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MAST CELL ACTIVATION DISORDERS
A LIAISON BETWEEN ANAPHYLAXIS AND MASTOCYTOSIS

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A Liaison between Anaphylaxis and Mastocytosis

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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The only constant is change

Heraclitus of Ephesus (535BC – 475 BC)

To Liza and Anton

ABSTRACT

The term mast cell activation disorders (MCAD) comprises a broad spectrum of heterogeneous conditions, such as mastocytosis, characterized by inappropriate mast cell activation/accumulation. The patients present with protean clinical manifestations and severity grade of symptoms may vary from case-to-case. The periodic or chronic symptoms that attributable to the local and systemic effects of mast cell mediators are common findings, where anaphylaxis appears to be one of the predominating clinical manifestations that generate a sensation of fear in most affected patients. The overall aim of this thesis was to provide data on the demographic, epidemiologic and clinical characteristic of patients with systemic mastocytosis, and to investigate the prevalence and features of mast cell mediator-induced symptoms, in particular, anaphylaxis. In addition, the complex interaction between anaphylaxis and mast cell activation disorders was explored by identifying risk factors.

In Paper I, the case presented highlighted the many faces of mastocytosis and proved also that there was a lack of recognition of mastocytosis symptoms among physician. In this particular case, a correct diagnosis required almost 20 years despite that the patient had consulted several doctors and underwent extensive medical investigations. The turning point for making right diagnosis was to confirm that this patient had an elevated level of baseline serum tryptase.

In Paper II, three puzzling cases of hymenoptera venom-induced anaphylaxis (HVA) with elevated levels of baseline tryptase were discussed. Although all three patients presented with demographically and clinically similar data and received diagnosis of HVA using traditional allergy work-up, investigation of bone marrow mast cells led to changes in final diagnosis. This paper lends further support to the hypothesis of a clear-cut association between severe HVA and clonal mast cell disorders.

In Paper III, we provided a comprehensive insight into patients with systemic mastocytosis (SM) with respect to allergological aspects of this disease. We reported the presence of mast cell mediator induced symptoms in 90% of SM patients, of these symptoms 63% were related to gastrointestinal symptoms. In addition, the prevalence of anaphylaxis in this cohort was found to be clearly increased (43%). Hymenoptera sting was the main elicitors (53%) followed by idiopathic anaphylaxis (39%). Anaphylaxis occurred more frequently in SM patients with atopic predisposition and patients without cutaneous engagement. Also, baseline tryptase levels were significantly lower in SM patients with anaphylaxis.

In Paper IV, we presented comprehensive data on the characteristics of patients with unexplained anaphylaxis (UEA), by investigating these patients' bone marrow mast cells. We found that 47% of patients had clonal markers of aberrant mast cells. Baseline serum tryptase levels were significantly higher (≥11.4 ng/ml) and conversely, total IgE levels were lower in patients with clonal mast cell disorders compared to patients with true idiopathic anaphylaxis.

In Paper V, we sought to examine whether mast cells of patients with mast cell disorders express hyperreactivity in the skin and lower airways compared to control subjects. We also analyzed different mast cell mediators (serum tryptase and urinary histamine and prostaglandin D_2 metabolites). Although we found elevated baseline levels of these mediators in patients with SM/MCAD, we found no evidence to support the hypothesis that a hyper-reactive mast cell phenotype would exist in the skin or bronchial airways of these patients.

In conclusion, the work presented in this thesis provides a better understanding of different phenotypes of patients with mast cell disorders. We observed a high prevalence of anaphylaxis in these patients. Our findings, thus, support that all patients with mast cell activation disorders should undergo comprehensive allergy work-up providing personal risk assessment before considering treatment and preventive measures. Our data also indicates that clonal mast cell disorders are present in a substantial subset of patients with UEA.

LIST OF SCIENTIFIC PAPERS

I. **Gülen T**, Hägglund H, Dahlen SE, Sander B, Dahlen B, Nilsson G. Flushing, fatigue, and recurrent anaphylaxis: a delayed diagnosis of mastocytosis. Lancet. 2014 May 3; 383(9928):1608.

II. **Gülen T**, Dahlen B, Sander B, Hägglund H, Nilsson G. The significance of diagnosing associated clonal mast cell diseases in patients with venom-induced anaphylaxis and the role of bone marrow investigation. Clinical and translational allergy. 2013;3(1):22.

III. **Gülen T**, Hägglund H, Dahlen B, Nilsson G. High prevalence of anaphylaxis in patients with systemic mastocytosis - a single-centre experience. Clin Exp Allergy. 2014 Jan;44(1):121-9.

IV. **Gülen T**, Hägglund H, Sander B, Dahlen B, Nilsson G. The presence of mast cell clonality in patients with unexplained anaphylaxis. Clin Exp Allergy. 2014 Sep;44(9):1179-87.

V. **Gülen T,** Möller-Westerberg C, Lyberg K, Kolmert J, Bood J, Öhd J, Dahlén S-E, Nilsson G, Dahlén B. *In-vivