be stabilising in particular among the under-10s, but it is too early to say if this plateau will continue. The same study also reports that obesity is starting earlier in life [18]. Eustace De Sousa, national lead for children, young people and families, at Public Health England, said: "Almost one in 10 children in Reception are obese - but what's even more shocking is that by the time they leave primary school, this doubles to nearly one in five."



Source: King's College London.

Figure 1. percentage of children who are overweight by age (UK statistics). Courtesy of http://www.bbc.co.uk/news/health-32797769.

It is important to consider the aetiology of childhood obesity. There is a genetic pre-disposition to obesity, but this interacts with the balance between activity levels (energy expenditure) and nutritional intake (energy intake). Some health conditions can cause weight gain however these problems are uncommon and account for less than 1% of cases of childhood obesity. Certain

medications, such as steroids and antidepressants, may also cause weight gain. Over the years, there has been a move to an increasingly sedentary lifestyle. Children play less outdoors, and spend more time indoors. Recreational activity is now focused on television, video games and gaming apps – with a phenomenal increase in 'screen time'. Changes in societal eating habits are also an important factor affecting recent trends in obesity. There is a greater consumption of processed foods, refined sugar and food portions are also generally larger.

Treating the children who are already overweight and obese remains a huge challenge. The majority of children who are obese and remain obese by school-age will become obese adults [19, 20] with a consequent decrease in life expectancy of anywhere between 5 to 20 years [21-23]. Overweight and obesity also contribute to significant physical, mental and emotional morbidities [24], summarised in Table 2, below.

Table 2. Complications of Overweight/Obesity

Physical		**Psycho-social**
Cardiovascular: Hypertension Heart disease Respiratory: Obstructive Sleep Apnoea Exacerbation of asthma Others: Benign Intracranial Hypertension Fatty Liver Disease/Cholelithiasis Obesity-related cancers	Metabolic: Hyperlipidaemia Insulin resistance Type 2 diabetes Polycystic Ovarian Syndrome Orthopaedic: Slipped Upper Femoral Epiphysis Blount disease	Social discrimination Bullying Poor self-esteem Depression Poor school attendance Eating disorders

The cost implications of obesity on health systems cannot be over-emphasized. In a systematic review by Muller-Riemenschneider et al. (2008), the authors sought to summarise the cost estimates and compare the costs attributable to obesity across different European cities. Of 797 publications meeting their search criteria, 13 studies were felt to be relevant to the review.

These studies investigated the health economics of 10 Western European countries in relation to obesity. The results showed obesity-related burdens of up to 10.4 billion euros. Reported relative economic burdens ranged from 0.09% to 0.61% of each country's gross domestic product [25]. In 2006/2007 obesity and obesity-related ailments cost an estimate £148 million for in-patients admissions in England [26]. The National Obesity Observatory's report on the economic burden of obesity (2010) quoted the estimates of the direct National Health Service (NHS) costs of treating overweight and obesity, and related morbidity in England as ranging from £479.3 million in 1998 to £4.2 billion in 2007. The estimated indirect costs (arising from the impact of obesity, for example due to a loss of productivity) ranged from £2.6 billion to £15.8 billion [27]. The most recent data suggests that with the current rate of obesity and overweight conditions, the cost to the NHS could increase from between £6bn and £8bn in 2015 to between £10bn and £12bn in 2030 [28]. Healthcare costs in the US relating to obesity are similarly alarming. In 2010 reported estimates for these costs were in the region of $315.8 billion per year [29].

Managing childhood obesity has become a priority for the NHS [30, 31] and also for many public health bodies across the world. It is recognised that 'prevention is better than cure' and indeed many campaigns have been focused on raising the awareness among families, school and health professionals in identifying overweight/obesity and how to prevent it. It is beyond the scope of this chapter to discuss all the public health interventions that have been proposed in preventing obesity rates, and we shall focus instead on treating obesity. An important aspect of managing childhood obesity is identifying which children need treatment and referral to specialist service providers. The Obesity Services for Children and Adolescents (OSCA) network published a consensus statement on assessment of childhood obesity in secondary care [32]. The paper delivers an expert opinion on which children should be seen in secondary care, and provides an algorithm for investigation. The options for treating obese children include lifestyle modification programmes (diet, exercise and behaviour modification), pharmacotherapies, and more recently – surgical interventions [33].

LIFESTYLE MODIFICATION PROGRAMMES

Introduction

Many healthcare providers and public health workers believe that the focus of management strategies for childhood obesity should target lifestyle interventions. An essential aspect of promoting such interventions is early, accurate identification of overweight and obese children. In the UK the National Child Measurement Program (NCMP) was established to identify children at the start of school life. [34] This program involves measuring children and providing comprehensive data on obesity among children. Children are measured in the reception year, age 4 to 5 and in the final year of primary school aged 11. The findings of the NCMP are used to inform local planning and delivery of services for children and gather population-level surveillance data to allow analysis of trends in weight. One of the key findings of the NCMP for England is that there has been a strong positive relationship between deprivation and obesity prevalence for children in each school year, with obesity prevalence being significantly higher in deprived areas. The prevalence of obesity is also found to be higher in urban areas than rural areas. The following section focuses on the outcomes of various community and government initiatives for weight-loss management in children in the UK.

MEND

One of the first, and probably most successful community weight programmes, was the MEND (Mind, Exercise, Nutrition, Do it) program study. The intervention was designed to be run by non-specialists in community settings and was a family-based obesity intervention, aimed at children aged 7-13 years, and it was funded by a variety of partners including corporate organisations and government agencies. In a randomised controlled study [35] obese children (BMI $\geq$ 98th percentile, UK 1990 reference data) from five different sites in the UK were randomly assigned to intervention or waiting list control (6-month delayed intervention). Parents and children in the intervention group attended eighteen 2- hour group educational and physical activity sessions. These sessions were held twice weekly in sports centres and schools, followed by a 12-week free family swimming pass. Waist

circumference, BMI, body composition, physical activity level, sedentary activities, cardiovascular fitness and self-esteem were assessed at baseline and at 6 months. Children were followed up at 12 months from baseline (0 and 6 months post-intervention for the control and intervention group, respectively). Participants in the intervention group had a reduced waist circumference z-score (−0.37; $P < 0.0001$) and BMI z-score (−0.24; $P < 0.0001$) at 6 months when compared to the controls. Significant between-group differences were also observed in cardiovascular fitness, physical activity, sedentary behaviours, and self-esteem. At 12 months review, there was a sustained reduction in the parameters measures in children in the intervention group with a reduced had waist and BMI z-scores by 0.47 ($P < 0.0001$) and 0.23 ($P < 0.0001$), respectively. The benefits in cardiovascular fitness, physical activity levels and self-esteem were also sustained.

A key strength of the MEND Program was its acceptability to families with a good uptake of the program with a mean attendance of 86%. This high-attendance rate suggests that families found this intensive community-based intervention acceptable. One of the limitations was the relatively short follow-up of only twelve months from baseline for the intervention group only which limits conclusions about the long-term effects of the intervention [35]. Overall, MEND remains one of the most successful community weight intervention programmes in the UK.

Change4Life

In January 2009, Change4life was launched with major advertising campaigns on television and on billboards, newspapers and online. The aim was to address the sensitive subject of obesity. The approach was sympathetic, not blaming the individual but highlighting the role of modern life such as abundant food choices and sedentary lifestyles [36].

By March 2009, the advertising began to show real people making changes to their lives – for instance, changing to sugar-free snacks, cutting back on fat, joining walking groups and generally being more active. The public were encouraged to join in and be part of the movement online or by telephone.

The vital objective was making parents more aware of the serious health implications of being overweight or obese. The group worked to identify realistic and achievable behaviours which could be changed. Examples were as shown in Table 3 below.

Table 3. Recommended initiatives for Change4Life

1. Sugar Swaps – swapping sugary snacks and drinks for ones that are lower in sugar, so as to decrease children's overall calorie intake.
2. Meal Time _ encouraging kids to have regular, proper meals as growing bodies respond better to routine.
3. Snack Check - keeping a careful check on snacks that kids have. Many snacks are packed with sugars, salt, fat and calories.
4. Me Size Meals – encouraging children and parents to look at the amounts of food in relation to a child's age. Too big or too small portions are not good.
5. A Day – this is to encourage children to have at least five portions of fruit and vegetables a day.
6. Cut Back Fat – we all know too much fat is bad for us. But it's not always easy to tell where it's lurking and hence is important to look at food labels.
7. 60 Active Minutes – kids are encouraged to have at least 60 minutes of activity a day to help them stay happy and healthy.
8. Up and About – keeping children and families from leading a sedentary life style and spending too long sitting down.

To create a united message, change4life encouraged co-branding. The Change4Life logo could be used on your materials (posters, leaflets or promotional items) as a co-brand or as the main brand on any healthy living initiatives around improving diet and increasing physical activity levels. Any individuals, councils or companies involved in developing healthy living initiatives could also use the sub brands from Change4Life, such as Walk4Life, Cook4Life or Bike4Life.

Brilliant Futures

A programme linked to the change4life was Brilliant Futures. This aimed at increasing healthy eating among children aged 2 to 11 in Portsmouth [37]. It was commissioned by Portsmouth city council and NHS Portsmouth's healthy Pompey programme. The Portsmouth Healthy Pompey programme aims to make activity and healthy food choices easier for people in Portsmouth The

overall aim of the programme was to become a city where all people are healthier, feel well, and are empowered to take responsibility for their health.

The lessons learnt from this programme were that family structure is a key driver of behaviour. There is a major disparity between claimed and actual behaviour and many parents believe they are already doing many of the desired behaviours and don't see the need to do more of them. Many of the behaviours identified in the Change4Life programme (portion size, snacking, fat content) were prevalent in the target wards. Some issues are more readily recognised as problems by parents (e.g., fat content). In terms of physical activity, there is a need to allay perceptions around safety in public spaces. There is a lot of importance placed on cost and convenience – reliance on the freezer came through as a strong theme in the research. The competition, and services that are working well, recognised the need to look after and provide benefits for the parent as well as the child. Many of the parents reported they cooked but in reality were simply heating up frozen food or ready meals.

PhunkyFoods

The PhunkyFoods mission is aimed to improve the dietary and physical activity habits of young children (aged 2-11 years) across the UK by helping to create supportive learning environments for health in early year's settings and primary schools. Rapid increases in childhood obesity prevalence means that we must help empower children, alongside their families and communities, to take control over their wellbeing to enable them to live longer, healthier and more fulfilled lives. The PhunkyFoods Programme aims to help early years settings and primary schools to deliver a whole-school approach to healthy lifestyles and to engage with all pupils, and their families, in promoting tangible health behaviour changes in a fun, lively and positive manner. As well as over 400 curriculums linked lesson plans, a number of parent and pupil workshops and class and school assemblies are conducted. As part of the phunkyfoods initiative, all staff were provided with the training they need to deliver top quality, fun healthy lifestyles activities in their setting. There was a range of training options including innovative training films. A fabulous resource box containing everything a setting will need to run the programme including large eat-well guide floor mat, food photo cards,

interactive whiteboard DVDs, big books and games, music CDs, workbooks and posters was developed and provided [38].

Alive'N'Kicking

Alive'N'Kicking (ANK) is another programme aimed to promote a healthier lifestyle for families. This was a highly successful children's lifestyle weight management service that helps overweight children and young people and their families to reach and maintain a healthier weight. The service provides age specific programmes for families with children aged between 2 to 19 years old. Each categorised programme is specifically designed to provide age appropriate messages, activities and behavioural change strategies that will benefit the whole family.

The latest report of ANK in 2015 showed that if the 4742 families enrolled in this programme and 1687 children passed through the schools obesity prevention programme. 81% of children had a reduced BMI z score with 65% of children increasing their 4 hour cardiovascular fitness over the duration of the programme. 70% of children also improved their self-esteem [39].

'Make It Count'

'Make it count' is an obesity treatment and prevention programme for children aged 4 to 7 years in primary schools in Walsall. Make It Count is a 12-week programme and uses a whole family approach to increase their physical activity levels and knowledge of healthy eating. The programme's objectives include making parents more aware of their current lifestyle behaviours and how to make small changes to become healthier. Children are encouraged to be more physically active and parents are encouraged to become active with their children too. The programme aims to increase the knowledge of children and their parents about healthy and unhealthy foods and it familiarises them with the Eat Well Plate and with Change4Life resources.

Workshops for parents are also included as part of the programme to educate them on preparing healthy meals and tips on how to reduce salt, sugar and fat. The children are assessed for height, weight, knowledge of healthy eating, and emotional development with a POMS (Profile Of Mood States)

activity which assesses how a child is feeling at each session, so the effect activity has on mood can be monitored.

The parents also provide feedback on home-life before and after the programme. Currently the programme has helped over 200 families by working with 20 different schools across Walsall. Two groups of programmes have been evaluated so far, and a further one in the evaluation stage. The results so far have been very positive with the evaluation of the pilot programmes showing that 42 children were involved across four schools. 93% of the participants in the first group reduced or maintained their BMI. The second group of programmes showed that 85% of parents reported that they had made at least one healthier lifestyle change [40].

Snack Right

The Snack Right campaign was started to encourage parents and carers of pre-school children in deprived areas of Cheshire and Merseyside. The families were taught to replace at least one unhealthy snack in their child's daily diet with a healthy one.

The campaign targeted interventions for parents of pre-school aged children and involved social marketing techniques. Interactive events were organised for children and parents to try fruit and veg, create healthy snacks, sign up for Healthy Start vouchers, engage with life size banana characters, and play games. A range of resources including a story book, sticker book, branded stationary, plastic snack bowl and leaflets were developed and used. Partnership with 'good competitors' to help promote the campaign and avoid mixed messages were used. The partners included parenting groups, SureStart and breastfeeding groups [41].

Summary

The Public Health England obesity, knowledge and intelligence group are aware of the need to improve the evidence base for the effectiveness of interventions to prevent obesity. This requires pragmatic evaluations of on-going prevention programmes in addition to formal academic research.

To improve the quality and consistency of evaluations, the Obesity Knowledge and Intelligence team in Public Health England (formally National Obesity Observatory) developed a Standard Evaluation Framework (SEF) for

weight management interventions. This has been widely used and has been considered a mandatory requirement by some commissioners.

The aim is to improve the evaluation of both physical activity and dietary interventions, through the development of two specific SEFs that could be used to help evaluators collect standardised data to allow comparison between similar programmes. The SEFs for diet and physical activity were developed in conjunction with leading academics and public health practitioners, to ensure that the document was evidence-based and suitable for practical application by public health practitioners.

MEDICAL MANAGEMENT

Introduction

Where conservative measures such as lifestyle changes and weight loss programmes have failed, clinicians can consider drug therapy. However, choices of drug therapy for paediatric obesity are considerably limited, and drugs may have significant side effects. This section will cover indications for medical management of obesity, current drug therapy options, advantages and limitations of specific medications, and possible future avenues for development.

The Food and Drug Administration (FDA) published obesity drug guidance in 2007 [42] which outlined requirements for clinically significant results of obesity medications. For a drug's result to be clinically significant, the difference in mean weight loss between active-treated and placebo-treated groups needs to be at least 5%, or the proportion of subjects who lose at least 5% baseline body weight in the active-treated group needs to be at least 35% and approximately double the proportion who lost at least 5% in the placebo-treated group. The FDA also states that the drug should show an improvement in metabolic biomarkers, for example, blood pressure and lipids.

The 2007 Draft Obesity Guidance also made several recommendations regarding obesity drug therapy for children and adolescents specifically [42]. Before being investigated in the paediatric population, all obesity medications must first be studied in adults. Initial studies of a drug's pharmacokinetics in paediatric subjects should also be considered prior to full trials, enabling correct drug doses to be established. Ideally trials should be randomized controlled double-blinded trials, of one-year duration. Before trials in children

aged less than 12 years are initiated, studies should first be carried out in adolescents aged 12-16 years old. Only once drugs have been shown to be safe in this age group can they then be investigated in children under 12 years old. Furthermore, due to changes in linear growth in children, the FDA recommends change in BMI should be the primary efficacy parameter of obesity drugs in children, as opposed to weight loss [42].

There is currently only a narrow range of pharmacological treatment options available for childhood obesity. There are several reasons for this, including a lack of good quality trial data and ethical considerations regarding clinical trials involving children. There has also been debate regarding specific weights for drug dosing in paediatric obesity, and several drugs have already been taken off the market due to their side effects, as we will discuss below [43].

In the UK, the use of drug therapy for childhood obesity is closely regulated. According to the National Institute of Health and Care Excellence (NICE) guidelines, drug therapy in children should only be considered if weight modification programmes have failed [44]. In children less than 12 years old, pharmacotherapy is only recommended in exceptional circumstances and if the child has severe co-morbidities. In children over 12 years old, NICE recommends drug therapy only if the child has severe psychological or physical co-morbidities (for example, orthopaedic problems or sleep apnoea). Orlistat is the only drug recommended by NICE for paediatric obesity. In all cases, drug therapy must be initiated and monitored by specialist paediatricians, working within highly experienced multidisciplinary teams. In cases where Orlistat is prescribed for children, NICE recommends an initial trial period of 6-12 months. Within this period the child must be reviewed regularly to assess the effectiveness of the drug therapy, monitor any adverse effects, and assess the child and family's adherence to the treatment. In children over 12 years old drug treatment may be continued in primary care where local circumstances, e.g., shared protocols, and licensing allow [44].

Scottish Intercollegiate Guidelines Network (SIGN) guidelines regarding pharmacological treatment in young people recommend Orlistat only for severely obese adolescents, with a BMI greater or equal to the 99.6th centile, with co morbidities, or those with very severe to extreme obesity who are attending a specialist clinic [45]. Again, SIGN like NICE, recommends regular reviews throughout the period of use, to assess effectiveness and monitor for any adverse effects.

The Past: Past Drug Therapies

Sibutramine

In considering the medical management of paediatric obesity, it is important to first remember Sibutramine - an inhibitor of noradrenaline, serotonin, and dopamine reuptake. This drug was withdrawn from the UK market in January 2010 due to its association with increased cardiovascular risk. Although the effectiveness of Silbutramine had been proved even in adolescent trials, the SCOUT trial (Sibutramine Cardiovascular Outcomes Trial) demonstrated that Sibutramine use in patients with cardiovascular conditions was associated with a 16% increased risk of myocardial infarction or stroke, although no greater risk of death overall [46]. However, due to these risks, the European Medicines Agency suspended use of Sibutramine. The initial use of Sibutramine and its subsequent withdrawal highlights the need for rigorous assessment of long-term outcomes in drug development.

The Present: Current Drug Therapies in Use

The following three drugs are possibly the most commonly used anti-obesity drugs in clinical practice within the UK – though their use for this indication is off-label.

Orlistat

Orlistat is the most commonly prescribed drug in the medical management of childhood obesity and is the only drug licensed by the FDA for use in children aged 12 years and over [47]. It is not licensed in the UK, with guidance from the Medicines and Healthcare Products Regulatory Agency (MHRA) advising its use only in adults aged 18 and over [48]. Orlistat is marketed in Europe as Xenical® and is available as 120mg capsules. It is also available as 60mg capsules under the brand name ‘alli’. Orlistat works by reducing absorption of fat - it irreversibly binds gastric and pancreatic lipases, thereby inhibiting the hydrolysation of dietary fats into the absorbable free fatty acids and monosaturates [49, 50]. Orlistat has a good safety record, owing to its low systemic absorption, and it is mostly excreted in the stools [49]. Studies show reduced fat absorption of up to 30% in patients taking Orlistat [51].

A meta-analysis study by Viner et al. [52] looked at two randomised controlled trials [53, 54] and concluded that combined lifestyle modifications with Orlistat could reduce BMI by 0.83 kg/m^2 compared to placebo, with an average weight loss of 2.3kg over a total period of 12 months. Another meta-

analysis carried out by Czernichow et al. [55] examined a different group of studies [53, 54, 56] and concluded that Orlistat resulted in an even greater reduction in BMI of 1.67kg/m^2, with weight loss averaging 6kg.

Orlistat is typically given at a dose of 120mg three times a day with meals [43]. The main side effects associated with Orlistat are gastrointestinal, occurring in up to 50% patients [53, 12]. These include: flatulence; oily loose stools; oily spotting; faecal urgency; and faecal incontinence [57]. Not surprisingly, this side effect profile is known to lead to poor compliance amongst some adolescent patients [43]. Another important effect is that Orlistat can lead to poor absorption of some fat soluble vitamins, in particular of beta-carotene and Vitamin E, meaning supplementation is recommended [58].

Fewer studies have investigated use of Orlistat in younger children. Norgren et al. carried out a pilot study in Sweden examining the effects of Orlistat in 11 severely obese children aged 8.3-12.3 years, for a 12-week period [59]; their findings indicated that the participants tolerated the treatment well and were able to reduce their fat intake to manage side effects. A median weight loss of 4kg was reported, which was significantly correlated with a decrease in body fat. The authors reported that all children and guardians expressed an interest in continuing with Orlistat after the trial period ended [59].

Overall, whilst Orlistat remains the most commonly prescribed medication in the management of childhood obesity, its use in children in the UK is often limited by its side effect profile and only modest effects on weight loss. Subsequently, much time and effort is being invested in developing alternative medical treatment options.

Metformin

Metformin is a biguanide derivative, which acts as an oral antihyperglycaemic agent to reduce blood sugar levels. It achieves this by improving sensitivity to insulin in peripheral tissues, and both reducing hepatic glucose production and absorption of glucose in the intestines [60]. Metformin is used widely in treatment of diabetes mellitus, and is approved by the FDA for treatment of type 2 diabetes mellitus in children over 10 years of age. Metformin is not currently licensed for use in treating obesity in children, however a UK population-based study published in 2012 analysed prescribing data over a period of 10 years from 2000-2010. The results showed an increase in Metformin prescribing, particularly between girls aged 16-18 years. Metformin was prescribed to manage obesity alone in 7.6% of patients (n=22).

Other indications for prescribing Metformin included polycystic ovarian syndrome (PCOS) and diabetes [61].

A meta-analysis and systematic review was reported by Bouza et al. (2010) to assess the efficacy and safety of Metformin for childhood obesity [62]; their results identified 7 trials which met the inclusion criteria. These trials compared Metformin with placebo and employed behavioural co-interventions, with an average follow-up of 6 months. Metformin was shown to provide a significant decrease in BMI of -1.90(-3 to -8). The main side effects reported were gastrointestinal, and no serious adverse effects were reported.

The MOCA trial was a UK based double-blind, placebo-controlled study, carried out across 6 paediatric endocrine centres. This trial investigated the effects of Metformin versus placebo in obese children and young people with hyperinsulinemia and/or impaired fasting glucose or impaired glucose tolerance. The results showed that Metformin therapy was associated with a significant reduction in BMI-SDS compared with placebo at 6 months [mean difference -0.1 SD (95% confidence interval -0.18 to -0.02), P = 0.02]. Significant improvements at 3 months were found in the Metformin group: fasting glucose, -0.16mmol/litre (-0.31 to -0.00), P = 0.047; alanine aminotransferase, 19% (5-36%), P=0.008; and adiponectin to leptin ratio, 32% (4-67%), P = 0.02. Study results suggest that Metformin is a safe and effective treatment for childhood obesity [63].

A further systematic review of randomised controlled trials assessing the use of Metformin in obese children without diabetes mellitus examined 14 studies, with a total of 946 children aged 10 to 16 [64]. Results showed that children taking Metformin had a pooled difference in BMI change of -1.16kg/m^2, and a pooled weight loss of -3.26kg. Weight loss was greater in children with an initial BMI >35, younger children aged 12 or under, and at 6 months, but there being no statistically significant difference in weight at 12 months. No adverse effects of Metformin were reported by the authors.

Another systematic review of Metformin in obese non-diabetic adolescents and children also concluded that Metformin had modest effects on BMI reduction [65]. Brufani et al. examined 11 trials, 9 of which concluded Metformin was associated with a small but significant reduction in BMI (from -1.1 to -2.7kg/m^2). Again results indicated that there was no statistically significant weight loss at 12 months [65]. These results suggest that there is possibly limited long-term impact on weight loss with metformin. These studies do not provide information on the long-term efficacy of metformin

beyond 12 months, or on considerations of duration of treatment or strategies for compliance.

Metformin has been shown to be relatively well tolerated by children and adolescents, with McDonagh's systematic review highlighting discontinuation of Metformin due to adverse effects occurring in just 1.6% of patients taking Metformin [64]. The most serious side effect of Metformin is lactic acidosis, although no known cases in children have been reported in the literature [64]. Common side effects of Metformin are mostly gastrointestinal, including nausea, abdominal pain and diarrhoea. Studies indicate mild side effects only, settling with temporary reduction of Metformin dose [63]d. In a randomised controlled trial, side effects were most common in the first month of treatment but at the end of the treatment period there was no significant difference in reported side effects between children on Metformin and children taking a placebo [66].

Octreotide

Octreotide has been noted to be effective in the treatment of paediatric patients with hypothalamic obesity [67]. Hypothalamic obesity is a well-recognised condition, which can occur following brain tumours or radiation to the brain. There are two main theories regarding hypothalamic obesity. The first is that structural damage to the ventromedial hypothalamus causes hyperphagia, since the ventromedial hypothalamus specifically is thought of as the satiety centre. The other commonly described theory is that damage to the ventromedial hypothalamus disinhibits the vagus nerve, resulting in stimulation of pancreatic beta cells, which in turn produces very large amounts of insulin. Octreotide is a somatostatin analogue, and so is able to bind to the somatostatin receptor-5 on the membrane of the beta cell. In this way, Octreotide limits the release of insulin. In 2003, results of a key double blind placebo controlled trial investigating use of Octreotide were published, demonstrating beneficial effects of Octreotide on both weight loss and insulin suppression in children with hypothalamic obesity [67].

The Future: Future Drug Therapies for the UK

There are a number of drugs that have been licensed for use in managing obesity in adults in the USA recently. These drugs represent the probable future of medical therapy for obesity in children. It is likely to be several years though before licensing is achieved in children and indeed in the UK.

GLP-1 Agonists

These drugs are glucagon like peptide-1 (GLP-1) receptor agonists, also known as incretin-mimetics. They are well established in the treatment of type 2 diabetes in adults. GLP-1 agonists work by increasing the secretion of insulin, whilst decreasing the secretion of glucagon**.** They also slow glucose absorption into the bloodstream by reducing the speed at which the stomach empties after eating, thus increasing satiety after a meal**.**

Exenatide

Data from adults indicates Exenatide suppresses appetite, resulting in the positive side effect of significant reduction in BMI, body fat and body weight. Weight loss is reported to be progressive and persistent for up to 2 years [68]. Kelly et al. carried out a 6 month, randomised, open-label, crossover pilot trial examining the effectiveness of Exenatide on reducing BMI in both children and adolescents with extreme obesity [69]. Twelve children and adolescents aged 9-16 years old were enrolled in the study, and Exenatide injections were administered subcutaneously twice a day. Results showed children taking Exenatide had significantly lower BMI (-1.7kg/m^2), and decreased body weight (-3.9kg) compared to the control group. However, reduction of percentage body fat was not statistically significant. The authors demonstrated Exenatide had few side effects and was well tolerated by children and adolescents, with mild nausea being the most common side effect in 36%. These symptoms resolved with temporary dose reduction. Although an injection regimen could be seen as a limitation of Exenatide's use in children and adolescents, compliance was quoted as greater than 94% [69].

Liraglutide

Liraglutide is another injectable long-acting GLP-1 analogue, and is also an established drug used for the treatment of diabetes in adults. Results from the SCALE-diabetes study showed that Liraglutide is associated with weight loss (up to -4.0% for 3mg and -2.71% for 1.8mg versus. placebo). Weight loss of 5% or greater was documented in 54.3% and 40.4%, respectively (compared to. 21.4% with placebo) [70]. A meta-analysis of trial data regarding the effects of GLP-1 receptor agonists concluded that they had significant effects on weight loss in both diabetic and non-diabetic obese patients. Furthermore, in this meta-analysis there was no significant difference in efficacy between Exenatide and Liraglutide [71]. The FDA approved the use of Liraglutide for management of weight loss in adults in September 2014 and the drug was subsequently launched for this indication. Approval has been

given for the UK and European Union (EU) and the launch is anticipated in 2016.

Anti-Epileptic Medications

Topiramate

Topiramate acts as a gamma-aminobutyric acid (GABA) agonist and is an established anti-epileptic drug. It is a carbonic anhydrase inhibitor that causes glutamate antagonism, resulting in prolonged satiety. Following reports of weight loss in patients taking Topiramate for treatment of epilepsy, Fox et al. carried out a retrospective study of 28 patients, who received treatment with Topiramate for 3 months, as part of a lifestyle modification programme [72]. The findings of this study indicate that the use of Topiramate, alongside life style modification programmes, is associated with a clinically significant reduction in BMI of 4-6% over 6 months. Importantly, Topiramate was well tolerated, with only 2 patients experiencing paraesthesia. No other side effects were reported [72]. Randomised controlled trials of Topiramate as a weight loss drug in severely obese adults demonstrated weight loss from 4.8-9.7% over a period of 24-64 weeks [73]. Side effects reported in adults were dose dependent and included paraesthesia, dizziness and fatigue. The exact mechanism by which Topiramate causes weight loss is still unclear. It has been hypothesised that Topiramate may result in decreased stimulation of the lateral hypothalamus, thereby reducing appetite. In children, Topiramate has also been associated with decreased levels of the appetite stimulating neurohormone Neuropeptide Y [72]. In children with epilepsy, treatment with Topiramate has been shown to increase the protein hormone adiponectin, which has an important role in metabolic regulations. However, use of Topiramate has not yet been investigated in children with simple obesity [73, 74, 75, 76, 77, 78].

Centrally-Acting Noradrenergic Agents

Phentermine

Phentermine is a centrally acting noradrenergic agent, which reduces appetite by activation of adrenergic and dopaminergic receptors. Phentermine was first approved by the FDA for treatment of obese adults in 1959. Later, in 1973, a requirement for short-term treatment only was added (use for less than

12 weeks), due to concerns regarding the potential addictiveness of Phentermine [79]. Although approval of Phentermine preceded the FDA's requirements for obesity drugs published in 2007, Phentermine remains approved and is used widely in the USA, where it is the most commonly prescribed obesity medication in adults [80]. Despite being only approved by the FDA for short term use in obesity, Phentermine is commonly taken by patients for longer periods off label. However, despite its widespread use, there are limited studies investigating the long-term effects of Phentermine on weight and risk factors for cardiovascular disease. Meta-analysis of the results of 6 separate studies following patients up from 2 to 24 weeks showed that taking 15-30mg of Phentermine per day could reduce weight by an average of 3.6 kg compared to placebo [80]. One study investigating the use of Phentermine either continuously or intermittently for a longer period of 36 weeks in obese adult female patients, showed a weight loss of 12.2kg in participants taking the drug continuously and 13.0kg in participants taking Phentermine intermittently, compared to weight loss of 4.8kg with placebo. However this study was widely criticised for potentially misleading analysis, presenting data for only participants completing the trial [81]. Side effects of Phentermine include insomnia, irritability and anxiety, altered taste, dizziness, tremors and restlessness, as well as changes in pulse and blood pressure [43, 81].

Diethylpropion

Diethylpropion has been shown to have a similar effect to Phentermine, but is less commonly prescribed. A meta-analysis of 9 small studies investigating Diethylpropion use for 6-52 weeks found that use of Diethylpropion 75mg/day was associated with an average weight loss of 3.0kg compared to placebo [82].

Phendimetrazine

Although there is poorer trial data supporting use of Phendimetrazine, it is used more widely than Diethylpropion for treatment of obesity in adults. Observations of Phendimetrazine in the 1960s showed a comparable weight loss to other noradrenergic drugs [83, 84].

Benzphetamine

Benzphetamine is the least commonly prescribed FDA approved noradrenergic drug in the treatment of adult obesity in the USA. Benzphetamine is also the least investigated, with a poor evidence base for its

use [81]. Although noradrenergic agents are widely used in the USA for the treatment of obesity in adults, in Europe, they remain unavailable.

Serotonin-Receptor Agonist

Lorascerin

Lorascerin is another obesity drug approved by the FDA. It acts as an agonist at serotonin receptors (5-HT_{2C}) within the propiomelanocortin neurons of the hypothalamus. This results in decreased food intake and increased feelings of satiety. Importantly, Lorascerin is highly selective for the 5-HT_{2C} receptor, with very little activation of 5-HT_{2A} or 5-HT_{2B} receptors. This is significant, since activation of the 5-HT_{2A} receptor has been associated with neuropsychiatric effects, and activation of the 5-HT_{2B} receptor can cause valvulopathy and pulmonary hypertension [85].

Lorascerin has been tested in two Phase 3 clinical trials, BLOOM [86] and BLOSSOM [87]. In the BLOOM trial, participants either received lorascerin 10mg twice a day or a placebo pill for 52 weeks, alongside diet and exercise interventions. After 52 weeks of treatment the average weight loss in the Lorascerin group was 5.8%, compared to 2.2% in the placebo treated group. Furthermore, the researchers were also able to show significant improvements in markers of cardiovascular risk [86]. In the BLOSSOM trial, 22.6% of participants receiving Lorascerin 10mg twice daily achieved a weight loss of at least 10%, compared to 9.7% of patients in the placebo group [87]. In both trials, echocardiography showed no increase in valvulopathy in those undergoing treatment with Lorascerin compared to those in the placebo group. Side effects noted from these trials include headache, dizziness and nausea [88].

Combination Therapy

Phentermine/Topiramate

A combination therapy of Phentermine and Topiramate, (Phen/TPM) was approved by the FDA in July 2012 for patients aged 16 years and over and subsequently became available in the USA in September 2012 [89]. This combined therapy consists of immediate release Phentermine and delayed-release Topiramate in a capsule, and has been shown to result in an average 12.2kg weight loss over 52-104 weeks, in three different clinical trials [90, 91, 92]. The idea is that combining these drugs allows smaller doses of each drug

to be given (so minimising side effects), whilst enabling two different anti-obesity pathways to be targeted. Phen/TPM, however, was rejected for use in the European Union. The European Medicines Agency cited concerns over the long-term effects of the combined medication on the cardiovascular and central nervous systems, and also the known teratogenic potential of Topiramate [89]. Other concerning side effects of this combined treatment are cognitive and psychiatric effects, and the risk of metabolic acidosis.

Naltrexone/Bupropion

Naltrexone/Bupropion is another combination drug that was approved by the FDA in September 2014 for use in the treatment of obesity. Bupropion is more commonly used in the treatment of depression and smoking cessation, and is a dopamine and norepinephrine reuptake inhibitor. Naltrexone is an opioid receptor antagonist used to treat addiction to alcohol and opioids. Whilst both drugs have been investigated as monotherapies in the treatment of obesity, their individual use resulted in only minimal weight loss. Combined together, however, they are understood to have a synergistic effect, with naltrexone reducing the activation of the hypothalamus in response to food stimuli and Bupropion improving self-control [93]. A recent review of trial data investigating Naltrexone/Bupropion concluded its use led to a moderate weight loss of at least 5%, with minimal side effects, mostly nausea. However, its cardiovascular effects are still not yet completely clear [93]. Naltrexone/Bupropion has been shown to result in an elevated blood pressure and heart rate, and as such further trials to assess cardiovascular risk have been required by the FDA.

Conclusion

In summary, obesity is a highly complex disease, whose presence in any given child can be due to a combination of different factors. Therefore, development of one effective drug, that will only target one such pathway, is inevitably a huge challenge. Whilst Orlistat remains the only obesity medication approved for use in children (used off-licence in the UK), it is only modestly effective and its use is limited by its side effects. Not surprisingly, in the hunt for a better alternative, we have seen different drugs being combined to enable a multi-faceted approach to this complex disease. Of course, for effective treatment of obesity, medical management alone will not suffice. Successful treatment requires specialist multidisciplinary support, with a

combination of different modes of management, including weight loss intervention programmes and in some cases surgery. Ultimately, the development of different drugs for use in obesity should help deliver the goal of personalised, effective treatment, with a patient's management plan being tailored to their own unique disease aetiology.

SURGICAL MANAGEMENT

Introduction

With the recognised limited outcomes in managing childhood obesity with community based weight programmes and drug therapy recent thoughts have now turned to bariatric surgery. Bariatric surgery is an established and well-recognised therapeutic option for obesity in adults, with encouraging results showing a sustained and significant weight loss, as well as a reduction in obesity-related co-morbidities [94]. This review discusses bariatric surgery in children with a review of the international literature available, and incorporates data from the UK.

Background

Recognised forms of bariatric surgery include gastric bypass, gastric band, gastric sleeve [95]. Gastric bypass surgery (Figure 2) is typically done laparoscopically and is sometimes called laparoscopic Roux-en-Y gastric bypass (LRYBP). It is often considered the gold-standard of bariatric surgery and it represents a permanent change in the anatomy of the upper gastrointestinal tract. Laparoscopic gastric band (LGB) surgery involves placing an inflatable band around the upper part of the stomach, thereby creating a small stomach pouch above the band, with the remainder of the stomach below the band (Figure 3). Laparoscopic sleeve gastrectomy (LSG) surgery is a non-reversible single step operation in which 80% of the stomach is removed (Figure 4).

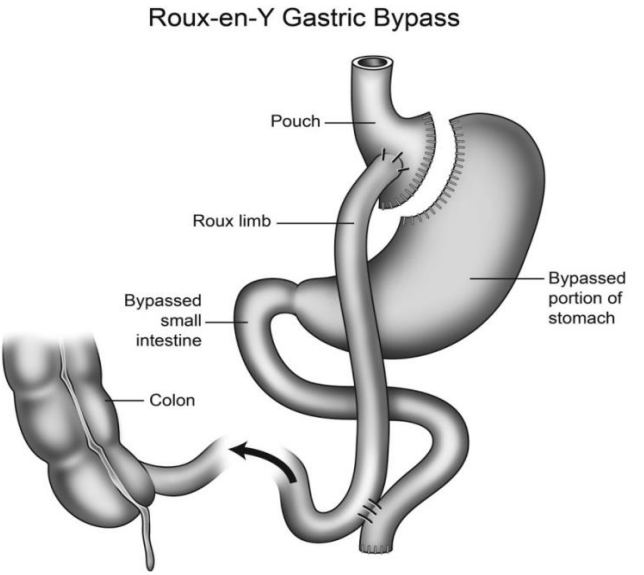


Figure 2. Courtesy of: http://www.thexraydoctor.co.uk/2015/06/xrayoftheweek-25-why-is-this-woman-in.html#.VxgFfHX2bIU.

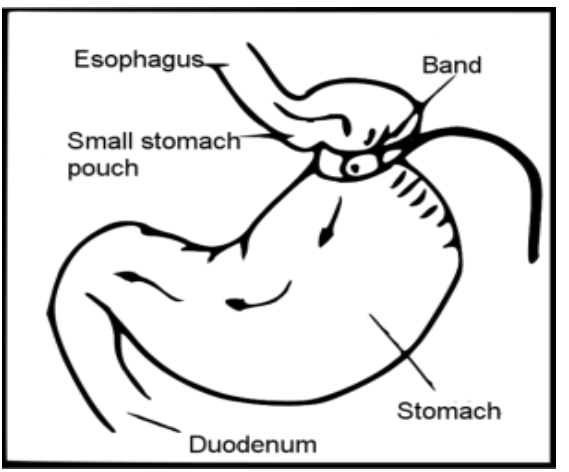


Figure 3. Gastric band. Courtesy of: http://afairgo.net/wp-content/uploads/2014/07/Gastric-Banding-pd-300x281.png.

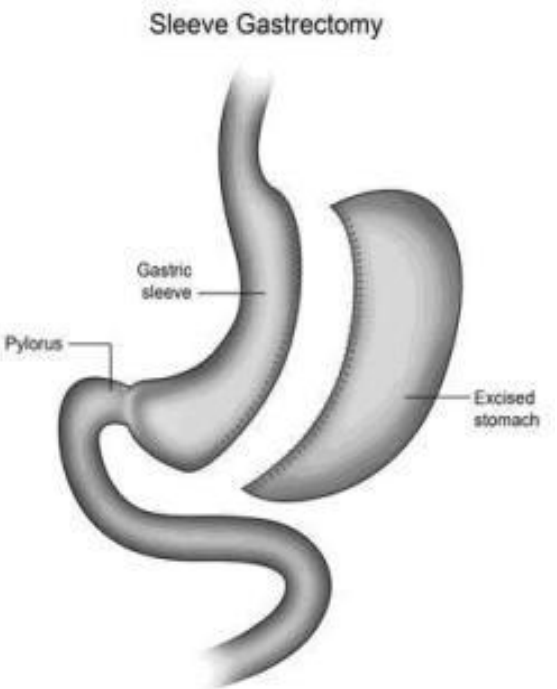


Figure 4. Gastric sleeve. Courtesy of: http://www.theholly.com/uploads/library/hosp7/images/in-page-content/20110518140224-Gastric-Sleeve.jpg.

LRYBP is considered the most effective form of bariatric surgery but it is also a technically more complex procedure with a higher proportion of side effects compared to LSG and LAGB. Gastric banding is generally considered the safest option in terms of rate of complications [96]. Figures from the National Bariatric Surgery Register (NBSR) in 2014 showed that on average, three years after their primary bariatric surgery patients lost 59.6% of their excess weight -65.4% for gastric bypass, n=536; 52.9% for gastric banding, n=453; and 59.0% for sleeve gastrectomy, n=40. The report analysed the cohort of bariatric surgery patients having procedures during the financial years 2011-2013 inclusive, and examines 16,956 primary and 1,327 revisions or planned second stage procedures. The summary below (Table 4) is an extract from the Executive Summary of the NBSR document [97]:

Table 4. Extract from the NBSR Second report executive summary

'In overview:

- 161 surgeons from 137 hospitals recorded 32,073 operations; 18,283 in the three financial years ending 2011, 2012 and 2013.
- In 2011-2013 76.2% operations were funded by the National Health Service; 22.6% were independently funded and a tiny proportion were paid for by private insurers.
- The majority of the analyses include data on operations carried out in the financial years 2011-2013, and include information on 9,526 gastric bypass procedures, 4,705 gastric band operations and 3,797 sleeve gastrectomy operations.
- 95.4% of all primary operations were performed laparoscopically over the last three financial years 2011, 2012 and 2013.
- The observed in-hospital mortality rate after primary surgery was 0.07% overall (and just 0.07% for gastric bypass), much lower than that for many other planned operations.
- The recorded surgical complication rate overall for primary operations was 2.9%.
- These figures compare to the best internationally available outcome benchmarks. Thus, surgery in the United Kingdom and Ireland, in the hands of the contributors, is safe.
- The average post-operative stay was 2.7 days, indicating efficient use of resources.

Follow-up data derived from some 30,933 follow-up entries for the 2011-2012 patients show:

one year after primary surgery:

- On average, patients lost 58.4% of their excess weight (36.6% for gastric banding, 68.7% for gastric bypass and 58.9% for sleeve gastrectomy).
- Over half of patients (64.0%) with pre-operative functional impairment returned to a state of no impairment one year after surgery, meaning they could climb 3 flights of stairs without resting.
- 61.0% of patients with sleep apnoea were able to come off treatment.

two years after primary surgery:

- 65.1% of patients with type 2 diabetes returned to a state of no indication of diabetes, meaning, in practice, that they were able to stop their diabetic medications.

three years after primary surgery for the 2006-2011 cohort:

- On average, patients lost 59.6% of their excess weight (52.9% for gastric banding, n=453; 65.4% for gastric bypass, n=536; and 59.0% for sleeve gastrectomy, n=40).'

An updated systematic review and meta-analysis, 2003-2012, by Chang et al. [94] looked at the effectiveness and risks of bariatric surgery. The analysis included a total of 164 studies (37 randomized clinical trials and 127 observational studies), with 161,756 patients -mean age of 44.56 years and body mass index of 45.62. The results showed a reduction in BMI at 5 years post-surgery of 12 to 17. The complication rate was 17% (95% CI, 11%-23%), and the reoperation rate was 7% (95% CI, 3%-12%). Gastric bypass was noted to be more effective in weight loss but it was associated with more complications. Adjustable gastric banding had a lower mortality and complication rate; yet, the reoperation rate was higher and weight loss was much lower than for gastric bypass surgery. Results for sleeve gastrectomy showed more effective in weight loss than adjustable gastric banding and comparable rates with gastric bypass.

The Cochrane systematic review by Colquitt et al. (2014), -which was an update of previous reviews in 2003 and 2009- reviewed randomised controlled trials (last search November 2103) comparing surgical interventions with non-surgical management of obesity or overweight or comparing different surgical procedures. A total of 1798 participants from 22 studies were included, with sample sizes ranging from 15 – 250. Their results were consistent with previous published data, showing that surgical interventions result in greater weight loss outcomes and more improvement in weight associated comorbidities when compared with non-surgical interventions, irrespective of the type of procedures employed. Outcomes were better for LRYGB and LSG compared to LAGB. Most studies reported follow-up of upto 2 years, therefore there is still a lack of long-term follow-up data, in addition adverse event rates and reoperation rates were generally poorly reported across all the studies [98].

Of all the bariatric surgery procedures done in the UK according the NBSR report, 1.6% of the publically funded procedures (and 2.4% of privately funded surgery) represented gastric balloon insertion. This is a relatively new form of bariatric surgery and the procedure involves inserting an. It is discussed in further detail below.

Bariatric Surgery in Children and Adolescents

Considerations

Bariatric surgery in children has proved to be a largely controversial issue in many settings, including in the UK [99-101]. Perception amongst the healthcare professionals is much more supportive however. In a survey of

members of the British Obesity and Metabolic Surgery Society and groups of primary care practitioners based in London two thirds (66%) of professionals believed that adolescents with a BMI >40 or BMI >35 and with significant co-morbidities could be offered surgery. Parental psychological counselling was felt to be one of the most important pre-requites. Other pre-requisites were 6 - 12 months of being involved in weight management programmes (58% stated 12 months as appropriate, and 24% regarding 6 months as sufficient. The majority of healthcare professionals stated that surgery should only be offered at an age over 16 years, while 17% of bariatric surgeons did not specify a minimum age limit. More than 80% of the professionals surveyed considered bariatric surgery in adolescents as acceptable [102]. Recent reports in the UK media and newspapers also reflect the shift in support of bariatric surgery for children and adolescents, a particular advocate being Dr Ashish Desai, a paediatric surgeon specialising in childhood obesity at King's College Hospital [103]. The argument for surgery is that having a surgical procedure is better and cheaper than treating the chronic effects of complications of obesity such as type 2 diabetes and high blood pressure- this is despite the typical cost for a gastric band being about £7000, but up to £12 000 [104]. Some data shows that morbidly obese people with T2DM who undergo bariatric surgery fully recover the costs of surgery in 2-3 years [105]. This is in part due to a decrease in need for obesity related medications. Obesity and associated illnesses such as type 2 diabetes are estimated to cost the Health Service £5 billion a year. However these conclusions about cost effectiveness should be interpreted with caution as they are somewhat limited due to lack of long term data.

The 2014 UK NBSR was the first in-depth description of bariatric surgery in patients under the age of 25 years old within the UK. For all those aged <25 years, the initial BMI ranged between 31-81 kg m^2 (average of 48.7 kg m^2). There were 62 patients aged ≤18 years having bariatric surgery during the three-years between 2011 and 2013 [97]. The youngest patient was aged 12, with 17 patients aged 16 or less. Media coverage surrounding the fact that a child as young as 12 had received bariatric surgery reflected the shock of society that children that young were requiring surgical intervention to manage obesity [104].

Those who speak strongly against bariatric surgery in children argue several points. A particular concern is that parents/carers are making a very complex decision on behalf of the young person – this is a particular concern if non-reversible surgery is contemplated. The other major concern is the psychological impact of the surgery on the young person. Food, and how we consume food, has a major role in how we fit in with our peers and with

society. Eating provides pleasure and there is a social reward associated with sharing food [106]. The drastic limitation in how much children can consume after having bariatric surgery could further limit their ability to interact socially – on a likely background that they are already socially isolated from a lack of confidence. Micronutrient deficiency is a major concern in considering the health outcomes of children who have had bariatric surgery [107, 108].

In a very elegant paper [109], Bjørn Hofmann (2013) explores the morally relevant challenges surrounding bariatric surgery in children including:

Beneficence and Bariatric Surgery

He cautions that extrapolating results from adults might be flawed, with a definite lack of concrete evidence. The conditions under which bariatric surgery is performed are highly relevant for the outcome, for example preoperative evaluation by a multidisciplinary team, bariatric surgery competency with children and adolescents, postoperative care and follow up and overall family support. Timing of surgery is also crucial as operations may be performed too early -with a poor benefit/harm ratio; and too late -missing relevant health benefits. There is also the question of "benefit for whom?".

Safety and Risk

Bariatric surgery is associated with potentially serious risks and recognized complications, and long-term safety and efficacy in children remains largely unknown. Therefore, surgery should be reserved for only the most severely obese and considered with extreme caution. Adverse events are reported to be frequent even with very experienced surgeons therefore surgery should be performed in specialised, high volume 'quaternary' centres. Very few studies have investigated psychosocial effects of bariatric surgery, with small numbers and short-term follow-up. Some studies have indicated high rates of depression scores and negative self-acceptance in adolescents following LAGB. Interestingly one study reported a significant rate of unplanned pregnancy within the first 2 years following bariatric surgery in adolescent female [110].

Autonomy and Compliance

Interventions aimed at managing obesity can be considered in the best interest of the common good, since childhood obesity is expected to result in significant health problems. However, some argue that such interventions may become and infringe on personal autonomy and become paternalistic. Children and adolescents can give assent to bariatric surgery while the adult with

parental responsibility gives consent. The general assumption is that parents and responsible adults are best placed to know what is best for their child but, for some obese children this can be questioned.

Informed Consent

There is an argument that it is difficult to provide information to families in a way that ensures valid informed assent or consent because there is limited evidence of long-term effectiveness and safety of bariatric procedures. Children and adolescents suffering from depression may also have difficulties with understanding and assessing information. Information provided can be biased and parents and surgeons may be overly optimistic. There may be a withholding of relevant information such as how surgery may change the life of the person in significant ways, variability in treatment outcomes, and the sometimes close relationships between health care professionals and industry e.g., with regards devices and surgical banding techniques. Families often seek surgery because they are desperate, have become scared, and feel judged by society.

The above are only some of considerations that are very important when bariatric surgery is being considered. In the UK NICE produced strict criteria for which children should be considered for bariatric surgery [111]. The guidelines were initially published in 2006, and were updated in 2014, and are outlined in Table 5 below.

Table 5. NICE guidance on surgical interventions for obese children

Surgery
When to consider surgery * Surgical intervention is not generally recommended in children or young people. *Bariatric surgery may be considered for young people only in exceptional circumstances, and if they have achieved or nearly achieved physiological maturity. *Surgery for obesity should be undertaken only by a multidisciplinary team that can provide paediatric expertise in: • preoperative assessment, including a risk-benefit analysis that includes

preventing complications of obesity, and specialist assessment for eating disorder(s)

- information on the different procedures, including potential weight loss and associated risks
- regular postoperative assessment, including specialist dietetic and surgical follow up
- management of comorbidities
- psychological support before and after surgery
- information on or access to plastic surgery (such as apronectomy) when appropriate
- access to suitable equipment, including scales, theatre tables, Zimmer frames, commodes, hoists, bed frames, pressure-relieving mattresses and seating suitable for children and young people undergoing bariatric surgery, and staff trained to use them.

*Coordinate surgical care and follow‑up around the child or young person and their family's needs. Comply with the approaches outlined in the Department of Heath's A call to action on obesity in England.

*Ensure all young people have had a comprehensive psychological, educational, family and social assessment before undergoing bariatric surgery.

*Perform a full medical evaluation, including genetic screening or assessment before surgery to exclude rare, treatable causes of obesity.

Outcomes

Overall there is still relatively limited long term data on outcomes in paediatric bariatric surgery. LRGBP surgery was the earliest surgical intervention used in paediatrics. One of the earliest reports from 1975 describes a series of 25 adolescents (< 20 years) who underwent gastric bypass surgery. There was an average weight loss of 15% of body weight at 6 months, and 25% at 36 months [112]. More recent reports have described substantially larger weight loss outcomes. In a systematic overview published in 2015 Canoy and Yany et al. [113] looked at the effectiveness and risks of bariatric surgery in children. The main purpose of the review was to seek evidence from randomised controlled trials (RCTs) to assess physical, psychosocial, and quality-of life outcomes of bariatric surgery in childhood obesity compared to appropriate control (i.e., no intervention, usual care, or waiting list control), or of different types of bariatric surgery compared with each other. They searched Medline, Embase, The Cochrane Library, and other important databases up to August 2014 and after filtering for inclusion and exclusion

criteria, 48 articles were fully reviewed, and two systematic reviews fulfilled the criteria. The reviews included mostly non-RCTs, and did not identify any RCT comparing bariatric surgery with no treatment, usual care, or waiting list control. Findings from the review of mostly non-RCTs suggested that surgical intervention in the management of severe obesity can lead to a substantial weight loss. However, the actual magnitude of weight loss is generally unclear. In one RCT, laparoscopic adjustable gastric banding resulted in substantial weight loss, improvements in cardiovascular risk factors, as well as improved quality of life when compared to an intensive lifestyle intervention [114] with a suggestion that if the comparison group was given usual or conventional care for obese children, the magnitude of the effect could have been greater in this study. However, overall evidence for the safety and effectiveness and of surgical interventions in treating of paediatric obesity remains poor. Uncertainties around indications, patient selection criteria, post-operative complications, and beneficial versus harmful outcomes beyond weight loss in the short and long term still remain. The other question is How long is 'long term' to assess benefits and harms associated with bariatric surgery in paediatric populations needs further clarification especially when considering that, depending on the age at surgery, a 24-month follow-up would still mean assessing outcomes during childhood in certain patients.

Next Canoy and Yang looked at the different types of bariatric surgery against each other. They found no direct information from RCTs on the effects of different types of childhood or adolescent bariatric surgery. Of the surgical techniques that have been used on of the key considerations to the technique of choice is the reversibility or permanence of the procedure on the gastrointestinal intestinal tract. They found no RCTs comparing the effectiveness between surgical techniques or evaluating the benefits versus harms of the different surgical techniques.

Canoy and Yang's meta-analysis highlights the fact that there are very few RCTs out there to assess childhood bariatric surgery. Until recently most data has been gathered from case series reports, and most of these with fewer than 100 children. A recent pooled data analysis from a well-conducted meta-analysis by Black et al. in the UK, published in August 2013 [115] in the United Kingdom in August 2013, includes studies involving children aged between 6 and 18 years and looked at outcomes across LRYGB, sleeve gastrectomy (SG), and adjustable gastric band (AGB) Findings were noted to be largely in keeping with previous meta-analytical results [116]. The mean change in BMI at 12 months was −13.5 kg/m2 (95% confidence interval (CI) −15.1 to −11.9). The authors were not able to provide summary estimates for

complications in this meta-analysis, but data extracted from currently available reports show complication rates of between 22%−33% for LRYGB [117, 118], 4.3% for LSG [119] and 10%−48% for LAGB [114, 120]. Additional outcomes, summarized from the meta-analysis by Black, and the wider literature, are shown by procedure and combined in Table 6 adapted from a paper by Beamish et al. [121].

Table 6. Short-term outcomes following bariatric surgery in adolescents

Variable	LRYGB	LSG	LAGB	All procedures (95% CI)
BMI reduction (kg/m2)	13.3–22.5	13.0–17.2	8.5–11.7	13.5 (15.1–11.9)
T2DM resolution (%)	67-100	0-68	80–100	0-100
OSA resolution (%)	100	56-80	20–100	20-100
HTN resolution (%)	82-100	69–100	50-100	50-100
Insulin resistance resolution (%)	100	50–96	44–77	44-100
PCOS resolution (%)	100	0	-	0–100
Dyslipidemia resolution (%)	87-100	0-58	35–100	0-100
Operative morbidity (%)	8-33	4.3	10–48	4–48

LRYGB: Laparoscopic Roux-en-Y gastric bypass; LSG: laparoscopic sleeve gastrectomy; LAGB: Laparoscoipc adjustable gastric band; CI: confidence interval; BMI: body mass index;T2DM: type II diabetes mellitus; OSA: obstructive sleep apnea; HTN: hypertension; PCOS: polycystic ovarian syndrome. Based on studies with mean 12-month follow-up

As available reports typically contain a few subjects and limited follow-up, the encouraging figures given above should be treated with caution. In the study with the most long-term follow-up, the sparsely performed malabsorptive procedure, biliopancreatic diversion (BPD) was used. A longitudinal observation of 76 adolescents aged between 14−18 years (mean 16.8) for a mean duration of 11 (range 2–23) years showed impressive prolonged weight loss (78% excess weight loss at longest follow-up), and equally impressive comorbidity resolution: 100% resolution of diabetes and dyslipidemia, and >80% resolution of hypertension [122]. In addition the paper gave a good insight into the evolutionary process of a bariatric procedure and associated improvements in outcomes as the authors concluded that the incidence of protein malnutrition improved across the study. Biliopancreatic diversion is not routinely used in childhood bariatric surgery in the UK.

In a case series report from Sheffield in the UK, Sachdev et al. [123] in 2014 reported on six adolescent patients operated upon between 2004 and

2012. Six patients (4 male) aged 14-16 years (mean age 15.10) underwent bariatric surgery. The mean preoperative body mass index (BMI) was 62.7 kg/m^2 and BMI SDS +4.4. Four patients had LRYGBP, one LAGB and one had a LGS performed. The mean percentage of weight loss, as a percentage of total body weight at 6 and 12 months, was 22 and 27%, respectively (Figure 5). Average absolute weight loss at the most recent follow-up was 54 kg. Mean BMI at 12 months post=procedure was reported at 46.5 kg/m^2-a mean fall of 16.2 kg/m^2. The mean BMI SDS fell from +4.4 to +3.8 at 12 months and +3.1 at 2 years.

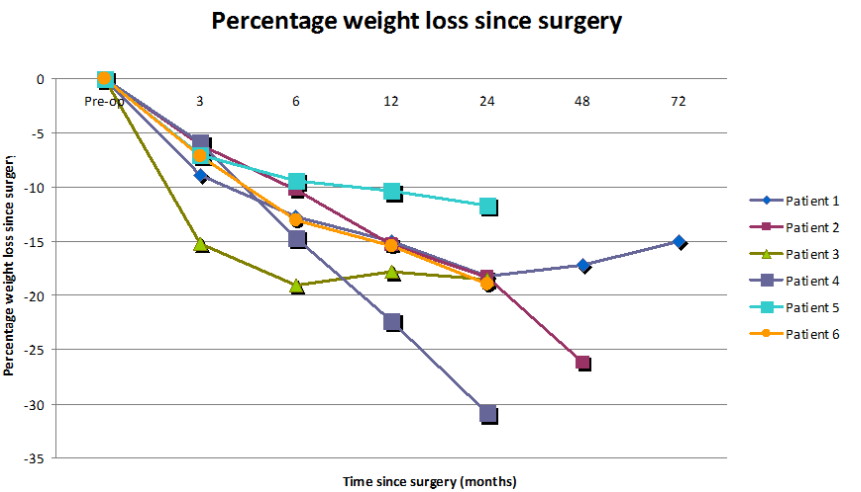


Figure 5. Percentage weight loss since surgery. Courtesy of Sachdev et al, 2014.

Resolution of hypertension, improved attendance at school and no progression to T2DM were the other benefits identified in this report.

Possibly the most recent data we have on outcomes on childhood bariatric surgery is from the Teen-LABS 2016 report published in the New England Journal of Medicine [124]. Teen-LABS is a prospective, multisite observational study which enrolled patients 5 US sites, from February 28, 2007, through to December 30, 2011, with a final analysis cohort of 242. The mean (SD) age of participants was 17.1 (1.6) years and the median BMI was 50.5. Laparoscopic Roux-en-Y gastric bypass, LSG and LAGB were performed in 66%, 28%, and 6% of patients, respectively. Three years after the procedure, the mean weight had decreased by 27% (95% confidence interval [CI], 25 to 29) in the total cohort, by 28% (95% CI, 25 to 30) among participants who had gastric bypass, and by 26% (95% CI, 22 to 30) in those

who had undergone LSG. By 3 years post-procedure, remission of type 2 diabetes occurred in 95% of patients (95% CI, 85 to 100) of participants who had diabetes at presentation, remission of abnormal kidney function occurred in 86% (95% CI, 72 to 100), remission of prediabetes in 76% (95% CI, 56 to 97), remission of hypertension in 74% (95% CI, 64 to 84), and remission of dyslipidaemia in 66% (95% CI, 57 to 74). Weight-related quality of life was also reported to improve significantly.

Complications

Surgery is not without risk and the mortality in adults across all procedures is 0.22%–0.34% (95% CI) [125, 126]. A similar risk in the adolescent population would be more difficult to accept. There is one report so far of a death following bariatric surgery in the "adolescent" population [127], however this was a 19-year old male and could therefore actually classed as an adult. In any case, the death of a young patient provided lessons on cardiovascular disease in bariatric surgery in the young.

Other huge concerns are nutritional deficiencies which commonly already exist in adolescents with severe obesity prior to bariatric surgery and frequently often persist in the postoperative population [105]. Macronutrient deficiencies include protein deficiency. However, micronutrient deficiencies are more common, including trace elements, essential minerals, and water-soluble and fat-soluble vitamins. Surgical sequelae, such as small intestinal bacterial overgrowth, can promote such deficiencies, especially in patients with T2DM. Dumping syndrome is another worrying complication. Concerns have been raised concerning the potential impact of micronutritional deficiencies in the preconception period and during pregnancy, with an impact on both maternal and fetal health. There is concern over the impact of bariatric surgery on the skeleton. Evidence shows reduced bone mineral density and bone mineral content within a year following gastric bypass [121].

The Teen-Longitudinal Assessment of Bariatric Surgery (Teen-LABS) study reported in 2014 on perioperative outcomes of adolescents undergoing bariatric surgery [128]. There were no deaths during initial hospitalization or within 30 days of the operation. Major complications -eg, reoperation were seen in 19 patients (8%) while minor complications -eg, readmission for dehydration- were noted in 36 patients (15%). All reoperations and 85% of readmissions were related to the bariatric surgery. These results are clearly very reassuring. Similarly in the case series by Sachdev et al. there was no mortality reported and the only complication noted was a single port rotation [123]. The Teen-LABS 2016 report quoted earlier [124] adds further 3-year

data - hypoferritinemia was found in 57% (95% CI, 50 to 65) of the cohort participants, and 13% (95% CI, 9 to 18) had undergone one or more additional intra-abdominal procedures. This data re-emphasizes the fact that on-going longterm data is essential for evaluating eventual health and well-being in children and adolescents who undergo bariatric surgery.

Bariatric Surgery in Children and Adolescents in the UK

There is limited experience with bariatric surgery in the UK. As quoted earlier the NBSR 2014 report [97] was the first in-depth description of bariatric surgery in patients aged less than 25 years of age in the UK: n=570. There were 62 patients aged ≤18 years who underwent bariatric surgery during the three-year period 2011-2013 (Figure 6) Unfortunately further analysis including primary operations for female patients: Initial BMI and age; and primary operations for male patients: Initial BMI and age was made for the age group <25 years without isolating the <18 age group. This is clearly one short-coming in this report, which will hopefully be addressed in future editions of the report.

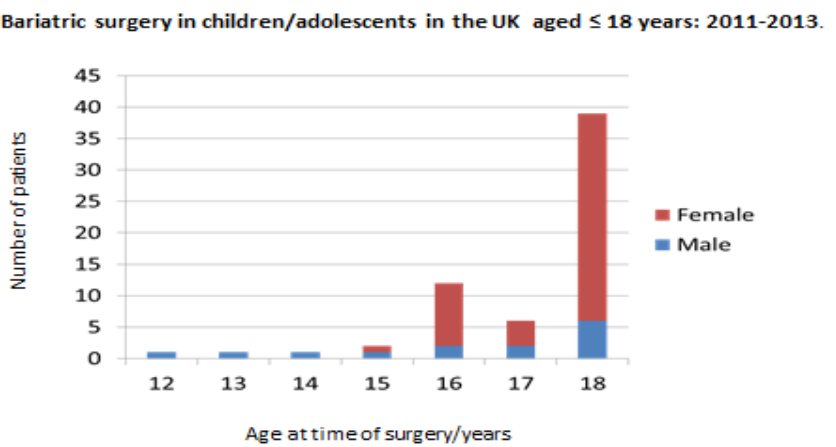


Figure 6. Bariatric surgery in UK children, 2011-2013. Courtesy of NBSR 2014 report, http://nbsr.co.uk/2014-report/.

As discussed above, there are clear guidelines which recommend when children should be considered for bariatric surgery [111]. However it is generally noted that there is chronic under-referral of to the bariatric services [129]. In 2009 around 1 million people met NICE criteria for bariatric surgery with around 240,000 wanting surgery, yet only 4300 operations were performed 2009 [130], see Figure 7, below.

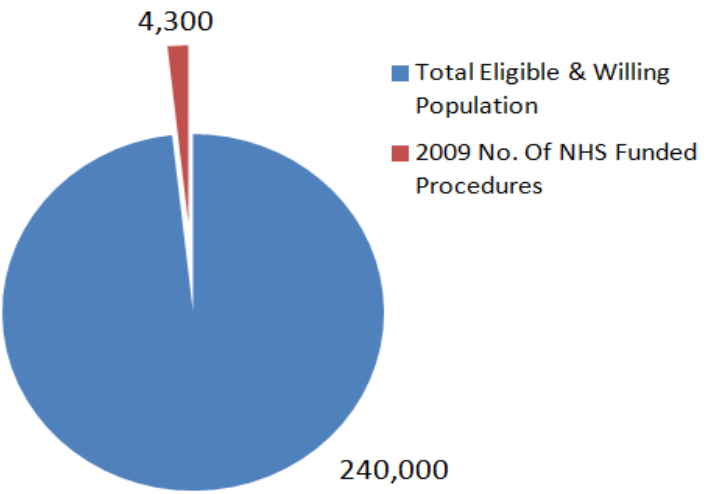


Figure 7. Bariatric surgery in 2009. Courtesy of http://bariatrictimes.com/the-provision-of-bariatric-surgery-in-the-united-kingdom%E2%80%94past-present-and-future-considerations-the-road-to-excellence/.

A similar attitude of under-referral has been evident with respect to childhood surgery. It is believed that this is due in part to the reservations of some clinicians and families to consider bariatric surgery on children, but also certain hospital trusts in conjunction with the Clinical Commissioning Groups (CCGs) – who provide the money and funding for healthcare services provided by the Trusts- have adopted stricter referral criteria – most likely with the aim of deterring referrals and ultimately saving money. To help understand this it is necessary to point out that the majority of bariatric surgery performed in the UK (adult and paediatric) is publically funded under the NHS. The NHS is a healthcare system funded through national taxation. It was established in 1948 with the aim of providing an ideal healthcare system available to all. Care in the NHS remains free to all UK and European Union Nationals at the point of delivery. The NHS has grown to become the world's largest publicly funded health service, and it the world's 5th largest employer after the United States Department of Defence, The People's Liberation Army (China), Walmart and MacDonalds (Chinese army, The Indian National Railway [131]. The CCGs are allocated budgets from the government, and they procure services for provision of healthcare therefore they have a level of discretion at how they spend some of these funds. The NHS has been in financial crisis over the last few years and efficiency savings are high on the

agenda of all CCGs. To support decisions on spending priorities, measurements of outcome rather than process are often required. An economic perspective led to the development of the 'quality adjusted life years' (QALYs) [132]. This is essentially a measure used by cost-utility analysts to calculate cost effectiveness of a particular medical intervention. It is a health status index which measures disease burden by including both the quantity and quality of life the lived. In general a beneficial health care activity is one that generates a positive level of QALYs, and an efficient health care activity is one where the cost per QALY is as low as it can be [133]. Comparisons are made between interventions, and priorities can be made based on those interventions that are low cost per QALY (relatively inexpensive) and those that are high cost per QALY (relatively expensive).

It is not surprising therefore that some CCGs are not convinced that spending thousands of pounds on one individual for what is viewed by many as a 'life-style disease' is the best option. There is still a great belief in many areas that obesity should be managed within the community. Another point of view may simply be that some CCGs are still being cautious about bariatric surgery in childhood and they are awaiting further, longer-term outcomes before committing to this as a standard treatment option.

There are 4 centres in England which perform bariatric surgery in children: Sheffield Children's Hospital (SCH), University College London Hospital (UCLH), Bristol Royal Hospital for Children (BRHC) and King's College Hospital (KCH).

Sheffield published their data in 2015 [123]. Between 2004 and 2012 six patients (4 male) aged 14-16 years (mean age 15.10) underwent bariatric surgery. Bristol has not yet formally published their data but over the last 10 years they have operated on 8 children (personal correspondence via Email). UCLH have performed several operations (data unpublished), while Kings College have the highest number of surgeries (data unpublished). In 2014 King's College London and King's College Hospital – part of King's Health Partners Academic Health Sciences Centre (AHSC) established the first university chair in metabolic surgery when they appointed Professor Francesco Rubino as Professor of metabolic Surgery [134].

Over the years it has become apparent there is not enough density of bariatric surgery in children in individual centres to allow sufficient information gathering. The Paediatric Research in Obesity Multi-modal Intervention and Service Evaluation (PROMISE) initiative was established to facilitate information gathering and sharing for childhood obesity [135]. There are several studies running under this umbrella programme including Study E:

Evaluation of an adolescent bariatric surgery programme. The objectives of study E are to 'investigate the acceptability, safety and short-term outcomes of adolescent bariatric surgery in the UK and to investigate predictors (BMI, psychological, surgical, patient-centred measures) of outcome'. The study aims to recruit 40 sequential patients aged 13-17 who undergo bariatric surgery at UCLH, with assessment at surgery, 1, 3 6 and 12 months post-surgery. The Decision making study has been an additional project, linked to Study E, looking at the decision making process within the adolescent bariatric teams in 3 different hospitals in England as well as how adolescents and their family decide upon bariatric surgery.

There has been a call to consolidate services for bariatric surgery in children, with the establishment of 'quaternary services' and specialist commissioning where these centres would have official designation and direct funding from the government (NHS England) to perform this surgery (Tier 4). This would facilitate referral pathways, information sharing, research and data collection. Providers, surgeons, premises, on site services and obesity surgery throughput should meet the minimum IFSO Guidelines for Safety, Quality, and Excellence in bariatric surgery. There is currently a consultation taking place for service set-up [136].

CONCLUSION

There is clear evidence of the effectiveness of bariatric surgery as a management option for obesity in adults. There is limited long-term data on outcomes and complication in the paediatric group – with a limitation in RCTs and limited data beyond 3 years. Currently the UK is lagging behind the US in terms of numbers of surgical interventions and trials. There is however an initiative to centralise bariatric surgery services in the UK and this should lead to more straight-forward referral pathways and more robust studies and information gathering. This should in turn help build public and physician confidence in adopting bariatric surgery as a viable and realistic treatment option in managing childhood obesity.

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Chapter 6

DYNAMIC AND INTEGRATED SYNAPTIC PROCESSING IN THE SEX-STEROID SENSITIVE MEDIAL AMYGDALA OF FEMALE RATS

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ABSTRACT

The posterodorsal medial amygdala (MePD) is a sexually dimorphic area that contains one of the highest concentration of gonadal hormone receptors in the rat brain. It modulates the timely display of reproductive behavior in both male and female rats. Dynamic changes affect the local synaptic processing along the estrous cycle. These changes are evident in the number of synapses made directly on dendritic shafts or the complex modulation of the density and the shape of dendritic and somatic spines during the short-term proestrus phase, when the levels of estradiol and progesterone reach a peak in circulation. That is, the MePD of females in proestrus has an increase in the number and diversity of somatic spines. In contrast, there is a marked reduction in the density of dendritic spines during this same phase. Spines are specialized postsynaptic neuronal

elements that modulate mainly the excitatory neurotransmission. These findings indicate that the female rat MePD modulates the synaptic input in a complex and dynamic way prior to influencing other interconnected brain areas relevant for the neuroendocrine secretion needed for the ovulation and the display of proceptive behavior. Furthermore, substitutive hormonal therapy to adult ovariectomized females do not resemble the physiological variations in the normally cycling rat. This synaptic organization changes once more when females have the experience of motherhood. In mothers, the reduced density of dendritic spines is likely involved with less avoidance behavior toward pups and for other adaptive skills developed for the new demands from the environment, for nursing behavior and memory elaboration about this life event. These data provide additional insights about the important role of the rat MePD as a model for the study of the link between sex steroids, the cellular specializations for synaptic processing, and the functional organization of the nervous tissue in females.

INTRODUCTION

The medial nucleus of the amygdala (MeA) is a subcortical component of the extended amygdala in the ventral forebrain of rats (de Olmos et al., 2004). According to morphological, neurochemical, hodological, and functional criteria, the MeA can be divided into the anterodorsal, anteroventral, posterodorsal (MePD), and posteroventral subnuclei (Canteras et al., 1995; Petrovich et al., 2001; Dall´Oglio et al., 2008a,b). The MePD contains one of the highest expression of gonadal hormone receptors in the rat brain, which is comparable to some hypothalamic nuclei that control neuroendocrine secretion for reproduction. The MePD has both estrogen receptors α (ER-α) and β (ER-β), progesterone receptors, and androgen receptors (Simerly, 1990; Gréco et al., 1998, 2001; De Vries and Simerly, 2002). These receptors have a complex interaction and dynamics along the estrous cycle and/or after ovariectomy (OVX). As reviewed in Rasia-Filho et al. (2012a), "In the MePD of female rats there were high concentrations of both ER-a and ER-ß (Simerly et al., 1990; Shughrue et al., 1997; Gréco et al., 1998, 2001, 2003). Different regional distribution of these ERs occurs in the dorsal and in the ventral parts of this subnucleus, but both ERs can be co-localized in the same neurons (Gréco et al., 2001, 2003). The replacement treatment with estradiol to castrated females decreased the number of MePD cells immunoreactive to ER-α and those co-expressing ER-α and ER-ß (Gréco et al., 2001). In castrated and hormone primed female rats, the early gene activation expression of Fos-

immunoreactivity following mating occurred in cells co-expressing ER-a or both ER-a/ER-ß in the dorsal MePD (Gréco et al., 2003). This same Fos detection occurred in cells only co-expressing ER-ß in the ventral MePD (Gréco et al., 2003). Progesterone receptors also showed complex dynamics in the female MePD along the estrous cycle or after castration (Gréco et al., 2001; Isgor et al., 2002). In addition, some MePD cells co-express ER-ß and progesterone receptors (Gréco et al., 2001)".

These data indicate the complexity of actions that sex steroids can have in the MePD of females along normal ovarian cycles or during the reorganizational period of the brain following OVX. Indeed, there is a variable hormonal modulation of the postsynaptic dendritic spines or the direct axonal contacts on dendritic shafts in the neurons of the female MePD (Rasia-Filho et al., 2004, 2012a; Brusco et al., 2014). In conjunction, the interface between gonadal steroid actions and neuronal function in the MePD is important for the interpretation of the social relevance of both olfactory and vomeronasal stimuli (Pro-Sistiaga et al., 2007; Westberry and Meredith, 2016) and motivation to socially investigate a conspecific (Dumais et al., 2016), for the response to genitosensorial stimulation and the modulation of sexually receptive behavior (Coolen et al., 1997; Pfaus and Heeb, 1997; Newman, 1999), for avoidance of the offspring or the maternal display (Fleming et al., 1980; Sheehan et al., 2001), for anxiety and conditioned or innate fear (Adamec and Morgan, 1994; Ducan et al., 1996) and the emotional processing involved in neuroendocrine responses to stressful stimuli (Dayas et al., 1999; Singewald et al., 2008; Lin et al., 2011). In this regard, although glutamate microinjection in the MePD induced only a subtle increase in carbohydrate food consumption in starved rats (Rosa et al., 2011), the local administration of corticotrophin releasing hormone indicates that the MePD affects stress-induced food intake (Hu et al., 2016). Therefore, the finding that various social behaviors can be modulated by the MePD suggests "that local cells integrate different demands from specific pathways. Each pathway provides inputs that are then temporally and spatially integrated within neural networks to trigger the most appropriate action according to the animal's context…" (Rasia-Filho et al., 2012b).

The rat MePD regulates the timing of puberty independently of changes in body weight and caloric intake, with local glutamatergic systems advancing the timing of puberty and GABAergic activation delaying it (Li et al., 2015). At adulthood, estrogen action in the MePD of estrous female rats plays a critical role for the arousal of interest, sniffing, and expression of preference for male odors (Fujiwara et al., 2016). The MePD modulates the timely display of proceptive and lordosis behavior in females at the same time that it

participates in the secretion of gonadotrophin releasing hormone (GnRH), by its connection with the hypothalamic anteroventral periventricular nucleus, during a short period along the estrous cycle (Simerly, 2002; Rasia-Filho et al., 2004 and references therein). In addition, from de Castilhos et al. (2008), "…in normally cycling females, the activation of glutamate NMDA receptors in the MePD is relevant to initiate long-term changes in prolactin secretion needed for pregnancy/pseudopregnancy or mnemonic events at the time of mating (Polston and Erskine, 2001; Lehmann et al., 2005). It is possible that these local NMDA receptors can show main effects when there are two concomitant physiological conditions: the variation in the hormonal milieu in the proestrus phase and the occurrence of repeated and temporally patterned overthreshold vaginocervical stimulation coming to specific MePD neurons (Polston et al., 2001; Lehmann and Erskine, 2005; Lehmann et al., 2005). Interestingly, small amounts of vaginocervical stimulation facilitate lordosis, moderate ones stimulate pacing behavior and neuroendocrine secretion, and large amounts induce the termination of estrus behavior (Pfaus and Heeb, 1997)".

It is remarkable that ovarian steroids have region-specific effects in the female brain (Hansberg-Pastor et al., 2015) and the MePD is sexually dimorphic and/or affected by sex steroids in rodents (Rasia-Filho et al., 2012a,b and references therein). For example, male and female differ in the spatial orientation of dendrites within the MePD neuropil, a finding likely involved with different axonal input properties (Dall´Oglio et al., 2008a). It is also evident that sex steroids can remarkably alter the function of the MePD cells by concomitantly change the number of dendritic spines (reviewed in Rasia-Filho et al., 2012a) and the synaptic inputs in both dendritic shafts and spines (Brusco et al., 2014). Dendritic shafts receive most synaptic contacts (~76%) in the MePD of males and cycling females (Brusco et al., 2014). The complexity of dendritic contacts involves the proportion of inhibitory synapses on dendritic shafts in the right MePD of proestrus females, higher than in the left MePD, and higher than in the right MePD of males or diestrus and estrus females (Brusco et al., 2014). Synapses made directly on dendritic shaft synapses can be stronger and make larger synaptic currents recorded at the soma than axo-spiny synapses, with the exception for those contacts on stubby spines (Segal, 2010). Dendritic spines form usually asymmetric contacts assumed as excitatory ones, although some receive inhibitory terminals and others are multisynaptic spines (Brusco et al., 2014). Then, most dendritic spines are specialized units with excitatory postsynaptic properties that modulate the occurrence, strength, and plasticity of signal transmission

according to the synaptic demand in the integrated neural networks. Specifically, "… the density of proximal dendritic spines in the MePD of adult Wistar rats is sexually dimorphic (higher in males than in proestrous, estrous or metaestrous females) and is affected by the normal fluctuations in plasma ovarian steroids along the estrous cycle (near 35% of reduction during the transition from diestrous to proestrous)... In the sampled MePD neurons, diestrous females had approximately 51% of thin spines, 31% of stubby/wide, 12% of mushroom-like, and 6% belonging to the other spine shapes. Proestrous females showed near 53% of thin spines, 28% of stubby/wide, 10% of mushroom-like, and 9% belonging to the other spine shapes. Estrous females showed around 47% of thin spines, 34% of stubby/wide, 14% of mushroom-like, and 5% belonging to the other spine shapes…" (Rasia-Filho et al., 2012a).

The structure of dendritic spines, with a continuum of shapes and sizes, would impact on the strength of synapses and, ultimately, the neuronal function (Kasai et al., 2010; Segal, 2010; Yuste, 2013; Tønnesen and Nägerl, 2016). Considering the morphological features of stubby/wide spines, thin spines with long necks or mushroom-like spines, it is likely that each spine type have: (1) a variable area for receptor trafficking and the presence of postsynaptic density; (2) local electrical resistance; (3) degree of biochemical compartmentalization or coupling with parent dendrites, and, then, (4) capacity to alter synaptic strength, integration, and cellular enduring effects (Arellano et al., 2007; Yuste, 2013; Tonnesen et al., 2014). Synaptic processing might also occur in microdomains in ramified spines or in more complex and atypical forms (Verzi and Noris, 2009). This can occur at the level of each single spine and depending on the brain region, the neuron type, and the functional implication for the synaptic demand (Chen et al., 2011; Chen and Sabatini, 2012; Dalpian et al., 2015).

Further data indicated the modulation of dendritic spines by ovarian steroids. That is, "In females, the dendritic spine density observed after 1 week following OVX is comparable to the lower values obtained in virgin rats from estrus to metaestrus… This suggests that MePD neurons can display a minimum basal number of dendritic spines that cannot be affected by the actions of ovarian steroids or be even reduced after OVX. On the other hand, the increase in the number of spines detected in OVX+estrogen-treated rats resembled that one found in normal females during the afternoon of diestrus... This effect became supra-physiological following progesterone injection, when the highest spine data were observed. Progesterone can induce complex actions (Guerra-Araiza et al., 2003; Villamar-Cruz et al., 2006) and, notably,

results after progesterone injection contrast with those observed in the proestrus (when endogenous estrogen+progesterone peak in circulation). The density of dendritic spines in proestrus females was comparatively low in the MePD… In this sense, current estrogen+progesterone substitutive treatments might not mimic exactly the daily physiological variations in the plasma concentrations of gonadal steroids (and also prolactin) across the different phases of the estrous cycle" (de Castilhos et al., 2008).

The MePD cellular and synaptic organization changes once more when females have the experience of motherhood. Different from the other MeA subnuclei, there is a decrease in the density of proximal dendritic spines after motherhood in the MePD. That is, dendritic spines are reduced approximately 24% in postpartum diestrus females when compared to age-matched virgin females also in the diestrus phase (Rasia-Filho et al., 2004). In these rats, the lasting reduced density of dendritic spines is likely involved with both the decrease of avoidance behavior toward pups and the development of adaptive skills in the mother for the new demands from the environment, for nursing behavior, and memory formation about this life event (Kinsley et al., 1999; Rasia-Filho et al., 2004).

Furthermore, there is a complex interaction between sex steroid-responsive neurons and glial cells in the MePD. According to Zancan et al. (2015), "glial processes can dynamically change their extension, possibly restructuring the astrocytic cytoskeleton and folding properties to accompany the changeable available surface of perikaryal membrane. In proestrus, a decreased surface would obligate glial processes to partially retract and condense, possibly contributing to increase the glial fibrillary acidic protein-immunoreactivity in the MePD neuropil. This effect cyclically changes along hours to days and starts to recover from estrus to diestrus. In addition, the MePD neuronal activity in the late proestrus phase would involve the intrinsic biophysical properties of the perikaryon for computing synaptic inputs and generate somatic action potentials associated with the modulatory function of new somatic spines with increased density and different shapes. In estrus, when ovarian hormones drop in circulation, these effects are no longer found."

Given the number of synapses that are made upon the available cell membrane surface, it could be concluded that the perikaryon is not the principal site of synaptic input to nerve cell (Peters et al., 1991). This does not mean that axo-somatic synapses cannot generate important consequences for the neuronal activity. It is well demonstrated the functional role of engulfing glial layers isolating axons from the perykarial membrane in the arcuate nucleus of the hypothalamus for the feedback control of the GnRH release

(Garcia-Segura et al., 1994). Accordingly, "…the emergence of perisomatic axospine synapses provides an additional amount of perikaryal surface to establish dynamically regulated contacts, in a place closer to the axon hillock, that can strategically influence the neuronal output (Peters et al., 1991). This was evident in late proestrus females, with a higher density of spines along the perikaryal perimeter, which could represent the type of labile spines with plastic capacities for local information processing and behavior modulation along the estrous cycle… The shape diversity increased in the night of proestrus with 33% stubby/wide spines; 22% thin spines; and 36% with a ramified, transitional, or atypical aspect…The existence of spinules is also suggestive of plastic properties for the somatic spines in the MePD of proestrus females. Spinules on dendritic spines were associated with an enhanced cellular activity because they are rapidly formed after synaptic stimulation (Applegate and Landfield, 1988; Schuster et al., 1990; Tao-Cheng et al., 2009; Brusco et al., 2014; Stewart et al., 2014), serve for transferring large molecules between pre- and postsynaptic elements (Tarrant and Routtenberg, 1977), and serve in intercellular signaling between active spines and the presynaptic element (Spacek and Harris, 2004)" (Zancan et al., 2015). These somatic spines would provide additional modulatory possibilities to the synaptic activity than contacts made directly on the perykarion.

The complex somatic and dendritic synaptic processing in the female MePD along the estrous cycle may occur with a concomitant switch in the local neurotransmission (Micevych et al., 1988; Oro et al., 1988; Polston and Erskine, 2001; Lehmann and Erskine, 2005; Lehmann et al., 2005; de Castilhos et al., 2008). It is likely that, in the proestrus phase, the impact of putative excitatory inputs in the cell body can be altered by the higher amount of somatic spines, whereas, on dendrites, excitatory input decreases due to a reduction in the density of proximal dendritic spines. We hypothesized (Zancan et al., 2015) "that the morphological and synaptic rearrangement in the left MePD can change the inhibitory impact of output projections on hypothalamic circuits during the proestrus phase to disinhibit both the activity of the ventromedial nucleus, involved in lordosis behavior, and in the medial preoptic area, for the display of proreceptive behavior (Pfaus and Heeb, 1997; for additional data and comments see Rasia-Filho et al., 2012a; Brusco et al., 2014). One possible reason for this hemispheric lateralization is that the left MeA would be specialized for chemosensory and/or steroid negative feedback regulation of the secretion of hypothalamic luteinizing hormone (discussed in Cooke and Woolley, 2005). These changes in the MePD occur during the proestrus phase and, therefore, some hours prior to those reported in the

hypothalamic arcuate nucleus of estrus females relevant for GnRH surge and ovulation". In contrast, males show no difference between hemispheres for the density of dendritic spines (Arpini et al., 2010) or dendritic shaft and spines synapses (Brusco et al., 2014).

These dendritic spines, somatic spines, and direct axonal contacts on dendritic shafts and perikarium are important sites for the integration of information coming from different, but overlapping networks in the MePD. Spatial and temporal processing in spines affect the impact of each circuit for the ultimate display of neuroendocrine secretion and behavioral display. A robust example of this synaptic organization was provided recently. According to Keshavarzi et al. (2015), "The MeA is a central hub in the olfactory neural network. It receives vomeronasal information directly from the accessory olfactory bulb (AOB) and main olfactory information largely via odor-processing regions such as the olfactory cortical amygdala (CoA)… Using the GAD67-GFP mouse, we show that MeA principal neurons receive convergent AOB and CoA inputs. Somatically recorded AOB synaptic inputs had slower kinetics than CoA inputs, suggesting that they are electrotonically more distant. Field potential recording, pharmacological manipulation, and Ca^{+2} imaging revealed that AOB synapses are confined to distal dendrites and segregated from the proximally located CoA synapses. Moreover, unsynchronized AOB inputs had significantly broader temporal summation that was dependent on the activation of NMDA receptors. These findings show that MeA principal neurons process main and accessory olfactory inputs differentially in distinct dendritic compartments… suggesting that this dendritic segregation leads to distinct input integration and impact on neuronal output; hence, dendritic mechanisms control olfactory processing in the amygdala".

Finally, sex steroids actions in the MePD are not restricted to the modulation of reproductive behavior. For example, ER-α in the MeA prevents stress-induced elevations in blood pressure in female mice (Hinton et al., 2016). Previously, it was reported that "the MePD modulates the occurrence of sexual and aggressive behaviors in both males and females (Newman, 1999), for which blood pressure has to be concomitantly regulated. In this sense, it is an interesting working hypothesis to consider that… the MePD might be changing baroreceptor- and chemoreceptor-reflex responses, broadening their ranges, to make homeostatic responses intentionally adjusted for the proper execution of an intended behavior… it is likely that the MePD might be coordinating sympathetic/parasympathetic responses with ongoing behavior. This would provide the animal with a dynamic mechanism of cardiovascular

control suitable for the display of social behaviors, for which the MePD is a relevant component in differently integrated brain circuits..." (Quagliotto et al., 2008).

In conclusion, the MePD is part of the neural network for the elaboration of social behaviors with the important property of being modulated dynamically by the levels of gonadal hormones in circulation (expanding data from Newman, 1999). The data reviewed here provide new insights for the comprehension of sexually dimorphisms and the feminine specialization of neural circuits, neuroendocrine secretion, and cyclic behavioral display in a current animal model. They provide additional insights about the important role of the rat MePD as a model for the study of the link between sex steroids, the cellular specializations for synaptic processing, and the functional organization of the nervous tissue in female rats.

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Chapter 7

CHARACTERIZATION OF ANTI-COXSACKIE VIRUS B3 CONSTITUENTS OF *SCABIOSA ARENARIA* FORSSK BY RP-HPLC-UV METHOD

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ABSTRACT

In the present work, the bioassay-guided fractionation strategy associated with ethnopharmacological information has been used. Thus, various plants belonging to the *Scabiosa* genus (Dipsacaceae) are used to treat skin diseases. In this study, the cytotoxic effect and the antiviral activity of *Scabiosa arenaria* Forssk. extracts were investigated against coxsackievirus B_3 (CVB3) in Vero cells. The cell viability was carried out using 3-(4, 5-dimetylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) assay. The best antiviral activity was shown with the roots ethyl acetate fraction (EtOAc) (IC_{50} = 722.22 ± 0.15 µg/mL and Selectivity index (SI) = 5.53). Its further fractionation yielded 4 subfractions. The most potent anti-coxsackievirus B_3 activity was obtained for SR1 and SR4 subfractions with SI of 6.45 and 5.73, respectively. Chemical analysis of these subfractions revealed the presence of flavonoids and phenolic acid, which could be attributed to this biological activity. The good anti-coxsackievirus B_3 of ethyl acetate roots of *S.arenaria* support the traditional use of many species of *Scabiosa* genus in the treatment of skin diseases of viral origin.

Keywords: scabiosa arenaria, phenolic compounds, anti-coxsackievirus B_3, RP-HPLC

ABBREVIATIONS

DMSO (dimethylsulfoxide)
MTT: 3-(4, 5-dimetylthiazol-2-yl)-2
5- diphenyltetrazolium bromide
RP-HPLC: Reversed Phase-High-Performance Liquid Chromatography
H_2SO_4: Sulfuric acid
RPMI: Roswell Park Memorial Institute medium
SI: selectivity index.

1. INTRODUCTION

Infectious skin diseases including those of viral origin are highly prevalent throughout the developing countries [1]. Febrile rash illness and hand, foot, and mouth syndrome are virus-induced skin diseases. They are caused by

enteroviruses, for instance coxsackie B_3 viruses [2, 3, 4]. This virus is also one of the major causative agents of myocarditis as well as pancreatitis and aseptic meningitis [5].

To date, effective treatment is not available for these infections. Patients are treated only symptomatically. There are several medicinal plants used traditionally or as complementary/alternative medicines to treat skin disorders. The use of such medicinal plants for the treatment of skin disorders arguably has been based largely on historical/anecdotal evidence [6, 7]. Based on long time experience, various plants belonging to the *Scabiosa* genus (Dipsacaceae) are used to treat skin diseases. The information about these plants is provided by ethnomedicine. In Iberian Peninsula, an infusion prepared from the flowers of *Scabiosa atropurpurea* L. was used for the treatment of acne, measles and furuncles [8], the roots of *Scabiosa columbaria* mixed with *Bakerianus aster* treat skin rashes [9]. An ointment made from the roots and leaves of this species was used in South Africa to treat the wound healing [10]. The inflorescences and roots of *Scabiosa ochroleuca* L. were used externally to eliminate warts, scabies, rash, callus and snake bites [11]. The roots of *Scabiosa sussica* L. was used in Europe in the middle ages to treat the poisonous insect bites, external wounds, herpes, ringworm, thrush and ulcers [12,15]. *Scabiosa* species have been reported to contain flavonoids, coumarins, irridoids, saponins and terpenes [16, 23]. Previously, we have shown that *Scabiosa arenaria* Forssk extracts contain several active phenolic compounds [24, 27]. As continuity of these works, and in order to rationalize the traditional use of *Scabiosa* genus in the treatment of skin disorders of viral origin. For the first time, the cytotoxic effect and the antiviral activity of this plant extracts were reported here. The anticoxsackie B_3 activity-guided fractionation using the MTT assay was reported as well as the characterization of the phenolic compounds from the most active fraction using RP-HPLC.

2. MATERIAL AND METHODS

2.1. Reagents and Standards

All solvents used in the experiments (ethyl acetate, butanol, methanol and dimethylsulfoxide (DMSO)), Silica gel 60 F_{254} (thin layer chromatography plates), Silica gel (60-120 Mesh) (Column chromatography) and sulfuric acid (H_2SO_4) were purchased from Merck (Darmstadt, Germany). Authentic standards of phenolic compounds were purchased from Sigma and Fluka.

Stock solutions of these compounds were prepared in HPLC-grade methanol, wrapped in aluminum foil then stored at 4°C. Ribavirin (Sigma) was used as the positive control in the anti-CVB3 test.

2.2. Samples

Scabiosa arenaria was collected in May 2010 from the area of Monastir (Tunisia) at the flowering stage. The plant material was kindly identified by Pr. Fethia Harzallah Skhiri, High Institute of Biotechnology of Monastir, Tunisia. A voucher specimen (*S. arenaria*) (Sa 110) was deposited in the Laboratory of Bioorganic Chemistry and Natural Products at the Faculty of Sciences of Monastir, Tunisia. The flowers, the fruits, the (stems and leaves) and the roots were air-dried at room temperature for two weeks and reduced to coarse powder. The powdered samples (250 g) were separately, extracted by maceration with MeOH–H_2O 80:20 (v/v) for 72 hours at room temperature. A repetitive extraction of the same material three times with solvent was carried out. After filtration, a portion of obtained extracts was dried completely for further biological activities and the residual aqueous layer was further subjected to a successive extraction with ethyl acetate (EtOAc) and butanol (n-BuOH) to yield dried fractions for each organ, knowing that, the extracts was evaporated in a rotary evaporator and dried by vacuum pump. Sixteen extracts were obtained and maintained at 4°C before analysis.

2.3. Cell Line

The Vero cells were derived from a kidney of normal, adult, African green monkey (Cercopithecus) by Yasumura and Kawakita at Japan Chiba University in 1962. This cell line has been extensively used for virus replication studies and plaque assays. Vero cells (kindly provided by Pr. Bruno Pozzetto, Laboratory of Bacteriology-Virology, Saint-Etienne, France) were used for culturing enterovirus strains. Vero cells were maintained in RPMI 1640 (Roswell Park Memorial Institute medium) supplemented with 10% fetal bovine serum (FBS), L-Glutamin (2 mM), penicillin (100 U/mL), and streptomycin (100 µg/mL). Cells were incubated at 37°C in a 5% CO_2 humidified atmosphere.

2.4. Virus Strain

Coxsakievirus B_3 Nancy strain (kindly provided by Pr. Bruno Pozzetto, Laboratory of Bacteriology-Virology, Saint-Etienne, France) was propagated in Vero cells. In brief, 100 µL of the virus suspension were used to infect a confluent monolayer of Vero cells in 75 cm^2 culture flask and adsorbed for 1 h to allow viruses entry into the cells. Non-adherent particles were washed off using 2% RPMI 1640 medium and the infected cells overlaid with 20 mL of 2% RPMI 1640 and incubated again until full cytopathic effect was observed in five to six days. When a cytopathic effect CPE in the virus-infected cells was observed microscopically, $TCID_{50}$ (the 50% tissue culture infective dose) was determined by the method of Reed and Muench [28]. The harvested virus was stored at −70°C until used.

2.5. Chemical Analysis: Bioassay Guided Fractionation

The bioguided chromatographic fractionation process began with the evaluation of anti-coxsackie B_3 screening of these sixteen samples. The roots EtOAc fraction which has the best anti-coxsackie B_3, was then submitted to chromatographic separation processes. This fraction (4.2 g) was chromatographed on silica gel column (4 cm x 80 cm) and eluted with ethyl acetate: methanol gradient mixture starting from 100% ethyl acetate and increasing the polarity up to 100% methanol to afford 216 fractions (25 mL). The fractions were monitored by thin layer chromatography (TLC silica gel 60 F_{254}) using ethyl acetate/methanol as eluent. After drying, the plates were visualized under UV light at 254 and 365 nm and then were sprayed with 10% sulfuric acid in methanol, and those with similar profiles were combined resulting in 4 subfractions (SR_1-SR_4), which were analyzed by RP-HPLC.

2.6. Cytotoxicity Assay

The evaluation of the cytotoxic effect of samples is based on the reduction of MTT (3[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide), by the mitochondrial dehydrogenase of viable cells, to give a blue formazan product which can be measured spectrophotometrically at 540 nm.

The MTT colorimetric assay was performed in 96-well plates [29]. Cells were seeded in 96-well plates at a concentration of 5×10^4 cells per well and incubated for 24 h at 37 °C in a 5% CO_2 humidified atmosphere. After treatment with various concentrations of the test compound (15.6, 31.25, 62.5,

125, 250, 500, 1000, 2000, 4000 and 8000 µg/mL), the cells were incubated for an additional 48 h at 37°C. The cells were examined daily under a phase contrast microscope to determine the minimum concentration of compounds that induced alterations in cell morphology.

After that, the medium was removed and cells in each well were incubated with 100 µL of MTT solution (0.5 mg/mL) for 3-4 h at 37°C. Fifty microliters of dimethyl sulfoxide (DMSO) were then added to dissolve insoluble formazan crystal and the plates were incubated at 37°C for 30 min.

Optical density (OD) was measured at 540 nm. Cell viability was expressed with respect to the absorbance of the control wells (untreated cells), which were considered as 100% of absorbance. The percentage of cytotoxicity is calculated as [(A - B)/A] x 100, where A and B are the OD_{540} of untreated and of treated cells, respectively. The percentage of viability was carried out using the formula: *100 - % cytotoxicity*.

The 50% cytotoxic concentration (CC_{50}) was defined as the compound's concentration (µg/mL) required for the reduction of cell viability by 50%, which were calculated by regression analysis [y = f(x); where y =% viability and x = concentration of extract, µg/mL] [30].The used definition of the cytotoxicity, as supported by other reports [31]was CC_{50} < 1 µg/mL – high cytotoxicity CC_{50} = 1-10 µg/mL – moderate CC_{50} = 10 - 20 µg/mL –mild cytotoxicity and CC_{50} > 20 µg/mL – non cytotoxic.

2.7. Virus Inhibition Assay

The antiviral activity of the samples was determined using the same method as previously described method [32], with some modifications. Briefly, the growth medium of confluent Vero cells, prepared at 96-well plates, was removed and replenished with 100 µL/well of Coxasckievirus B3 suspension (5.10^4 $TCID_{50}$/mL), except in the untreated cell controls (200 µL/well of culture medium). After 1 h of adsorption at 37°C, the monolayers were washed with phosphate buffered saline (PBS). Two-fold concentrations of samples (100 µL/well) were added for tests. For virus controls, the same volume of culture medium was added. After that, plates were incubated for 72 h and the same procedure in Section 2.6 was performed. Ribavirin was used as positive control for Coxsackie B3 inhibition. The 50% inhibitory concentration (IC_{50}) was defined as the concentration that inhibited 50% of viral replication when compared to virus controls. The selectivity index (SI), which is an important parameter to evaluate antiviral activity, was calculated from the ratio CC_{50}/IC_{50} [33].

2.8. Analysis of Individual Phenolic Compounds by Analytical RP-HPLC

The dried subfractions from the more active extract were hydrolysed according to slightly modified method of Proestos et al. [34]. Forty millilitres of methanol containing BHT (1 mg/ml) were added to 0.5 g of dried subfractions. Then, 10 ml of 6 M HCl. The mixture was stirred carefully and then sonicated for 15 min and refluxed in a water bath at 90°C for 2 hours. The obtained mixture was filtered through a 0.45 µm membrane filter and injected to RP-HPLC. The separation of phenolics was performed with an Agilent 1100 series HPLC system equipped with in-line degasser (G 1322A), quaternary pump (G 1311A), a thermostatic auto sampler (G 1313A), column heater (G 1316A), and diode array detector (G 1315A).

Instrument control and data analysis was carried out using Agilent HPLC Chemstation 10.1 edition through Windows 2000. The separation was carried out on a reverse phase ODS C18 (4 µm, 250 × 4.6 mm, Hypersil) column used as stationary phase at ambient temperature. The mobile phase consisted of acetonitrile (solvent A) and water with 0.2% sulfuric acid (solvent B). The flow rate was kept at 0.5 ml/min. The gradient program was as follows: 15 A/85 B 0–12 min, 40% A/60% B12–14 min, 60% A/40% B 14–18 min, 80% A/20% B 18–20 min, 90% A/10% B 20–24 min, 100% A 24– 28 min. The injection volume was 20 µl and peaks were monitored at 280 nm. Peaks were identified by congruent retention times compared with standards.

2.9. Statistical Analysis

The results were given as the average ± SD for at least three replicates for each sample. The 50% cytotoxic concentration (CC_{50}) was defined as the compound's concentration (µg/mL) required for the reduction of cell viability by 50%, which were calculated by regression analysis [y = f(x); where y =% viability and x = concentration of extract, µg/mL]. The 50% inhibitory concentration (IC_{50}) was defined as the concentration that inhibited 50% of viral replication when compared to virus controls. The data were subjected to ANOVA, and Duncan's multiple range test was used to compare means. Statistical analyses were performed with the SPSS statistical software program (SPSS v.16). P values <0.05 were regarded as significant. To evaluate the antiviral activity *in vitro*, the selectivity index ($SI = CC_{50}/IC_{50}$) was determined. The selectivity index describes the ratio between the cytotoxic and the antiviral activity of a tested compound.

3. RESULTS AND DISCUSSION

3.1. Cytotoxicity Test

Before conducting antiviral activity assays, the cytotoxicity of the extracts on the Vero cells was studied. An antiviral drug should be active against the virus inducing significant toxicity on the host cell. Therefore, the concentration range in which the extracts will not induce significant toxicity to the host cells was estimated and the 50% cytotoxic concentration (CC_{50}) was determined for each extract. As shown in Table 1, the 50% cytotoxic concentrations of the majority of the 16 extracts tested is between 500 µg/mL and 4000 µg/mL. We can note that, the lowest cytotoxicity was observed with the two water fractions of (stems and leaves) and fruits (CC_{50} = 500 µg/mL). Results revealed significant differences ($P < 0.05$). The cytotoxicity of our studied extracts was very low ($CC_{50} > 20$ µg/mL) [31].

The cytotoxicity of plants belonging to the dipsacaceae family was studied for the first time in our work.

3.2. Antiviral Activity

All extracts tested were used at their non-cytotoxic concentrations (Table 1). To evaluate the activity of antiviral agents *in vitro*, the selectivity index ($SI = CC_{50}/IC_{50}$) was determined. The selectivity index describes the ratio between the cytotoxic and the antiviral activity of a substance. The roots EtOAc fraction showed the better antiviral activity (IC_{50} = 722.22 ± 0.15 µg/mL and SI = 5.53) followed by the flowers butanol fraction which presented also an interesting anti-coxsackie B_3 activity (IC_{50} = 750 ± 0.12 µg/mL and SI = 2.66). While, the roots water fraction showed an IC_{50} value greater than the two previous extracts (IC_{50} = 938.59 ± 0.1 µg/mL and SI = 2.13) (Table 1). We found that the selectivity index of the positive control, Ribavirin, (SI = 7.03) was close to that of the roots EtOAc fraction. This fraction showing the best anti-coxsackie B_3 activity was fractionated into 4 subfractions. The anti-coxsackie B_3 activity of the four subfractions was shown in Table 1; the subfraction SR_1 showed the best result with IC_{50} = 620 ± 0.05 µg/mL and selectivity index = 6.45 followed by the subfraction SR_4 with value of IC_{50} = 697.34 ± 0.04 µg/mL and selectivity index = 5.73.

These two subfractions demonstrated an anti-Coxsackie B_3 activity more important than the crude extract (roots EtOAc) (IC_{50} = 722.22 ± 0.15 µg/mL

and SI = 5.53). The subfractions SR_2 and SR_3 showed moderate antiviral activity: IC_{50} = 1750 ± 0.02 µg/mL; SI = 2.28 and IC_{50} = 1876 ± 0.1 µg/mL; SI = 2.13, respectively. Results revealed significant differences ($P < 0.05$). Our work is the first study on the antiviral activity of a species belonging to the Dipsacacae family. So we will compare our results with other works that have treated anti-coxsackie B_3 activity of plants following our protocol. Guo et al. [35] showed that the aqueous extracts of Chinese medicinal plants like *Sargentodox acuneata* (Oliv.) Rehd. et Wils., *Sophoraton kinensis* Gapnep., *Paeonia veitchii* Lynch, *Spatholobus suberectus* Dunn. and *Cyrtomium fortunei* J. sm. have activity against Coxsackie virus B_3 and the SI ranged from 3.9 to 9.8. Edziri et al. [36] showed that methanol, butanol and ethyl acetate extracts of *Marrubium deserti* showed significant anti-coxsakie B_3 activity with IC_{50} values ranging from 100 to 135 µg/ml and with a selective index (SI) above 3.

These results were more interesting than ours. Another recent study showed that the ethyl acetate extract of *Dodonaea viscosa* leaves has moderate anti-coxsackie B_3 activity (SI = 1.2) [37].

3.3. Phenolics Identification by RP-HPLC

Roots EtOAc fraction of *S. arenaria* exhibited an interesting anti-coxsackie B_3 activity. This result is motivating to simplify this fraction by chromatography process, further identify the most constituents in each subfraction by RP-HPLC and try to localize the eventual researched activities. The free forms of phenolic compounds are rarely present in plants. More often, they occur as esters, glycosides or are bound to the cell wall. For this reason, before HPLC analysis, hydrolysis of glycosides or esters was necessary, so that phenolic compounds can be identified, since a considerable fraction is in bounded form. Moreover, BHT, was added to prevent degradation of phenolics during hydrolysis [38]. RP-HPLC coupled with UV–vis multiwave length detector was employed to separate phenolic compounds. Compounds have been identified according to their retention times and spectral characteristics of their peaks against those of standards, as well as by spiking the sample with standards. The compounds identified in the four subfractions (SR_1 – SR_4) are reported in Table 2 and Figure 1 (A-D).

Table 1. Anti-coxsackievirus B_3 activity of *S. arenaria* extracts in Vero cells

Extracts	CC_{50} (µg/mL)	IC_{50} (µg/mL)	SI
Roots			
Crude extract	$4000^{b} \pm 0.19$	nd	nd
EtOAc fraction	$4000^{b} \pm 0.09$	**$722.22^{d} \pm 0.15$**	**5.53**
n-BuOH fraction	$2000^{a} \pm 0.3$	Nd	nd
Water fraction	$2000^{a} \pm 0.23$	**$938.59^{f} \pm 0.1$**	**2.13**
Flowers			
Crude extract	$4000^{b} \pm 0.31$	Nd	nd
EtOAc fraction	$4000^{b} \pm 0.05$	Nd	nd
n-BuOH fraction	$2000^{a} \pm 0.03$	**$750^{e} \pm 0.12$**	**2.66**
Water fraction	$2000^{a} \pm 0.07$	Nd	nd
Fruits			
Crude extract	$4000^{b} \pm 0.08$	nd	nd
EtOAc fraction	$4000^{b} \pm 0.09$	nd	nd
n-BuOH fraction	$2000^{a} \pm 0.43$	nd	nd
Water fraction	$500^{a} \pm 0.13$	nd	nd
(Stems and leaves)			
Crude extract	$4000^{b} \pm 0.24$	nd	nd
EtOAc fraction	$4000^{b} \pm 0.21$	nd	nd
n-BuOH fraction	$1000^{a} \pm 0.09$	nd	nd
Water fraction	$500^{a} \pm 0.05$	nd	nd
Subfractions			
SR1	$4000^{b} \pm 0.05$	**$620^{b} \pm 0.05$**	**6.45**
SR2	$4000^{b} \pm 0.07$	**$1750^{g} \pm 0.02$**	**2.28**
SR3	$4000^{b} \pm 0.07$	**$1876^{h} \pm 0.1$**	**2.13**
SR4	$4000^{b} \pm 0.2$	**$697.34^{b} \pm 0.04$**	**5.73**
Ribavirin (positive control)	**1576^{a} (6453.6 µM) ± 0.09**	**224^{a} (917.26 µM) ± 0.02**	**7.03**

CC_{50}: 50% cytotoxic concentration; IC_{50}: 50% inhibitory concentrations; Selectivity index (SI = CC_{50}/IC_{50}); nd: not determined, the different letters (a–h) indicate a significant difference between the extracts ($P < 0.05$)

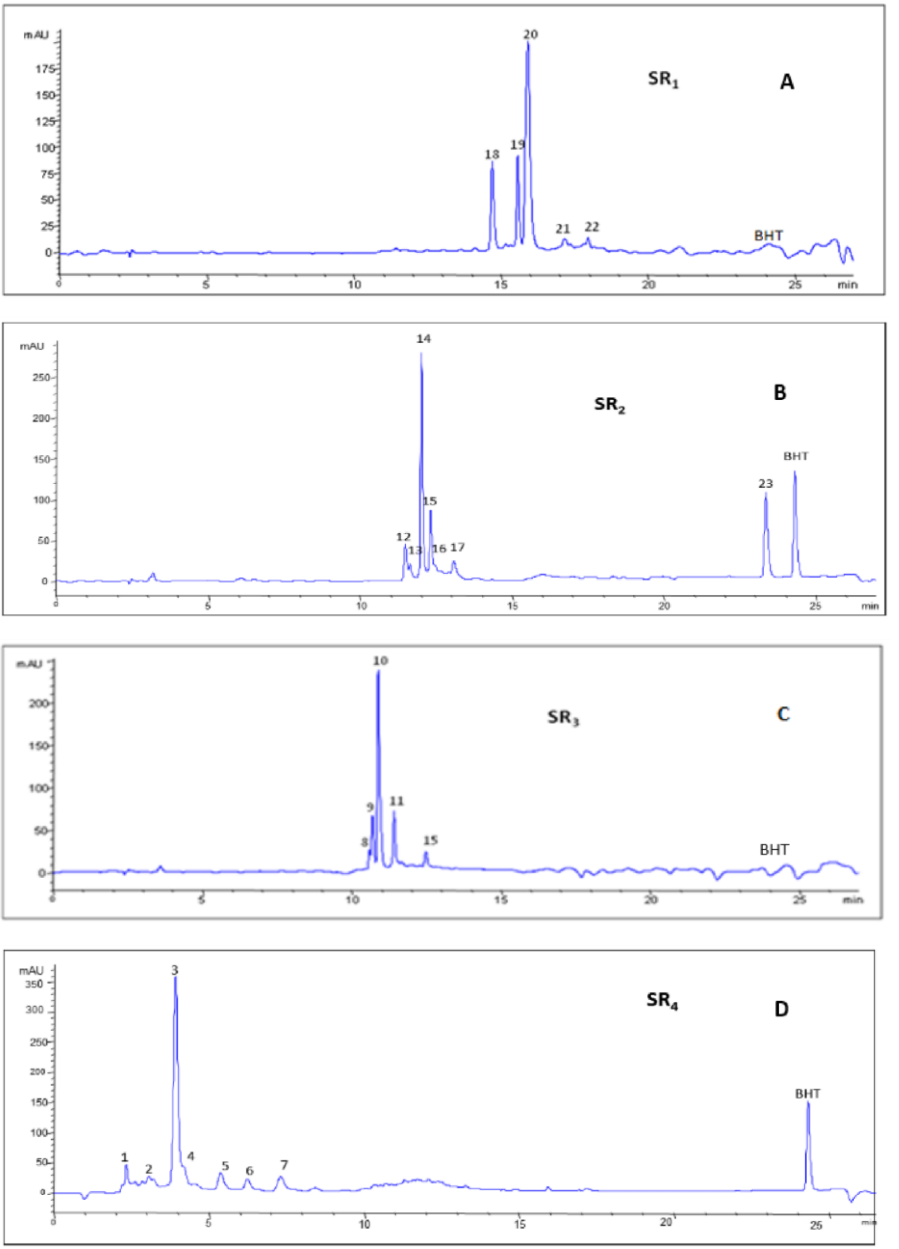

Figure 1. RP-HPLC chromatograms of SR_1 (A), SR_2 (B), SR_3 (C) and SR_4 (D) after acid hydrolysis. Signal was recorded at 280 nm. Numbering of peaks refers to their identification as shown in Table 2.

These identified compounds comprise tannic acid (1), catechin (2), protocatechuic acid (3), resorcinol (4), methyl gallate (5), vanillic acid (6), *p* coumaric acid (9), taxifolin (10), naringin (12), naringenin-7-*O*-glucoside (13), rutin (14), methyl-4-hydroxybenzoate (15), morin (17), hesperetin (18). In contrast, (7), (8), (11), (16), (19), (20), (21), (22) and (23) are unknown compounds. The most abundant phenolic compounds were protocatechuic acid in SR_4 (71.94%), rutin in SR_2 (41.46%) and taxifolin in SR_3 (59.42%). Tannic acid, catechin, protocatechuic acid, resorcinol, methyl gallate, *p*-coumaric acid, taxifolin, naringin, naringenin-7-*O*-glucoside, methyl-4-hydroxy benzoate, morin and hesperetin were identified for the first time in the *Scabiosa* genus. Rutin was identified previously in *Scabiosa atropurpurea* L. [16], vanilic acid was identified also in *Scabiosa hymettia*Boiss. et Spruner [21]. As far as we know, there are no relevant reports on the anti-coxsackie B_3 of the phenolic compounds found in the EtOAc fraction of *S. arenaria* roots. The significant anti-coxsackie B_3 activity of SR_1 can be explained by the presence of hesperetin in important percent 18.09%, this flavonoid was shown to inhibit replication of poliovirus type 1, which belong to the family Picornaviridae like coxsackie B_3 [39]. Concerning, the subfraction SR_4, presented also an interesting anti-coxsakie B_3, the tannic acid, belong to hydrolysable tannin may contribute to this activity. In literature, this type of polyphenols showed potent anti-coxsackie B_3 activity [40]. Taxifolin, represent the main compound in the subfraction SR_3 (59.42%), this can be the cause of the decrease of its antiviral activity, in fact and according to Beladi et al. [41], this compound hasn't any effect on poliovirus types 2 and 3, which belong to the family Picornaviridae like CVB3.

The antiviral activity of SR_2 was also moderate; this result can be due to the presence of rutin and naringin, which constitute more than 50% of the total SR_2 compounds. These two flavonoids were shown in the literature hasn't any effect on virus belonging to Picornaviridae family, as poliovirus type 1, type 2, type 3 and Coxsackie B strains [42, 39]. Catechin, protocatechuic acid, vanillic acid, trans-4-hydroxycinnamic acid and morin found in this active fraction have been reported to inhibit the replication of many viruses belonging to other family of virus [43, 46]. The isolation of phenolic compounds identified in SR_1 and SR_4 is required to confirm its good anti-coxsackie B_3 activity. In addition, up to 9 compounds were unknown and are the subject of future purification and characterization efforts.

Table 2. Main phenolic compounds identified in subfractions from EtOAc fraction of *S. arenaria* roots

N°	Compounds	RT (min)	% in SR_1	% in SR_2	% in SR_3	% in SR_4
1	Tannic acid	2.35	ND	ND	ND	3.01
2	Catechin	3.59	ND	ND	ND	1.11
3	Protocatechuic acid	3.95	ND	ND	ND	71.94
4	Resorcinol	4.19	ND	ND	ND	5.35
5	Methyl gallate	5.39	ND	ND	ND	7.61
6	Vanillic acid	6.26	ND	ND	ND	4
7	Unknown	7.32	ND	ND	ND	6.92
8	Unknown	10.59	ND	ND	4.12	ND
9	Trans-4-hydroxycinnamic acid	10.7	ND	ND	16.06	ND
10	Taxifolin	10.89	ND	ND	59.42	ND
11	Unknown	11.41	ND	ND	15.36	ND
12	Naringin	11.48	ND	9.25	ND	ND
13	Naringenin-7-*O*-glucoside	11.64	ND	3.51	ND	ND
14	Rutin	12.01	ND	41.46	ND	ND
15	a-methyl-4-hydroxybenzoate	12.31	ND	12.49	5.01	ND
16	Unknown	12.42	ND	1.41	ND	ND
17	Morin	13.08	ND	3.78	ND	ND
18	Hesperetin	14.71	18.09	ND	ND	ND
19	Unknown	15.58	14.3	ND	ND	ND
20	Unknown	15.92	64.87	ND	ND	ND
21	Unknown	17.16	1.75	ND	ND	ND
22	Unknown	17.96	0.97	ND	ND	ND
23	Unknown	23.35	ND	28.05	ND	ND

RT: Retention times (minutes).
ND: Not detected.

CONCLUSION

As far as we are concerned, the present work constitutes the first report studying the antiviral activity of *Scabiosa arenaria* Forssk. extracts against coxsackie B3. Furthermore, the antiviral activity of roots EtOAc, water fractions and the flowers butanol fraction showed the best activity. The roots EtOAc fraction was then submitted to chromatographic separation process.

The subfractions were analysed by RP-HPLC. The anti-coxsackie B_3 of SR_1 and SR_4 could be attributed to the phenolic compounds identified.

The obtained results might be considered sufficient to further studies for the isolation of the active principles and to evaluate possible synergism among components for their anti-coxsackie B_3 activity.

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Chapter 8

CONGENITAL UPPER LIMB ANOMALIES: RISK FACTORS, DIAGNOSIS AND MANAGEMENT

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ABSTRACT

Congenital upper limb anomalies (CULA) are a relatively common finding at birth. Recent advancements in developmental biology and clinical genetics have provided new insights into the mechanisms of limb formation and malformation. Depending on the aberration, it may be isolated or affect disparate groups of cells thereby affecting other organ systems. The etiologies of CULA are diverse and disorder-specific, but potential contributors include genetic, mechanical, and a variety of

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environmental factors. Careful examination, both prenatally and postnatally, is key to early identification and management. Progress has been made recently to improve the classification/diagnosis of CULA using language that is inclusive of both basic and clinical sciences. In this review, we incorporate a similar approach in providing a comprehensive overview of CULA combining information regarding the molecular landscape of limb development with the growing database of clinical genetics. We will also highlight recent advances in risk-factor association, diagnosis and management of congenital upper limb anomalies.

Keywords: limb development, limb dysmorphology, epidemiology, classification, etiology, molecular diagnosis, treatment, review

EMBRYOLOGY

Limb development begins in the fourth week of gestation (Figure 1A) and coincides with the development of other organs such as the heart, kidney, liver and lungs. Consequently, Congenital Upper Limb Anomalies (CULA) can occur in conjunction with other congenital anomalies. Vertebrate limb development is under tight molecular regulation directing the number, location and pattern of tetrapod limbs. Expression of Homeobox (Hox) genes along the cranial-caudal or anterior-posterior axis of the developing embryo provides segmental identity to the embryo and establishes presumptive limb fields within the lateral plate mesoderm from which the limb buds will emerge (Figure 1B) (Oliver et al., 1988; Burke et al., 1995; Wellik, 2009; Tanaka, 2016).

Limb Initiation

Limb bud initiation occurs as a complex interaction between wingless-type MMTV (WNT), fibroblast growth factor (FGF) signals and T-box (TBX) transcription factors in the mesenchyme of the presumptive limb field (Figure 1C). Murine *Fgf10* knockouts develop tetra-amelia or failure of limb bud initiation (Ng et al., 2002), whereas disruption of another member of the pathway, WNT3A, has been shown to cause tetra-amelia in humans (Niemann et al., 2004). TBX4 and TBX5 contribute to initiation and identity of the hindlimb and forelimb, respectively (Gibson-Brown et al., 1996). Holt-Oram

syndrome, which is characterized by abnormalities of the heart and upper limb, occurs in humans with mutations in the *TBX5* gene (Basson et al., 1997; Li et al., 1997; Mori and Bruneau, 2004). Through loss and gain of function experiments in chicken and mice, a role for *PITX1* in hindlimb specificity has also been identified (Lanctot et al., 1999; Logan and Tabin, 1999; Szeto et al., 1999). Misexpression of *PITX1* in wing-field of chicks leads to ectopic *TBX4* expression and results in hindlimb-specific changes to the limb (Logan and Tabin, 1999). Pitx1 overexpression in mice and misregulation of PITX1 in humans (Liebenberg syndrome) lead to similar phenotypes with partial transformation of the upper limb into a hindlimb (Spielmann et al., 2012; Al-Qattan et al., 2013; Mennen et al., 2014).

Limb Patterning along the Proximal-Distal Axis (Figure 1C)

Subsequent to limb bud initiation, limb development occurs along three axes: proximal-distal, anterior-posterior, and dorsal-ventral. Signaling centers are organized to govern growth and patterning along each axis. The apical ectodermal ridge (AER) forms as a thickened epithelium at the distal tip of the limb bud in response to mesodermal signals. FGFs, primarily FGF8 from the AER, direct growth and patterning along the proximal-distal axis (shoulder to fingers). A deficiency of Fgf8 and 4 (from the AER) (Boulet et al., 2004) or Fgf10 (from limb mesoderm) (Min et al., 1998) in mice results in the complete absence of fore- and hindlimbs. Interrupting AER-related FGF function either by AER removal in chick wings or conditional Fgf receptor knockouts at various developmental stages, truncates limbs along the proximal-distal axis corresponding to its progressive stage of distalization (Saunders, 1948; Mahmood et al., 1995; Lu et al., 2008; Yu and Ornitz, 2008). In addition, maintenance of AER integrity and FGF secretion is important for proper digit formation. Several molecules involved in AER maintenance and FGF8 production have been described including: TP63, DLX5, WNT10B and FBXW4. Mutations in target genes or regulatory elements associated with this pathway disrupt AER structure and function, producing a phenotypic spectrum of abnormalities known as split-hand/foot malformations (SHFM) (Naruse et al., 2007; Marinic et al., 2013; Restelli et al., 2014; Sowinska-Seidler et al., 2014).

Activation of FGF-specific intracellular signaling pathways involved in cell proliferation, growth and differentiation is mediated through FGF binding to specific receptors (FGFRs) that exhibit tyrosine kinase activity. Abnormal

limb development in humans as a consequence of deficiency/mutations in these receptors are well known and include Apert syndrome (FGFR2 mutation), Crouzon syndrome (FGFR2 mutation), Pfeiffer syndrome (FGFR1 and 2 mutations), achondroplasia (FGFR3 mutation), and thanatophoric dysplasia (FGFR3 mutation) (Toriello et al., 2008).

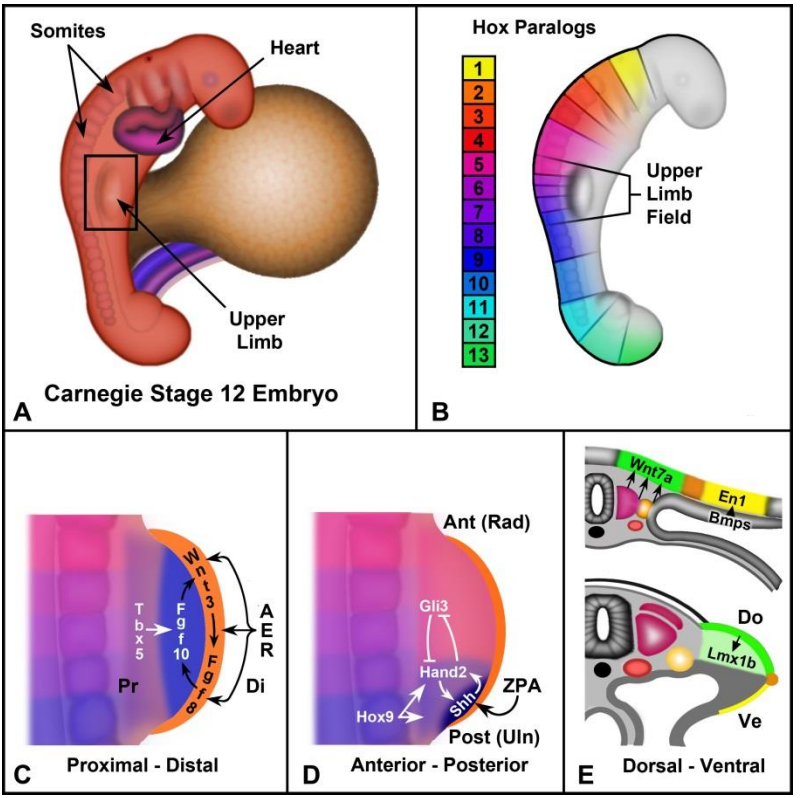

Figure 1. Early limb development and axis organization. A) Illustration of a human embryo at Carnegie stage 12 (~ 4 weeks gestation) showing an emerging upper limb bud. B) Evidence suggests that Hox genes establish upper limb position and polarity. C-E) Molecular pathways involved in forelimb initiation and patterning along the proximal-distal axis (C), anterior-posterior axis (D), and dorsal-ventral axis. Apical ectodermal ridge (AER) (orange), Pr – proximal, Di-distal, Ant(Rad)-anterior/radial, Post(Uln)-posterior/ulnar, zone of polarizing activity (ZPA) (dark purple), Do-dorsal, Ve-ventral, dorsal ectoderm and mesoderm (green). From Oberg et al., (Oberg et al., 2014) with permission.

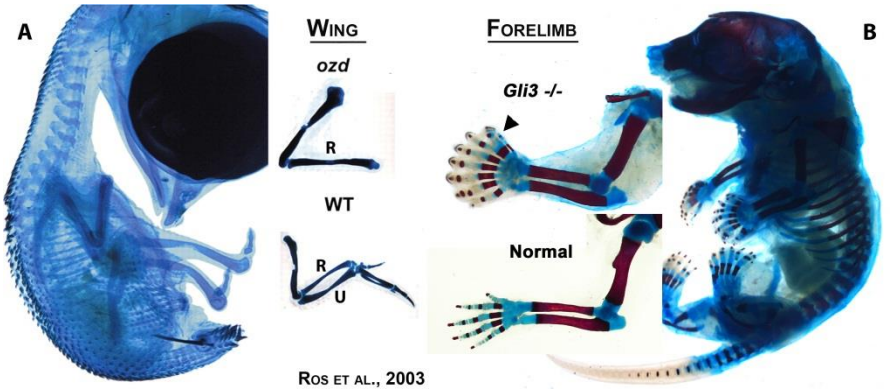

Figure 2. The influence of SHH/GLI3 on digit formation. This figure illustrates the range of SHH/GLI3 signaling in two common models of limb development, chick and mouse. A) A spontaneous mutation in chickens deletes the limb-specific enhancer of SHH generating the Oligozeugodactyly (ozd) phenotype. Without the enhancer, no SHH is expressed in the limbs and GLI3r function is unopposed. The unopposed GLI3r inhibits the formation of the ulna and digits. B) In contrast, if GLI3 expression is absent as seen in the GLI3 knockout mouse, the number of digits is excessive and the digits lack specific AP (radioulnar) identity, including a two phalangeal thumb. (Images courtesy of John F. Fallon, PhD, University of Wisconsin, and Maria A. Ros, MD, PhD, Instituto de Biomedicina y Biotechnologia de Cantabria; (A) adapted with permission from Ros et al. (Ros et al., 2003) and Oberg (Oberg, 2014).

Limb Patterning along the Anterior-Posterior Axis (Figure 1D)

The zone of polarizing activity (ZPA) is a cluster of cells in the posterior distal mesoderm of the developing limb that is responsible for patterning along the anterior-posterior axis. These cells secrete sonic hedgehog (SHH), a potent morphogen that directs posterior expansion and patterning (Towers et al., 2008; Zhu et al., 2008). In the absence of *SHH* expression, the posterior zeugopod bones (ulna and fibula) and posterior digits fail to develop (Chiang et al., 2001; Kraus et al., 2001). In contrast, ectopic anterior expression of *SHH* is associated with transformation of anterior zeugopod bone (radius) into a posterior bone (ulna) and mirror duplication of posterior digits (Riddle et al., 1993).

In the murine model, Gli3 transcription factor functions as a downstream effector of Shh signaling. In the absence of Shh signaling, Gli3 is processed into a truncated repressor variant (Gli3r) (Litingtung et al., 2002). Gli3 is expressed in the limb bud prior to Shh expression and acts antagonistically to

restrict *Hand2* expression to the posterior limb (te Welscher et al., 2002). Correspondingly, Hand2 restricts Gli3r to the anterior aspect of the limb. This reciprocal antagonism sets up the ZPA and *Shh* expression in the posterior distal mesenchyme. Shh subsequently counters Gli3r repression by blocking the local processing of Gli3, keeping it in its full-length activator form. In the developing autopod, Shh and Gli3 (Gli3/Gli3r ratio) regulate the number and identity of the digits. In the absence of Gli3, the digits increase in number, become syndactylous, and lose their identity (Litingtung et al., 2002). Mutations in Gli3 result in polydactyly in both mouse and humans (Hinchcliffe, 1980; Vortkamp et al., 1991; Vortkamp et al., 1992).

A conserved *cis*-regulatory element, the ZPA regulatory sequence (ZRS), is found within intron 5 of the LMBR1 gene and is required for limb-specific *SHH* expression (Clark et al., 2001; Lettice et al., 2002). Misregulation of SHH by microduplication of the ZRS and presumed anterior ectopic expression causes Laurin-Sandrow Syndrome with tetramelic posterior zeugopod duplication (ulnar/fibular dimelia) and mirror-image polydactyly (Lohan et al., 2014). In a chicken model termed oligozeugodactyly (ozd), there is a deletion of the ZRS resulting in the absence of SHH expression with a loss of the posterior zeugopod elements and the entire autopod of the three-digit forelimb/wing. In humans, a deletion mapping upstream of this region results in an autosomal recessive disorder *Acheiropodia* (Ianakiev et al., 2001) that causes longitudinal postaxial deficiencies resembling the limb phenotype of *ozd* chicks and *Shh* $^{-/-}$ mice (Ros et al., 2003). Apart from Acheiropodia in humans, pre-axial polydactyly (Lettice et al., 2003) triphalangeal thumb (Heutink et al., 1994) and acropectoral syndrome (Dundar et al., 2001), are other limb-specific disorders associated with mutations in this region.

Limb Patterning along the Dorsal-Ventral Axis (Figure 1E)

Limb dorsalization is accomplished by the release of WNT7a from the dorsal ectoderm which induces the expression of LIM Homeobox transcription factor 1 beta (LMX1B) in the underlying mesoderm. Expression of Engrailed 1 (EN1) in the ventral ectoderm restricts WNT7a to the dorsal ectoderm thereby establishing the dorsal-ventral aspects of the limb. In mice lacking Wnt7a or Lmx1b function, limbs develop with near ventral-ventral symmetry. In contrast, over expression of Wnt7a or Lmx1b in the ventral limb generates a dorsal-dorsal limb. Mutations that disrupt LMX1B function and cause

haploinsufficiency, impair limb dorsalization and cause Nail-Patella Syndrome in humans (Chen et al., 1998).

Coordination of Inter-Axes Patterning

The signaling centers further coordinate patterning during limb outgrowth by inter-axes regulation. AER-related FGFs and SHH regulate each other in a positive feedback loop during limb development (Laufer et al., 1994; Niswander et al., 1994). Loss of FGF signaling from the AER diminishes SHH expression (Laufer et al., 1994) and removal of SHH downregulates FGF expression in the AER (Chiang et al., 2001).

SHH regulates FGFs in the AER via Formin, Gremlin, and bone morphogenic proteins (BMPs). A recessive mutation in mice termed *limb deformity* (*ld*) is characterized by mutations in the *Formin* locus. Within this locus resides a *cis*-regulatory region that negatively affects *Gremlin* expression (Zuniga et al., 2004). Gremlin is a BMP antagonist that aids in maintaining the structural/functional integrity of the AER. In Gremlin-deficient mouse embryos, a morphologically distinct and functional AER fails to develop which negatively affects Fgf signaling, and in turn, leads to diminished *Shh* expression (Khokha et al., 2003; Michos et al., 2004). Homozygous mice for the *ld* mutation present with limb patterning defects characterized by synostosis of the zeugopod in combination with oligo- and syndactyly of metacarpal bones and digits (Zeller et al., 1999). Zuniga et al., in their 2004 report, hypothesize that homologous mutations in this *cis*-regulatory region, which maps to chromosome 15q13-14 in humans (Maas et al., 1991), may be linked to human congenital malformations such as Cenani-Lenz-like nonsyndromic oligosyndactyly which resembles the *ld* phenotype (Zuniga et al., 2004).

WNT7a links the dorsal-ventral axis to the anterior-posterior axis by promoting the expression of SHH (Yang and Niswander, 1995). Similarly, the loss of Lmx1b function in knockout mice causes a loss in distal ulna formation in addition to ventral-ventral limbs (Chen et al., 1998). In humans, homozygous missense mutations of WNT7A causes ulnar deficiency/absence with loss of one or more ulnar digits in a condition called Al-Awadi/Raas-Rothschild syndrome (AARRS) (Kantaputra et al., 2010).

Defining the Number and Identity of Digits

A Turing-type model is now being used to describe how the number of digits is determined in the autopod, as opposed to a simpler diffusion-gradient type model of the polarizing agent, SHH (Sheth et al., 2012). Recent reports show this periodic patterning model integrates an activator (BMP) and an inhibitor (WNT) to specify a *SOX9* expression pattern, which stimulates chondrogenesis and dictates digit number (Raspopovic et al., 2014). BMPs may play additional roles in defining digit identities by regulating SMADs and the signal transduction pathways of the phalanx forming regions (Suzuki et al., 2008).

Joint Formation

Disruption of genes integrally involved in muscle and joint development also result in congenital anomalies of the limbs. Joint-development is under the regulation of WNT proteins and growth and differentiation factor 5 (GDF5). In mice, a Gdf5 deficiency leads to brachypodism, an alteration in the length and number of bones in the limb. The axial skeleton is not affected (Storm et al., 1994). In humans, a missense mutation in the GDF5 gene leads to multiple synostoses syndrome (Dawson et al., 2006), whereas haploinsufficiency of the gene leads to brachydactyly (Polinkovsky et al., 1997; Robin et al., 1997).

Arthogryposis in humans is classified as congenital joint contractures in two or more areas of the body. Passive extension and flexion in the affected joint or joints is negatively affected (Kalampokas et al., 2012). Arthogryposis may be caused by environmental factors, but genetic etiologies are also well established. Defects in muscle migration/formation, neuromuscular connectivity, and general cell metabolism can impair joint movement. Mutations in at least five genes (TNN12, TNNT3, TPM2, MYH3 and MYH8) are associated with distal arthrogryposis (restricted movement of the hands and feet) (Bamshad et al., 2009).

CLASSIFICATION

Increased knowledge in clinical genetics and the molecular basis of limb development has aided clinicians and scientists to better define and classify the

anomalies resulting from mutations/disruptions in this molecular machinery. Congenital Upper Limb Anomalies (CULA) have since been described as malformations, deformations (including disruptions), or dysplasia of the upper extremities during embryonic development resulting in an atypical limb phenotype at birth.

The categories included in the definition above are the basis for the Oberg, Manske, Tonkin (OMT) classification of CULA and describe/convey etiological associations as well as phenotype (Table 1). Malformations are caused by intrinsically abnormal processes during limb development. Deformations are abnormal forms, shapes or positions of the limb that result from mechanical forces interfering with the normal developmental process. Dysplasia refers to the abnormal organization of cells into tissue. The Oberg, Manske, Tonkin (OMT) classification was proposed in 2010 and subsequently adopted (as of 2014) by the International Federation of Societies for Surgery of the Hand (IFSSH) (Oberg et al., 2010; Ezaki et al., 2014).

EPIDEMIOLOGY

The incidence of CULA in the US and worldwide is underestimated mainly because reporting these anomalies is not mandated in many jurisdictions as well as the fact that many musculoskeletal disabilities are grouped together. What has been studied and reported, however, highlight congenital malformations as the leading cause of infant mortality and also accounts for 12% of all pediatric hospitalizations (Egbe et al., 2015). Data from the European Surveillance of Congenital Anomalies (EUROCAT) show that congenital limb anomalies account for almost 16% of all anomalies. In Sweden, CULA have been reported with an incidence of 21.5 per 10,000 (~ 1 in 500) live births (Ekblom et al., 2014). Previous reports estimate the prevalence of congenital hand anomalies to be 11.4 (Lamb et al., 1982) in Scotland, and 19.7 (Giele et al., 2001) per 10,000 live births in Western Australia. These different studies could reflect biologic variability in regional populations, a relative increase in the apparent prevalence of CULA, or regional differences in reporting/recognition of hand abnormalities. Irrespective, the data suggest that CULA are one of the most common malformations recognized at birth.

Table 1. The OMT classification of congenital upper limb anomalies (CULA)

I. MALFORMATIONS

A. Failure of axis formation/differentiation— entire upper limb

1. Proximal-distal axis
- i. Brachymelia with brachydactyly
- ii. Symbrachydactyly
  - a) Poland syndrome
  - b) Whole limb excluding Poland syndrome
- iii. Transverse deficiency
  - a) Amelia
  - b) Clavicular/scapular
  - c) Humeral (above elbow)
  - d) Forearm (below elbow)
  - e) Wrist (carpals absent/at level of proximal carpals/at level of distal carpals)
  - f) Metacarpal
  - g) Phalangeal (proximal/middle/distal)
- iv. Intersegmental deficiency
  - a) Proximal (humeral – rhizomelic)
  - b) Distal (forearm – mesomelic)
  - c) Total (Phocomelia)

2. Radial-ulnar (anteroposterior) axis
- i. Radial longitudinal deficiency - Thumb hypoplasia (with proximal limb involvement)
- ii. Ulnar longitudinal deficiency
- iii. Ulnar dimelia
- iv. Radioulnar synostosis
- v. Humeroradial synostosis - Elbow ankyloses

3. Dorsal-ventral axis
- i. Ventral Dimelia
  - a) Furhmann/Al-Awadi/Raas-Rothschild syndromes
  - b) Nail Patella syndrome
- ii. Absent/hypoplastic extensor/flexor muscles

4. Unspecified axis
- i. Shoulder
  - a) Undescended (Sprengel)
  - b) Abnormal shoulder muscles
  - c) Complex
- ii. Arthrogryposis

B. Failure of axis formation/differentiation— hand plate

1. Proximal-distal axis
- i. Brachydactyly
- ii. Hypodactyly
- iii. Symbrachydactyly
- iv. Transverse deficiency
  - a) Wrist (carpals absent/at level of proximal carpals/at level of distal carpals)
  - b) Metacarpal
  - c) Phalangeal (proximal/middle/distal)

2. Radial-ulnar (anteroposterior) axis
- i. Radial deficiency (thumb - no forearm/arm involvement)
- ii. Ulnar deficiency (no forearm/arm involvement)
- iii. Radial polydactyly
- iv. Triphalangeal thumb
- v. Ulnar dimelia (Mirror hand – no forearm/arem involvement)
- vi. Ulnar polydactyly

3. Dorsal-ventral axis
- i. Dorsal dimelia (palmar nail)
- ii. Ventral (palmar) dimelia (including hypoplastic/aplastic nail)

4. Unspecified axis
- i. Soft tissue
  - a) Syndactyly
  - b) Camptodactyly
  - c) Thumb in palm deformity
  - d) Distal arthrogryposis
- ii. Skeletal deficiency
  - a) Clinodactyly
  - b) Kirner's deformity
  - c) Synostosis (carpal/metacarpal/phalangeal)
- iii. Complex
  - a) Cleft hand
  - b) Synpolydactyly— central
  - c) Apert hand
  - d) Not otherwise specified

II. DEFORMATIONS
- A. Constriction ring sequence
- B. Trigger digits
- C. Not otherwise specified

III. DYSPLASIAS

A. Hypertrophy

1. Whole limb
- i. Hemihypertrophy
- ii. Aberrant flexor/extensor/intrinsic muscle

2. Partial limb
- i. Macrodactyly
- ii. Aberrant intrinsic muscles of hand

B. Tumorous conditions

1. Vascular
- i. Hemangioma
- ii. Malformation
- iii. Others

2. Neurological
- i. Neurofibromatosis
- ii. Others

3. Connective tissue
- i. Juvenile aponeurotic fibroma
- ii. Infantile digital fibroma
- iii. Others

4. Skeletal
- i. Osteochondromatosis
- ii. Enchondromatosis
- iii. Fibrous dysplasia
- iv. Epiphyseal abnormalities
- v. Others

Adopted by the International Federation of Societies for Surgery of the Hand (IFSSH) in 2014 (Ezaki et al., 2014)

Using the OMT classification, "malformations" was found to be the most prevalent CULA category in Sweden (Ekblom et al., 2014) and also in midwestern United States (Goldfarb et al., 2015). Epidemiological data from Finland (Koskimies et al., 2011), Sweden (Ekblom et al., 2010), and Western Australia (Giele et al., 2001) show that males are more likely affected than females. Bilateral malformations were more common than malformations affecting either the left or right sides (Ekblom et al., 2014). The most common diagnoses in the midwestern states of the US were trigger digits and multiple hereditary exostoses (Goldfarb et al., 2015), whereas in Sweden the most common diagnoses were trigger digits and ulnar polydactyly (Ekblom et al., 2014).

Congenital anomalies can be categorized as "isolated" when only the limb is involved or "associated" when one or more additional major malformations are present. Of infants born with multiple congenital anomalies, the most common disorder associated with limb malformations is the VACTERL (Vertebral anomalies, Anal atresia, Cardiac malformations, Tracheo-Esophageal fistula, Renal anomalies, and Limb abnormalities) association (Robert et al., 1997; Stoll et al., 2010). The most prevalent organ system reported with limb reduction defects is the cardiac system (Robert et al., 1997; Rosano et al., 2000; Stoll et al., 2010) followed by the urogenital, central nervous, and digestive systems (Stoll et al., 2010).

RISK FACTORS

In many cases, the precise etiology of CULA is unclear or unknown. There are however a variety of risk factors that predispose an embryo to developmental anomalies. These fall into two large categories: Genetic and Epigenetic/Environmental.

Genetic Contributions

The molecules involved with limb development are the key targets of genetic disruption, although disruption of generalized growth and development may also manifest with associated limb abnormalities. The molecules involved with limb development are the key targets of genetic disruption, although disruption of generalized growth and development may also manifest with associated limb abnormalities.

Smith-Lemli-Opitz syndrome (SLOS), for example, is a human metabolic disorder caused by a mutation in the 7-dehydrocholesterol reductase (7-DHCR) enzyme, leading to an inborn error of cholesterol biosynthesis. SLOS is characterized by dysmorphogenesis of multiple organs including the limb. Noting that cholesterol interacts with hedgehog proteins and modulates their activity, Porter et al., postulated that there may be defective modification of the hedgehog proteins and perhaps other similarly processed proteins in SLOS (Porter et al., 1996).

In terms of limb phenotype, patients present with shortened limbs, postaxial polydactyly and proximally placed thumbs. Using an *in vivo* rat model for cholesterol deficiency, Gofflot et al., then chemically blocked cholesterol biosynthesis and were able to induce patterns of the autopod consistent with SLOS patients. Via *in situ* hybridization, they were also able to show modifications in downstream targets of Sonic and Indian hedgehog signaling thereby indicating that cholesterol plays a pivotal role in limb morphogenesis (Gofflot et al., 2003).

Apart from the apparent role of cholesterol in cell structure and signaling that could account for the SLOS phenotype, some of the pathologic characteristics may also be due to accumulation of cholesterol precursors (Merkens et al., 2004). Currently, dietary cholesterol is being considered as a therapeutic agent for SLOS (Elias et al., 1997; Irons et al., 1997; Nwokoro and Mulvihill, 1997) as reports show that dietary cholesterol not only stably increases plasma cholesterol levels but also decreases plasma concentrations of the potentially toxic cholesterol precursors through a feedback inhibition mechanism (Pappu et al., 2002; Merkens et al., 2004). Animal studies in pregnant rats fed an inhibitor of the enzyme defective in SLOS, and supplemented with cholesterol, suggest that there may be some benefit to prenatal therapy (Barbu et al., 1988).

A number of the targeted disruptions have already been discussed above; however, there are about 1200 different genes associated with just over 500 OMT related phenotypes (for review see OMIM, http://www.ncbi.nlm.nih.gov/omim; or CulaPhen, Reference in process).

Many of these genetic disorders occur as spontaneous mutations but many are inherited. For example, advanced age of parents has been linked to chromosomal abnormalities in their offspring. In Western Australia, the reported prevalence of congenital hand anomalies increased with maternal age. Mothers older than 40 years were found to be twice as likely as mothers younger than 30 years to have a child with a hand deformity (Giele et al., 2001). Advanced paternal age is also associated with a higher risk for

mutations, particularly in the fibroblast growth factor receptor family (FGFRs) (Crow, 2000).

Epigenetic/Environmental

There is a widely accepted notion that "we are products of our environment." The same holds true for a developing embryo in the womb. This is most clearly seen in Barker's hypothesis, also known as Fetal Programming (Barker, 1990). Fetal programming is an emerging concept that links environmental conditions during embryonic and fetal development with risk of diseases later in life.

Studies on fetal programming have mainly focused on healthy weight maintenance during gestation as a means of lowering adverse risks in infants such as chronic conditions in adulthood - obesity, diabetes, and cardiovascular disease. Other factors termed teratogens, such as maternal age, infection, drugs (pharmaceutical or recreational) may also play a role in fetal programming and have been linked to birth defects including CULA.

Teratogens

Prenatal exposure to a teratogenic agent accounts for approximately 10% of all birth defects (O'Rahilly and Müller, 2001). A teratogen refers to any agent that can induce or increase the incidence of congenital anomalies and includes radiation, infections, drugs and other chemicals. Many of these harmful agents cause damage only if exposure occurs during a sensitive period of prenatal development. In other words, the susceptibility of an embryo to a teratogen may depend on the stage of development. Furthermore, the organs undergoing the most rapid proliferation, differentiation or migration during exposure are the most vulnerable to the influence of the teratogen. The amount (dosage) and length of time of exposure to a teratogen are critical factors that influence the degree of harm the agent will cause. In general, the higher the dosage of teratogen the more deleterious the effects on the embryo. Prolonged exposure, even at low doses can cause cumulative harm.

Ionizing Radiation

Ionizing radiation can injure the developing embryo due to cell death or chromosome injury. There is no proof, however, that human congenital malformations have been caused by diagnostic levels of radiation (Gilbert-Barness, 2010). As it relates to congenital upper limb anomalies, Wang et al., report that *in utero* irradiation of mouse fetuses on embryonic day 11 (Thaller stages 18-19, during autopod development) induces a dramatic increase in autopod malformations. They detected a marked increase in the number of apoptotic cells in the predigital regions in the forelimb buds 4 hours after irradiation. Aphalangy (absence of the phalanx bone on one or more digits) and ectrodactyly (split hand/cleft hand) were the main types of anomalies observed on day 19 in the limbs of the fetuses irradiated with 5 Gy. (Wang et al., 1999). In humans, the average radiation dose from an abdominal X-ray is 1.2 mGy and from an abdominal CT scan is 10 mGy, dosages far lower than those required in the murine model (Lin, 2010). Dosage for radiation therapy of cancer cells is much greater and varies depending on the type and location of the cancer. For adjuvant radiotherapy in ovarian cancer, over 45 Gy may be administered over a one to two-month period (Biete et al., 2010). To avoid any potential exposure to a developing gestation, female patients of child bearing potential (~12-50 years old) must be screened and a pregnancy ruled out before the initiation of radiation therapy (Akintomide and Ikpeme, 2014).

Nutrition

Overnutrition leading to a high body mass index (BMI) can increase the risk of congenital anomalies. Stothard et al., reported an increased risk of limb reduction anomalies in gestations from obese women (BMI: >30) compared to women within the recommended BMI (BMI: 19-25) (Stothard et al., 2009). Nutrient deficiencies during pregnancy, including those of microminerals and vitamins as a result of undernutrition or maternal starvation, have been implicated in a variety of developmental defects (Polin et al., 2011).

Information regarding vitamin and mineral requirements, dietary intake, and metabolism during pregnancy tends to be lacking, thus making it difficult to form associations with fetal/maternal outcomes. Deficiencies in macrominerals such as phosphorous, magnesium, or calcium are rare during pregnancy (Ladipo, 2000), although the latter may exist in individuals who avoid a diet rich in dairy products. Low concentrations of magnesium and

calcium have been associated with gestational hypertensive disorders, which can increase the risk of birth defects (Li et al., 2011), although a causal relationship for the former has not been found (Maine, 2000). Some associations have been well-established, such as a maternal deficiency of folic acid and thiamine to neural tube defects in the fetus and neonatal death (Ladipo, 2000). The effects of micronutrient deficiency (including copper, zinc and manganese) are less clear in humans than in animals. Manganese deficiency in cattle, however, has been reported to include impaired growth, impaired reproductive function and overt congenital limb anomalies (de Carvalho et al., 2010; Allen and Dart, 2015).

Diabetes Mellitus

A correlation has been reported between elevated hemoglobin A1c (HbA1c) levels and the incidence of major congenital anomalies in infants of diabetic mothers (Miller et al., 1981). HbA1c levels during pregnancy that exceed 11.5% are associated with congenital abnormalities in 66% of the offspring, but levels below 9.5% are not associated with increased frequency of anomalies in infants of diabetic mothers (Nielsen et al., 1997). Defects of the heart, central nervous system (CNS), kidneys, and skeleton predominate, however, other anomalies including those observed in the VACTERL association have also been reported. (Gilbert-Barness, 2010).

Researchers from the National Birth Defects Prevention Studies (NBDPS) found that pre-existing diabetes was more common among mothers of babies born with a range of birth defects when compared to mothers with gestational diabetes. The birth defects include anencephaly, hydrocephaly, cleft lip (with or without cleft palate), absent kidney, limb deficiencies, and a variety of heart defects. Additionally, if the mother was overweight or obese and had pregestational diabetes, the risk of these birth defects was increased. (Correa et al., 2008)

Infections

Cytomegalovirus (CMV)

The most common viral infection of the fetus is congenital CMV infection, which can lead to spontaneous termination of pregnancy when the embryo is infected during the first trimester. Exposure later in the pregnancy

can result in intrauterine growth retardation, micromelia (shortened limbs), chorioretinitis, blindness, microcephaly, cerebral calcifications, mental retardation, and hepatosplenomegaly (Chung, 2004).

Varicella Zoster Virus (VZV)

Congenital VZV syndrome describes a group of defects that include scars, defects of muscle and bone, malformed and paralyzed limbs, small head size, blindness, seizures, and mental retardation. During the first 20 weeks of pregnancy, if a woman becomes infected with the varicella virus, there is a 2% increased chance that her baby will exhibit symptoms of this syndrome (Paryani and Arvin, 1986). This syndrome is rarely seen if the infection occurs after 20 weeks of pregnancy (Gilbert-Barness, 2010).

Pharmaceutical Drugs

Thalidomide

To date, the most notorious drug associated with CULA is thalidomide, which was commonly prescribed to treat nausea in pregnant women in the late 1950s and early 1960s (Bren 2001). During this period, the use of thalidomide before 42 days of gestation led to birth defects in over 10,000 individuals from 46 different countries, with only a 50% survival rate (Vargesson, 2015). Thalidomide impacts proximal-distal patterning, with the most common form of limb defects reported as shortened limbs, with the arms being more frequently affected (intersegmental defect). The range of birth defects included phocomelia, dysmelia, amelia, bone hypoplasticity, and other congenital defects affecting the ear, heart, and other internal organs.

Warfarin

Women with a history of thromboembolic disease or artificial heart valves often require long-term anticoagulant therapy. There is an estimated 25% risk for affected infants after exposure during the period from 8 to 14 weeks of pregnancy. Warfarin inhibits the formation of carboxyglutamyl from glutamyl residues, decreasing the ability of proteins to bind calcium (Pauli, 1988). Calcific stippling occurs primarily in the tarsals, proximal femurs, and paravertebral processes. Brachydactyly and small nails, with greater severity in the upper limbs, has been present in about one-half of affected infants (Gilbert-Barness, 2010).

Valproic Acid (VPA)

VPA is an anticonvulsant used to treat seizures and manic depression. The mechanism of action surrounding the teratogenic effects of VPA is unclear. Paradis and Hales hypothesized that VPA disrupts regulation of the expression of genes that are critical in chondrogenesis and osteogenesis during limb development. They tested this in mouse forelimbs and found it to be true as there was a downregulation in expression of *RUNX2* and *SOX9* (Paradis and Hales, 2013). Analysis of a case-control study done on data from Spanish Collaborative Study of Congenital Malformations (ECEMC) assessed the relationship between prenatal exposure to VPA and the presence of limb deficiencies in newborn infants. Of the total of malformed infants exposed to VPA, 36.8% (21/57) presented with congenital limb defects of different types, including overlapping digits, clubfoot, clinodactyly, arachnodactyly, pre- and postaxial polydactyly (Rodriguez-Pinilla et al., 2000).

Retinoic Acid

In very high doses, retinoic acid (a derivative of Vitamin A) may affect limb development. Isotretinoin, which is closely related to Vitamin A, is used by some as a treatment for severe acne. Isotretinoin (sold under several brand names, including Accutane) has been linked to birth defects and is not advised for women who are pregnant or who may become pregnant while taking the medicine.

Recreational Drugs

Alcohol

Alcohol is a common drug abused by women of childbearing age. Infants born to alcoholic mothers demonstrate prenatal and postnatal growth deficiency, mental retardation, and other malformations, including those of the limb (Spiegel et al., 1979; Herrmann et al., 1980; Cremin and Jaffer, 1981; van Rensburg, 1981). In the early 1970's a link between maternal alcohol intake and congenital upper limb anomalies was observed and Spiegel et al., reported the most frequent malformation associated with Fetal Alcohol Syndrome (FAS) is radioulnar synostosis (Spiegel et al., 1979). This group also reported that hypoplastic toenails, shortened fingers, camptodactyly of the fingers, clinodactyly of the toes, and flexion contractures of the elbow are observable phenotypes of FAS.

Cocaine

The complications of intrauterine growth restriction and limb defects, among other deformations, linked to maternal cocaine use appear to be related to vascular disruption. Cocaine may alter the availability and utilization of calcium and reduce blood flow from the uterus to the placenta. It is metabolized very slowly in the fetus due to low plasma cholinesterase activity. Cocaine-exposed fetuses also have increased incidences of prematurity, microcephaly, and sudden infant death (Gilbert-Barness, 2010).

Lysergic Acid Diethylamide (LSD)

Children born to mothers who used LSD before or during pregnancy have exhibited a variety of congenital anomalies. Defects of the central nervous system, eyes, and limbs (including arthogryposis) may be present (Gilbert-Barness, 2010). It is not uncommon for LSD users to be using other drugs simultaneously, which may compound the variety of anomalies observed.

Other Chemicals

Toluene Exposure

Toluene is an organic solvent used in gasoline and may be present in laboratory settings. A strict permissible exposure limit (PEL) for toluene has been set by the U.S. Occupational Safety and Health Administration (OSHA) to regulate the handling of this teratogen. Toluene embryopathy includes prenatal and postnatal growth deficiency, microcephaly, anencephaly, developmental delay, cardiac and limb defects, and craniofacial anomalies similar to fetal alcohol syndrome (FAS) (Gilbert-Barness, 2010).

Other Environmental Factors

Mechanical Constraint of Development

Mechanical forces can also act as teratogens. Abnormalities (deformations/malformations or disruptions) occurring as a result of mechanical forces are usually due to restriction of fetal movements. Malformations of the uterus, cervical lodgment of the embryo, and oligohydramnios (low amniotic fluid level) can produce similar phenotypes such as club hand/foot. Amniotic bands, which are fibrous strands associated

with amniotic membrane disruption and repair, can cause intrauterine amputations or deformations of the limbs. (Chung, 2004).

Hyperthermia

Hyperthermia is defined as a body temperature of at least 38.9°C and has been described as an antimitotic teratogen after exposure between weeks 4 and 14 (Gilbert-Barness, 2010). In a retrospective study, Smith and colleagues presented 21 patients who had been exposed during pregnancy to hyperthermia caused by infections or by sauna bathing. When the mothers were exposed to hyperthermia between 4 and 6 weeks' gestation, severe mental deficiency, seizures in infancy, microphthalmia, midface hypoplasia, and mild distal limb abnormalities were the most consistent manifestations observed in their offspring. Between 7 to 16 weeks' gestation, predominant features in the offspring were hypotonia, neurogenic arthrogryposis, and central nervous system dysgenesis (Smith et al., 1978).

PRENATAL DIAGNOSIS

There are many reasons supporting the use of prenatal testing to identify and diagnose CULA and other congenital anomalies. Foreknowledge will enable the parents to begin planning for a child with special needs, start addressing anticipated lifestyle changes, identify support groups and resources, and become aware of potential interventions that may be considered as well as the decision whether to carry the gestation to term.

Non-Invasive Screening Tools

Ultrasound scanning is offered to pregnant women around 11 to 14 weeks of gestation. An examination of fetal limbs is part of a routine scan. There are several potential benefits of routine prenatal ultrasound scanning at 10 to 20 weeks. It causes little discomfort for mothers and is simply and quickly performed. It has no adverse effects for the mother or fetus. It allows the instigation of therapeutic intervention or informed termination of pregnancy. Data from Kevern et al., used two different groups to study prenatal detection rates of limb anomalies and the accuracy of prenatal diagnosis. The data suggests low sensitivity of this technique (15/60 anomalies were detected);

however, if an anomaly is detected, then it is likely to be confirmed postnatally indicating a high positive predictive value (Kevern et al., 2003).

Limitations are imposed on technicians by fetal movement and the fact that some fetal positions can obscure limb details. The experience of the technicians may also affect low detection rates with more extensive examinations by senior staff being reserved for pregnancies with a high risk for abnormality (Kevern et al., 2003).

Cell-free fetal DNA (cffDNA) testing from the mother's blood may be used in prenatal screening for aneuploidy (abnormal number of chromosomes) and genetically inherited diseases. It is usually offered after 10 weeks' gestation and especially recommended for high-risk pregnancies. cffDNA originates from the placental trophoblasts which expel DNA fragments into the maternal blood. It is estimated that 2-6% of the DNA in the maternal blood is fetal in origin (Lo et al., 1998). cffDNA is significantly smaller than the maternal DNA in the bloodstream (approximately 200bp in size), which is the basis for separating fetal DNA from maternal DNA in the plasma (Li et al., 2004; Li et al., 2005).

Clinical genetic testing is increasingly becoming a standard practice for patients with congenital abnormalities including unexplained developmental delay/intellectual disability (DD/ID), autism spectrum disorders (ASD), and multiple congenital anomalies (MCA) (Miller et al., 2010). Published guidelines for testing such patients have emphasized (1) testing for chromosomal abnormalities by G-banded karyotyping and (2) testing for common single-gene disorders (Moeschler et al., 2006).

G-banded karyotyping allows a cytogeneticist to visualize and analyze chromosomes for chromosomal rearrangements, including genomic gains and losses. Molecular karyotyping, also known as a chromosomal microarray (CMA), performs a similar function, but at a much higher resolution for genomic imbalances. G-banded karyotyping has been the standard first-tier test for detection of genetic imbalance for more than 35 years, whereas CMA is not yet standard in all clinical settings (Miller et al., 2010).

Invasive Diagnostic Tools

Amniocentesis and chorionic villus sampling (CVS) are used to prenatally rule out chromosomal abnormalities. These tests are usually done if there is a family history of aneuploidy or if any of the screening tests gives a positive result. During amniocentesis, a small amount of amniotic fluid, which contains

fetal exfoliated squamous cells, is sampled (trans-abdominally) from the amniotic sac surrounding a developing fetus, and the fetal DNA is examined. This is usually done between the 15th and 20th weeks of pregnancy (and not earlier) to minimize risk to the developing fetus. The obtained fetal cells are separated from the amniotic fluid, cultured, fixed, stained, and examined microscopically for chromosomal abnormalities.

CVS is a procedure wherein a sample of the placental tissue (chorionic villus) is obtained (in a transcervical or transabdominal manner) and assessed for chromosomal abnormalities, usually with fluorescent *in situ* hybridization (FISH) or PCR. CVS usually takes place at 10–12 weeks' gestation, earlier than amniocentesis, and is the preferred technique before 15 weeks (Alfirevic and von Dadelszen, 2003).

Chorionic villi sampling and amniocentesis have both been linked to procedure-related miscarriages, with average risks of about 1 in 100 pregnancies and 1 in 200 pregnancies, respectively (Olney et al., 1995; Mujezinovic and Alfirevic, 2007). Apart from a risk of miscarriage, there is a risk of infection and amniotic fluid leakage. Leakage of the amniotic fluid can result in oligohydramnios (low amniotic fluid level), which is associated with developmental disorders including limb reduction defects (Froster and Baird, 1992).

POSTNATAL DIAGNOSIS

After delivery, the pediatrician may consult a congenital hand surgeon to confirm the diagnosis and with the family, develop a treatment plan. A detailed history of the pregnancy and history of any known family members with congenital anomalies is the first step of the assessment. Since CULA can be isolated or associated to anomalies of other organ systems, the history plays an important role in determining whether additional systems need to be evaluated and additional specialists consulted. The initial evaluation and examination will likely establish a tentative diagnosis and OMT classification and indicate whether other tests, e.g., genetic, x-rays, or functional, are needed to confirm the diagnosis. The precise structural and functional CULA impairment may require ongoing examinations and investigations as the child grows. The child at play is an exceptional diagnostic tool. How they use the affected limb and to what degree the affected structures function can largely be determined at distance by observing play with random or standardized toys. With time, handedness will become evident. X-rays are an important tool for

determining skeletal differences, but in the immature skeleton with abundant cartilage, the full extent of abnormality may not be evident. Magnetic resonance imaging (MRI) can provide soft tissue and vascular details but also requires sedation. MRI is more commonly used to plan and optimize an intervention rather than clarify a diagnosis.

MANAGEMENT

To the growing child, the CULA will be "normal" and the mind will develop with the body that is present. The perception of the limb as abnormal will occur over time, but a supportive family and clinical team can be an enormous help to the child as they navigate this adjustment. Because a number of professionals may be involved with the care of a child with CULA, clinic visits may be numerous and overwhelming to the child and family. Some institutions have implemented a team approach for a variety of congenital anomaly clinics, including hand clinic, in which pediatricians, geneticists, surgeons, therapists, and social workers converge to offer a one stop clinic where a team can assess, discuss and collaborate with the family regarding the treatment plan.

It is important to evaluate the child's structural and functional progression using developmental milestones to fully understand the impact of their CULA. For example, comparing the X-Ray appearance of secondary ossification centers and subsequent growth plate closures to standardized norms can indicate which structures are affected by the CULA and how the natural progression of their condition may manifest in adult life. Measuring the milestones of prehension from rudimentary grasp to the incorporation of fine thumb involvement can suggest how the structural aspects of the CULA has affected function (Erhardt and Lindley, 2000). These assessments allow caregivers to monitor the condition's progression and offer timely therapeutic options to the patient and family.

Conservative Management

For some disorders, monitoring, splinting and/or physical therapy are the preferred approach. Trigger fingers are a common CULA that often resolves over time without intervention (Baek and Lee, 2011). Conservative management may also be most appropriate for conditions in which outcomes

from operative interventions are generally poor such as camptodactyly (Watts and Hooper, 2006). Other conditions that have yet to declare the degree of their structural or functional impairment are likely best to monitor and manage conservatively. Conservative management allows a child to accommodate to a disorder and develop alternative ways to function, which may be problematic if operative intervention is desired in the future. When conservative management fails to correct or slow the progression of a condition, patients and caregivers turn to operative intervention.

Operative Intervention

If the patient and family opt for reconstructive surgery to address the CULA, three main factors influence decision-making for the operative plan: function, appearance, and timing.

Hand function requires a stable shoulder, upper arm and forearm. Within the hand, a mobile thumb is needed to accomplish opposition, radial digits for fine pinching, and ulnar digits for grasping. Adequate digit length, stability, and mobility are needed to optimize hand function. In a complicated preaxial polydactyly with 7 digits and near mirror image duplication, one of the extra digits should be pollicized (shortening with partial rotation) to form a thumb and the remaining polydactylous digits removed (Figure 3). Depending on the CULA, it may not be possible to attain normal function, particularly with a complex anomaly. Prioritization of functional objectives for reconstruction will depend on the complexity of the abnormality, the handedness of the child, and the function of the other upper limb. Cultural expectations must also be considered. For example, if the forearm must be fixed in a stable position to optimize hand function, having the hand fixed in supination would allow a child to hold their own bowl when eating, whereas in another culture maintaining the hand in a neutral position would accommodate more customs.

The appearance of a noticeable abnormality such as distal thumb duplication (Wassel type III) can be a source of considerable concern for the child and parents, even if it allows significant function. Moreover, loss of a structure is often less noticeable than an abnormal structure. Pollicization of the index finger in a hand with thumb aplasia produces a reasonable thumb equivalent with three additional digits, an outcome that passes as normal to the casual observer. In some complex cases, such as symbracydactyly, reconstruction to form a normal appearing hand is not possible. In these cases, adding a toe transfer to improve function may be seen as further disfiguring

the hand and calling more attention to the abnormality. If a hand with normal appearance cannot be attained, realistic goals need to be discussed and approaches to minimize attention-drawing features considered.

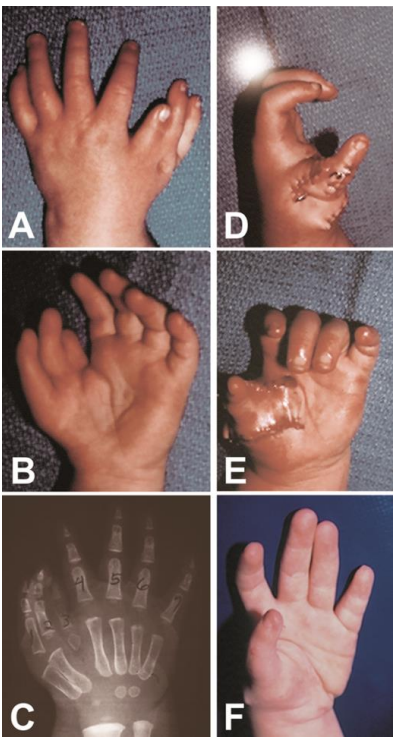

Figure 3. Reconstruction of a mirror hand (pre-axial polydactyly) with pollicization of one of the pre-axial digits. A-C) Preoperative view of the mirror hand with 3 additional pre-axial digits: Dorsal view (A), ventral view (B) and radiograph (C). D-E. Intraoperative views of the repair. View from the lateral (radial) aspect following rotation and reconstruction of the pollicized digit (D). Volar (anterior) view post-reconstruction of the "new" thumb (E). Several months post-operative (F). From Virchel Wood, "Reconstruction of mirror hands," Congenital Hand Anomaly Study Group (CHASG), 1995, Tampre, Finland and from Wood. (Wood, 1993).

The timing of conservative therapy or operative repair for most CULA is typically initiated within the first 18 months of life in an effort to optimize the anatomy for which the child will learn to use (Tonkin, 2004). There are several notable exceptions. Trigger fingers may resolve without intervention and observation or conservative management is typically preferred over more aggressive early intervention. The natural progression of camptodactyly and

the typically poor results from operative intervention prompts observation and consultations with patient and families regarding realistic expectations for any intervention. Reconstruction of a symbrachydactyly is complicated and may encompass a series of operations that include a toe transfer. Optimal adaption to the reconstructed anatomy is always considered; however, musculoskeletal and vascular development of the symbrachydactyly and toe for transfer are also important to ensure success. Thus, a delay until 2-3 years of age is considered more optimal. Although early intervention has benefits, there may be a number of reasons to defer intervention including parental apprehension. Honest open discussions with the parents about options and outcomes is the responsibility of the caregiver, and it may take some time to collectively develop a plan of treatment for the child that the parents can support with confidence.

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Chapter 9

EXFOLIATION SYNDROME AND EXFOLIATIVE GLAUCOMA: FROM GENETICS TO SURGERY

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ABSTRACT

Exfoliation syndrome (XFS) is a genetically determined systemic condition characterized by the production and accumulation of an abnormal protein, the exfoliative material. Though XFS is a systemic elastosis, the most significant clinical consequences occur in the eye (nuclear cataract formation, Zinn's zonule fragility, increased risk for cataract surgery complications and exfoliative glaucoma [XFG]). XFG is the most common type of secondary open-angle glaucoma, and is characterized by elevated intraocular pressure with high fluctuation, and rapid progression. XFG is typically a disease of the elderly. In many cases XFG starts as a clinically unilateral disease in eyes with XFS. This

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chapter summarizes the current knowledge on genetics, environmental factors, clinical characteristics, medical, laser and surgical treatment options. It also addresses the significance of systemic vascular diseases associated with XFS and XFG, and the need for consultation between the ophthalmologist and cardiologist or general practitioner during long-term management of the disease.

INTRODUCTION

Open-angle glaucoma is one of the most important causes of severe irreversible visual field deterioration, decrease of vision related quality of life, and blindness [1-4]. Due to global population ageing along with increased life expectancy, glaucoma will have an ever increasing impact in the next decades. Since open-angle glaucoma is a group of progressive diseases, end-of-life glaucoma related blindness is not uncommon [5,6]. This chapter deals with one type of the open-angle glaucoma, the most common secondary open-angle glaucoma, exfoliative glaucoma (XFG)[2-4,7]. XFG is different from primary open-angle glaucoma; it develops from exfoliation syndrome (XFS), a systemic condition. For several decades XFG was considered a rare type of glaucoma and frequently remained unrecognized by ophthalmologists. In the recent years, however, both research and clinical interest in XFG have dramatically increased so that awareness of XFG is heightened worldwide.

EXFOLIATION SYNDROME

Exfoliation (or "pseudoexfoliation") syndrome is a common genetically determined systemic condition characterized by the synthesis and accumulation of an abnormal protein, the exfoliation (or pseudoexfoliation) material (XFM). In XFS, elastin production and metabolism are disturbed, thus XFS is a systemic elastosis [3,4]. Clinically, the signs of XFS are best detectable with slit lamp examination in the anterior segment of the eye after pupillary dilation. XFM appears on the anterior lens capsule in a typical distribution ("classic" XFS): a homogeneous central XFM area ("central disc") in the pupillary area surrounded by a transition zone containing some XFM. The transition zone reflects the brushing effect of pupil movements on the XFM. Outside the transition zone a peripheral zone of XFM deposition is seen (Figure 1).

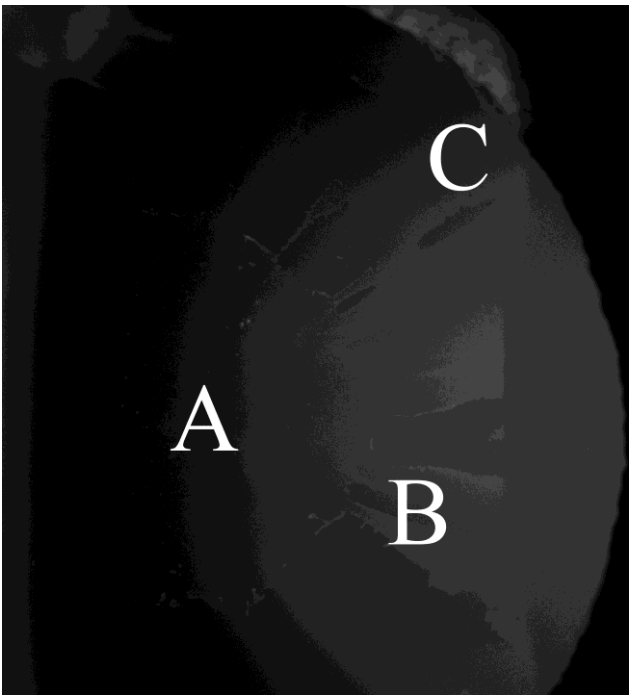

Figure 1.Distribution of exfoliation material on the lens surface. A, central disc; B, transition zone; C, peripheral zone.

Detection of XFM is difficult without pupil dilation, particularly because in the early stages of XFS the amount of XFM is small and its distribution less obvious. Clinically XFS is frequently unilateral: at the slit lamp it is detectable only in one eye. However, XFS is always a systemic disease: even in apparently unilateral cases, XFM is ultrastructurally detectable in both eyes and in several extraocular tissues and organs [3,4,8]. In approximately 38% of unilateral presentations, XFS becomes clinically detectable in the seemingly uninvolved fellow eye in a decade [9]. Clinically XFS (and XFG) typically appears after 60 years of age.

XFM is also frequently detectable on the pupil margin[10,11], the corneal endothelium[12], the zonules and the anterior chamber angle where pigment granules liberated from the iris tissue due to XFS are typically accumulated and distributed along undulating lines on and anterior to the Schwalbe's line (Sampaolesi' line) [3,4,11]. The loss of melanin granules results in the typical "moth-eaten" appearance of the pupillary margin in XFS. The zonules holding

the crystalline lens are also frequently damaged in XFS due to elastosis [3,4,8,11]. As a consequence, dislocation of the crystalline lens or a previously implanted artificial intraocular lens (in-the-bag IOL dislocation) is not uncommon in eyes with XFS. When the crystalline lens is dislocated towards the anterior chamber, pupillary block and secondary angle closure glaucoma can develop [13]. Development of nuclear cataract is also typical in XFS [14]. In addition, the disturbance of elastin metabolism diminishes the resistance of the lamina cribrosa [15, 16]; leads to decreased blood-aqueous barrier function in the iris, which results in localized vascular occlusions in the anterior iris layer [17]; and increases the postoperative inflammatory reaction after extracapsular cataract surgery[14]. However, the most significant complication of XFS is the development of intraocular pressure (IOP) elevation and XFG, the most common secondary open-angle glaucoma worldwide [8,18,19].

EXFOLIATIVE GLAUCOMA

XFG develops from XFS, but most XFS eyes do not eventually convert to XFG. Initially, IOP increases and exhibits high diurnal fluctuation [20]. Later, mild structural alterations (e.g., retinal nerve fiber layer thinning) become detectable with imaging devices [20-22]. Ultimately, the increased oxidative stress in the anterior chamber and pigment liberation from the iris lead to further, typically very significant IOP elevation, which (in part due to the elastosis-related weakening of the lamina cribrosa) results in severe glaucomatous neuroretinal rim damage, increased optic disc cupping, severe retinal nerve fiber layer loss and severe visual field deterioration, in many cases already prior to the first ophthalmology visit [18,19,23]. Since the presence of XFG is frequently unilateral or the severity of glaucomatous damage is remarkably asymmetrical, most patients do not realize unilateral visual field deterioration until it becomes very severe, or until the fellow eye suffers from central visual field deterioration [13,23]. The progression rate of XFG is almost 3 times faster than that of high-pressure primary open-angle glaucoma, and 4 times higher than that of normal pressure glaucoma [24,25].

ROLE OF GENETIC ALTERATIONS, ENVIRONMENTAL FACTORS AND OXIDATIVE STRESS IN THE DEVELOPMENT OF EXFOLIATION SYNDROME AND EXFOLIATIVE GLAUCOMA

XFS is a genetically determined condition. It has recently been shown that 2 common single nucleotide polymorphisms (SNPs) of the lysyl oxidase-like protein 1 (LOXL1) gene are responsible for almost 100% of the population-related risk of XFS [26,27]. In most white populations the risk alleles are G153D and R141L [26,27]. The LOXL1 protein is responsible for elastin production and turnover in the whole body, thus its disturbed translation and dysfunction explain several characteristics of intraocular and systemic alterations in XFS [16,28]. It is important to note, however, that there are different risk alleles in various populations and these are also common in healthy individuals in whom XFS never develops [26]. Thus, currently unknown environmental factors are also believed to take part in the development of XFS. Very recently a novel association between XFS and calcium signaling has been established: one SNP of the calcium voltage-gated channel subunit alpha1A gene (CACNA 1A) confers 1.16 times increased risk for XFS [29]. The exact mechanism of LOXL1 and CACNA1A polymorphisms causing XFS remains to be determined.

It is important to recognize that conversion from XFS to XFG is not genetically determined, thus any genetic screening for XFG is impossible. The conversion from XFS to XFG is considered a result of environmental factors [26,30-32]. The common mechanism of all environmental factors seems to be increased oxidative stress in the anterior chamber [28,30,33]. According to the current hypothesis, increased sunshine exposure provokes production of oxidative stress markers (e.g., 8-ioprostaglandin F2α, endothelin 1) and depletion of antioxidant protection molecules (e.g., ascorbic acid) in the anterior chamber [33]. The oxidative stress, which is greater in XFG than in XFS, is supposed to cause nuclear cataract, dysfunction of the trabecular meshwork and zonular damage. The trabecular meshwork dysfunction is considered responsible for the IOP elevation and the large IOP fluctuation typical in XFG, and the zonular damage for phacodonesis, lens dislocation, or secondary angle closure glaucoma (when the luxated lens causes pupillary block or directly blocks the anterior chamber angle) [4]. Recently, based on population-based retrospective studies, it has been suggested that the amount of time spent outdoor in bright sunshine without sunglasses in young age (a

proxy for ultraviolet light-induced oxidative stress in the anterior segment of the eye), high coffee consumption and decreased folate intake may be risk factors for the development of XFS and XFG in later life [30-32,34,35]. These hypotheses, however, require confirmation and further research. Figure 2 summarizes the proposed key mechanisms of the development of XFS and XFG.

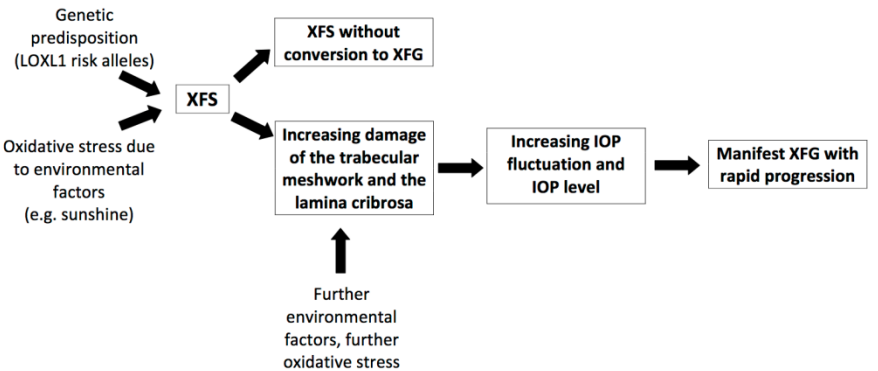

Figure 2. Key mechanisms involved in the pathophysiology of XFS and XFG.

GLOBAL HEALTH SIGNIFICANCE

It has been estimated that roughly 60 to 70 million people in the world have XFS. Approximately 25% of them have high IOP, and one third of them (i.e., 5-6 million people) have XFG in at least one eye [3,7,8]. The global impact of XFG becomes even more obvious considering the fact that approximately half of the patients with glaucoma remain undiagnosed [7,8,36]. This fact can have a profound effect on individual patients and healthcare resources alike, because XFG is often difficult to control and has a poor long-term prognosis. Irrespective of the risk for glaucoma, the global impact of exfoliation should also be seen in the light of increased risk for cataract formation and increased risk for complications during or after cataract surgery [14,37].

Numerous reports in the last several years have contributed to the rejection of the notion that XFS and XFG are entities peculiar to Scandinavia. In fact, these conditions have been identified in diverse populations worldwide and XFG may account for 20-25% of open-angle glaucoma cases globally [3,7,8].

In particular, over half of open-angle glaucoma cases may be due to XFG in countries such as Norway, Iceland, Greece, Turkey and Saudi Arabia [7,8].

The prevalence of XFS in the United States is generally comparable to that in Western Europe but significant racial variations exist, since the condition is much more prevalent in whites than persons of African origin, and Spanish-American men are almost six times more likely to have XFS compared to non-Spanish-Americans [36,39].

It is puzzling that local variations in prevalence of exfoliation can be remarkable. In Nepal, for example, XFS was detected in 12% of the members of the Gurung ethnic group, but only in 0.24% of age-matched non-Gurungs [39]. Similarly, in the Middle Norway survey XFS prevalence values of 10.2%, 19.9% and 21% were observed in three population cohorts from different municipalities who were examined simultaneously using the same criteria, even though the municipalities were lying only about 100 kilometers apart [40]. Obviously, the interplay of genetic and environmental factors (epigenetics) is a major determinant of exfoliation prevalence. Well-designed population-based studies rather that frequency reports or studies with convenience samples are needed in order to explore possible etiological associations.

CLINICAL CHARACTERISTICS OF XFG

Patients with XFG are typically older than patients with primary open-angle glaucoma (POAG) [13,19]. In fact, XFG is very uncommon before the age of 50. Because XFG has a fast rate of progression, serious visual deterioration or even terminal damage is often seen in some patients at diagnosis, occasionally with remarkable inter-eye asymmetry [13,23]. Since IOP is typically very high in eyes with XFG, subjective symptoms reported by patients may include dull ocular or retrobulbar ache, clouding of vision due to corneal edema, or sudden severe visual deterioration due to branch- or central retinal vain occlusion [41,42]. However, most XFG patients do not experience symptoms. IOP characteristics in XFG deserve particular mention: these eyes usually have higher mean, higher peak and larger 24-hour and intervisit IOP fluctuation compared to eyes with POAG [43-45]. Furthermore, IOP peaks in eyes with XFG often occur outside office hours and can therefore remain unnoticed in typical clinical settings [45,46]. As a consequence, single daytime IOP measurements every few months may be inadequate as a means of evaluating a patient’s overall IOP characteristics or the response to therapy. In

addition to the worse IOP characteristics in eyes with XFG, the purported elastosis-induced biomechanical alterations of the lamina cribrosa that may render the optic nerve more vulnerable to effects of IOP elevation are thought to underlie the poorer long-term prognosis compared to POAG [15,16,23,25]. It should be noted that gradual or sudden IOP elevation in treated and seemingly well-controlled eyes with XFG may occur. Thus, close follow-up over the long term is necessary even in apparently well-controlled XFG patients [3,19]. A notable difference between XFG and POAG is that the former is not associated with a higher risk of IOP elevation following steroid treatment [47].

Medical Treatment

Because XFG is a high-pressure glaucoma with unpredictable characteristics (e.g., high spikes outside office hours), achieving a sufficiently low IOP throughout the 24-hour cycle is frequently difficult [23,44,45]. Medical therapy should ideally be tailored to the particular needs of eye in XFG [48,49]. Prostaglandin analogues are common first choices in open-angle glaucoma due to their efficacy, convenient once-daily administration and favorable safety profile [50-52]. A 3-month, multicenter, single masked, prospective, parallel group study with XFG patients compared the diurnal efficacy of evening-dosed latanoprost 0.005% versus timolol 0.5% administered twice daily [53]. The authors found that latanoprost provided marginally superior diurnal IOP reduction and smaller IOP fluctuation compared to timolol. Although the efficacy difference between latanoprost (reduction from untreated baseline 24.9 mmHg to 17.4 mmHg) and timolol (reduction from untreated baseline 24.7 mmHg to 18.3 mmHg) for the diurnal IOP did not reach statistical significance (P=0.07), the IOP reduction at the 8 a.m. time-point was significantly greater with latanoprost (-8.5 mmHg vs. -6.0 mmHg).

The investigators of a 6-month, single masked, prospective, parallel group study with IOP measurements at 8 a.m., 10 a.m. and 4 p.m. compared the IOP lowering efficacy of evening-dosed latanoprost 0.005% and travoprost 0.004% and the dorzolamide 2%/timolol 0.5% fixed combination dosed twice daily, in patients with XFG [54]. The mean untreated daytime IOP in all groups varied between 23.8 mmHg and 25.7 mmHg and the mean IOP reduction was 8.2 mmHg for latanoprost, 9.3 mmHg for travoprost and 11.5 mmHg for the dorzolamide/timolol fixed combination. This result, however, may not be

representative for the true 24-hour IOP lowering efficacy of these medications in XFG since 2 of the 3 time points coincided with the post-instillation peak efficacy period of the dorzolamide/timolol fixed combination, thus overestimation of IOP lowering effect of the fixed combination was possible.

In a 24-hour, investigator-masked, crossover trial made on XFG patients, latanoprost 0.005% and travoprost 0.004% lowered the mean untreated 24-hour IOP (25.1 mmHg) by 7.3 mmHg (30.8%) and 7.8 mmHg (31.1%), respectively [55]. The efficacy difference between the two treatments was statistically significant mostly due to the IOP difference at the 6 p.m. time point (17.9 mmHg on latanoprost *vs*16.7 mmHg on travoprost, $p<0.01$).

In a 3-month, prospective, multicenter, investigator-masked, crossover trial comprising 129 XFG patients, the efficacy of latanoprost 0.005% and bimatoprost 0.03% was compared [56]. The mean untreated diurnal IOP was 26.9 mmHg. Bimatoprost provided statistically and clinically significantly lower mean diurnal and individual time point IOP than latanoprost. The mean diurnal IOP values were 18.6 mmHg for latanoprost and 17.6 mmHg for bimatoprost. Only 34 patients (26.4%) on latanoprost versus 55 patients (42.6%) on bimatoprost achieved mean diurnal IOP<17 mmHg. This difference is clinically relevant because a mean diurnal IOP<17 mmHg has been shown to lower the risk of progression in XFG [25].

A 2-month, crossover, prospective, investigator-masked study conducted on treatment-naive XFG patients assessed the efficacy of latanoprost 0.005% dosed in the evening and dorzolamide/timolol fixed combination dosed twice daily [57]. The mean untreated IOP (measured at 10 a.m.) was 31.2 mmHg, which decreased by 40.2% to 18.9 mmHg with latanoprost and by 42.8% to 18.1 mmHg with the fixed combination (P=0.21). The results indicate that the 2-hour post-instillation peak efficacy of the dorzolamide/timolol fixed combination and the 14-hour post-instillation efficacy of latanoprost monotherapy are comparable in XFG.

The efficacy of fixed combinations of a prostaglandin analogue (latanoprost 0.005%, travoprost 0.004%, bimatoprost 0.03% or tafluprost 0.0015%) and timolol 0.5% has also been investigated in XFG [58-61]. In general, the use of fixed combinations offers a number of advantages: convenient once daily dosing, decreased ocular tissue exposure to preservatives, reduced systemic absorption of timolol compared to twice daily administration, and increased efficacy compared to that of the individual constituents [52,62,63]. The investigators of a 3-month, 24-hour crossover study found that in XFG patients the evening dosed travoprost/timolol fixed combination reduced the mean 24-hour IOP from 28.5 mmHg (untreated

baseline) by 34.4% (9.8 mmHg), while the evening-dosed latanoprost/timolol fixed combination by 31.2% (8.9 mmHg) [58]. This difference was statistically significant (P< 0.001) [58]. In another crossover study, the mean 24-hour baseline IOP in a mixed population of POAG and XFG patients was 27.7 mmHg [59]. After 2 months of therapy, the mean 24-hour IOP was 19.2±3.5 mmHg when the travoprost/timolol fixed combination was dosed in the morning, and 18.4±3.3 mmHg when dosed in the evening (P=0.001) [59]. In a 3-month, multicenter, prospective, single-masked, 24-hour crossover study conducted on XFG patients [60] the bimatoprost/timolol fixed combination was administered in the morning in one study phase, and in the evening in the other phase. The mean 24-hour IOP reduction from the mean untreated 24-hour value (29.03 mmHg) was 33.9% (9.84 mmHg) with morning dosing and 35.3% (10.2 mmHg) with evening dosing. This difference was statistically significant (P=0.005). These results suggest that in XFG topical prostaglandin analog/timolol therapy is more effective when the drop is administered in the evening. Recently, the IOP lowering efficacy of morning-dosed preservative-free tafluprost/timolol fixed combination was evaluated in XFG patients who participated in a 6-month, randomized, double-masked, active controlled, parallel group, multicenter phase III study [61]. The mean untreated baseline IOP varied between 26.5 mmHg (4 p.m.) and 28.1 mmHg (8 a.m.). The mean time-wise IOP reduction ranged between 8.62 and 10.25 mmHg (31.8 to 36.7%), and the overall IOP reduction was 34.1%.The results discussed in this paragraph suggest that an IOP reduction of 31 to 36% from untreated baseline can be expected with once daily administration of all prostaglandin analogue/timolol fixed combinations. Currently there are no controlled data on the efficacy of combination therapy with 3 or more IOP lowering medications in XFG (e.g., a prostaglandin analogue and a topical carbonic anhydrase inhibitor/timolol fixed combination or a prostaglandin analogue/timolol fixed combination and a topical carbonic anhydrase inhibitor).

Laser Treatment

Argon laser trabeculoplasty (ALT) exerts its IOP lowering effect by enhancing aqueous humor egress through the trabecular meshwork. It was initially hypothesized that the mode of action is mechanical distention of the collapsed trabecular meshwork [64,65]. However, laser-inducedenhancement of extracellular matrix turnover due to stimulation of trabecular cells has also

been proposed [65]. Since energy absorption by melanin in the trabecular meshwork is necessary for ALT to be effective, patients with XFG are usually good candidates for this treatment because their eyes typically have debris laden, heavily pigmented anterior chamber angles.

ALT commonly provides significant IOP reduction in XFG eyes with high untreated IOP, at least in the first post-laser months or years [66-70]. Unfortunately, the hypotensive effect diminishes over time, conceivably due to the continuous accumulation of pigment granules and exfoliation material on the trabecular meshwork. A retrospective study evaluated the efficacy of ALT on 28 POAG and 26 XFG patients poorly controlled on medical treatment [70]. Failure was defined as IOP >22 mmHg after 4 to 6 months following ALT, or need for filtering surgery. The authors found that despite an initially better response to ALT, patients with XFG eventually failed faster than patients with POAG. For instance, the cumulative probability of success after approximately 8 months was 59% and 77% for the XFG and POAG groups, respectively.

Another retrospective study compared the efficacy of ALT on 29 XFG eyes (mean follow-up time 23 months) and 66 POAG eyes (mean follow-up time 27 months) [67]. XFG patients had a greater percentage of IOP reduction in the first post-laser year compared to POAG patients. The IOP reduction in the XFG group varied between 28% and 38% (mean pre-laser IOP 25.8 mmHg), whereas in the POAG group it varied between 19% and 27% (mean pre-laser IOP 23.2 mmHg). The 1-year rate of failure (need for glaucoma filtering surgery; or an additional laser treatment; or IOP ≥22 mmHg; or IOP elevations up to 15% of the baseline IOP value in at least 2 visits) was 40% for POAG and 18% for XFG, while the 3-year failure rate for the groups was 58% and 47%, respectively (P=0.89). The effect of ALT diminished over time and by three years both groups had comparable measures of success.

ALT was eventually proposed as primary treatment in patients with POAG [71]. A retrospective study also investigated the efficacy of ALT as primary therapy on 75 XFG and 93 POAG patients [66]. Using customized clinical criteria based on each patient's need for additional IOP lowering therapy, the probability of success (defined as no need for additional treatment) in the XFG group was 80% after 2 years, 54% after 5 years, and 36% after 8 years. The existing evidence suggests that in eyes with XFG and pre-laser IOP between 23 and 30 mmHg, clinicians can expect an approximately 20-40% pressure reduction from ALT during the first 4 post-laser years [66-68,70].

Selective laser trabeculoplasty (SLT) employs a frequency-doubled Q-switched Nd-YAG laser to deliver low energy in brief pulses with a large spot diameter (400μm) to the surface of the trabecular meshwork [72]. The precise mechanism by which SLT enhances aqueous humor outflow remains unknown. SLT is considered to selectively target the pigmented trabecular meshwork cells without causing obvious coagulative or disruptive changes. Therefore the technique can be repeated in eyes previously treated with ALT or SLT [72,73].

In eyes with POAG, the efficacy of SLT has been found similar to that of ALT [73]. However, there is limited information on the comparative efficacy of SLT and ALT in XFG. A 6-month randomized trial evaluated the efficacy of SLT versus ALT on 76 inadequately controlled XFG eyes and XFS eyes with ocular hypertension [74]. SLT and ALT resulted in similar IOP reductions (6.8 mmHg and 7.7 mmHg, respectively, P=0.56) from the corresponding pre-laser baseline values (SLT: 23.1 mmHg, ALT: 25.2 mmHg, P=0.03). Moreover, an identical proportion of patients (73%) achieved at least 20% IOP reduction with either treatment at the 6-month post-laser visit. Other reports suggest that XFG and POAG eyes respond to SLT similarly both when the procedures are used as primary [75] or adjunctive [76,77] interventions.

It is important to note that ALT and SLT are seldom adequate treatments for patients with high-pressure XFG, especially when considering that a low target IOP (<17 mmHg) is needed to prevent progression on the long term [25]. Nonetheless, these techniques can be considered in elderly XFG patients with suboptimal compliance, in XFG patients intolerant to medical therapy and in XFG patients who are poor candidates for filtration surgery.

Surgical Treatment

Glaucoma surgery is mainly reserved for eyes insufficiently controlled with medical and/or laser treatment; cases in which progression develops on maximal tolerated medical and laser treatment; and in patients intolerant or non-compliant to medical therapy. For several years trabeculectomy (typically with adjuvant 5-fluorouracil or mitomycin C) has been considered the gold standard surgical procedure in XFG [78,79]. Evidence suggests that the long-term survival of trabeculectomy in XFG may be longer than that in POAG [78,80,81], and glaucomatous progression after filtering surgery may be slower in XFG patients compared to POAG patients [19,81].

Although non-penetrating techniques rarely achieve sufficiently low IOP to defer progression in patients with an aggressive glaucoma like XFG, they may still be an attractive alternative in few selected patients due to their favorable safety profile [82,83]. In a randomized trial that comprised both POAG and XFG patients intraoperative use of mitomycin C increased the effectiveness of deep sclerectomy [84]. Thirty-six months postoperatively the mean IOP in eyes operated with and without adjunctive mitomycin C was 15.96 ± 1.71 mmHg and 18.71 ± 2.90 mmHg, respectively (P=0.001). In a retrospective study that contained data on XFG eyes, the efficacy of the newly developed technique of CO_2 laser-assisted deep sclerectomy was found comparable to that of conventional deep sclerectomy [85]. The complete success rates (IOP <18 mmHg without medication) were 73% in the CO_2 laser-assisted group and 71% in the control group, respectively, whereas the total success rate (IOP <18 mmHg with/without medication) was 96% in the CO_2 laser-assisted group and 89% in the control group. A prospective study conducted on 24 XFG and 25 POAG patients investigated the efficacy of deep sclerectomy combined with various space-maintaining implants [86]. With a mean follow-up period of 19.9 months for the XFG group and 16.2 months for the POAG group, the proportion of eyes with complete success (IOP <19 mmHg without medication) was 60.7% in the XFG group and 37.9% in the POAG group.

The available evidence on the efficacy of viscocanalostomy in eyes with XFG is limited. In a prospective, non-randomized case series comprising 57 glaucomatous eyes (14 of them had XFG) an IOP <21 mmHg without medication was observed in 60% of eyes after 60 months following viscocanalostomy [87]. Recently the efficacy of concurrent phacoemulsification and viscocanalostomy in 30 eyes of 22 patients with cataract and poorly controlled XFG has been investigated [88]. The mean preoperative IOP was 25.3 mmHg. After a mean follow-up period of 18.6 months, success (IOP <21 mmHg with/without medication) was achieved in all eyes and complete success (IOP <21 mmHg without medication) was achieved in 90% of the eyes. In a subsequent prospective trial the efficacy of phacoemulsification combined with viscocanalostomy was evaluated in cataract patients with medically uncontrolled XFG or POAG [89]. The mean preoperative IOP was 24.5 mmHg for the XFG group (n=30) and 21.7 mmHg for the POAG group (n=30). The mean follow-up period was 19.7 months for both groups. At the last postoperative visit the XFG patients had significantly lower IOP than the POAG patients (49.7% vs. 30.9% IOP reduction, respectively).

The recently developed technique of ab-interno trabeculotomy with the Trabectome™ (Neomedix Inc., Tustin, CA, US) has been approved for the treatment of open angle glaucoma. A 12 months, prospective, non-randomized study evaluated the outcomes of Trabectome™ surgery alone and in combination with cataract surgery and intraocular lens implantation in XFG and POAG eyes [90]. For the "Trabectome™ surgery only" analysis the preoperative IOP was 29.0 mmHg and 25.5 mmHg for the XFG (n=67) and the POAG group (n=450; $P<0.01$), and the IOP decreased by 12.3 ± 8.0 mmHg and 7.5 ± 7.4 mmHg in the XFG and POAG group, respectively ($P<0.01$). The cumulative probability of success for the XFG and POAG groups was 79.1% and 62.9%, respectively ($P=0.004$). In the "combined Trabectome™ plus cataract surgery" analysis the mean preoperative IOP was 21.7 mmHg and 19.9 mmHg for the XFG (n=45) and the POAG (n=263) group ($P=0.06$), and the IOP decreased by 7.2 mmHg and 4.1 mmHg in the XFG and POAG groups, respectively ($P<0.01$). The cumulative probability of success for the XFG and POAG group was 86.7% and 91.0%, respectively ($P=0.73$). The results suggest that ab-interno trabeculotomy offers superior IOP control in XFG compared to POAG.

Trabecular aspiration is a technique specifically developed for the treatment of XFG based on the notion that surgical removal of the exfoliation material and pigment granules deposited on the intra-trabecular spaces should enhance aqueous humor outflow. [91] In a prospective trial with 22 eyes of 19 patients with uncontrolled XFG the mean preoperative IOP on medication (31.3 mmHg) was significantly lowered to 16.8 mmHg 18 months after trabecular aspiration [91]. Following the procedure, 45% of the patients required no IOP lowering medication. In the same study, the authors evaluated the efficacy of trabecular aspiration combined with phacoemulsification or extracapsular cataract surgery in 42 eyes of 36 patients with uncontrolled XFG and cataract. For these eyes the mean preoperative IOP on medication (32.4 mmHg) significantly decreased to 18.7 mmHg 2 years after the combined procedure [91]. In this subgroup, 54% of the participants did not need medication at the end of the 2-year follow-up period. In another prospective, controlled study the same authors compared the efficacy of phacoemulsification, combined phacoemulsification and trabecular aspiration, and combined phacoemulsification and trabeculectomy on 74 non-operated eyes with XFG and cataract [92]. Failure was defined as the necessity for additional surgical or laser interventions (except for laser suture lysis), or the need for more than one medication to reach an individualized target pressure. After a 2-year follow-up period the proportions of successfully treated patients

in the phacoemulsification alone group, the combined phacoemulsification and trabecular aspiration group, and the combined phacoemulsification and trabeculectomy group were 36%, 64% and 78%, respectively. The proportions of medication-free well-controlled patients 2 years after the above procedures were 9%, 38% and 65%, respectively.

Cataract surgery is frequently necessary in XFG due to nuclear cataract induced by increased oxidative stress in the anterior chamber [28,30,33].In addition, cataract surgery offers a clinically significant IOP decrease in XFG and XFS eyes with elevated IOP [93-96]. It has been suggested that the IOP-lowering effect of cataract extraction in eyes with XFG and XFS is due to a combination of the following mechanisms: widening of the anterior chamber angle, removal of a large portion of the exfoliation-producing anterior capsule, decrease of irido-lenticular friction (which limits the release of pigment and exfoliation material postoperatively), aspiration of deposited debris from the trabecular meshwork during surgery, and enhancement of the trabecular outflow as a consequence of low-grade inflammation (the hypothesized mechanism being similar to that seen after argon laser trabeculoplasty [14,93]). In a 2-year, multicenter, prospective cohort study the IOP-lowering effect of phacoemulsification was assessed in 71 cataract patients with exfoliation (29 patients had XFG) and 112 without exfoliation [93]. Eyes with XFS and XFG had a significantly larger IOP reduction than POAG patients and non-glaucomatous controls. Remarkably, the investigators found that the ocular hypotensive effect of cataract surgery in the exfoliation group was associated with the volume of the irrigating fluid. This suggests that the IOP-lowering effect of cataract surgery can be attributed to the intraoperative removal of debris. The results of this prospective study fit well to earlier data from retrospective case series [94,95]. In another retrospective study conducted on 1122 XFS and XFG eyes followed for 7 years, the postoperative IOP reduction was proportional to the preoperative IOP [96]. A recent retrospective study found that the proportion of XFS patients who converted to XFG after phacoemulsification within the more than 6-year long follow-up period was lower than expected, and the mean IOP of eyes with exfoliation at the end of the follow-up period was 2.6 mmHg lower that the mean preoperative value [97].

However, cataract surgery in XFS and XFG is associated with several disease-specific surgical challenges [14,19,98]. These include Zinn's zonule laxity and/or fragility, phacodonesis, lens subluxation or luxation, inadequate mydriasis, hard nucleus, fragile capsule and increased risk of vitreous loss. In the postoperative period common complications include corneal edema due to

exfoliation-related endotheliopathy, transient IOP elevation, postoperative fibrinous inflammatory reaction (mostly after extracapsular cataract extraction), anterior capsule phimosis and delayed in-the-bag intraocular lens subluxation or luxation.

VASCULAR ALTERATIONS IN XFS

An association between XFS and systemic vascular dysfunction or even vascular disease (capillary, medium and large artery dysregulation, increased plasma homocysteine level, increased arterial stiffness and reduced distensibility, abnormal baroreflex sensitivity and heart rate regulation, increased risk for venous occlusions and aortic aneurisms) has been shown in several, but not all, populations [51,99,100,101]. The relationship between systemic vascular disease and XFS is independent from the presence of XFG. In XFS, retinal vein occlusion is a part of the systemic vascular abnormalities. Since management of the vascular diseases associated with XFS exceeds the scope of the ophthalmologist, consultation with the patient's cardiologist, internist or general practitioner, who cannot detect XFS, is necessary. The ophthalmologist needs to indicate that the patient has XFS, and that XFS may represent a special risk for systemic venous occlusions and vascular diseases, thus special attention and care may be necessary after the first signs of systemic vascular disease are detected.

UNMET NEEDS IN THE TREATMENT OF EXFOLIATIVE GLAUCOMA

Similarly to primary open-angle glaucoma, the current treatment of XFG entails IOP reduction. Though therapeutic measures to decrease IOP are essential for the successful long-term management of XFG, disease progression remains common even when both the treated long-term mean IOP is low and the between-visit IOP fluctuation is small [25]. As discussed earlier in this chapter, increased oxidative stress plays a key role in the IOP elevation in XFG [33]. Therefore decrease of oxidative stress in the anterior chamber of the eye would be a potential disease-specific treatment in XFG. Unfortunately, currently no such treatment or prevention option exists except for use of sunglasses during outdoor activity.

CONCLUSION

Since XFS is a common condition, and XFG is an especially severe, rapidly progressing and common secondary open-angle glaucoma, better understanding of the pathophysiological processes in XFS and XFG and establishing a disease-specific targeted therapy of XFG would be of great clinical importance. Currently XFS is not considered a disease to treat, but rather a genetically determined condition to observe for conversion to XFG. In contrast, XFG requires effective IOP lowering treatment which needs to be introduced early in the course of the disease to prevent progression. In XFG, IOP reduction is satisfactory when the diurnal IOP curve shows no high peaks but low mean value (IOP <17 mmHg) and low fluctuation, the visual field does not progress or the rate of progression is small, and the patient is adherent to the successful treatment. Even in such cases regular ophthalmological examinations are unavoidable, and ophthalmologists need to know that due to the continuous synthesis of XFM and the gradual buildup of pigment in the trabecular outflow system there is always a risk of sudden loss of IOP control, and a need to introduce a more complex IOP lowering medication, laser therapy or surgery. Since XFG is a disease of the elderly it is important to simplify therapy using fixed dose combination eye drops and minimal number of daily instillations, thus supporting adherence. Consulting with a glaucoma subspecialist is also important when optimal IOP control is not achievable in XFG because unnecessary delay prior to surgery results in unavoidable disease progression.

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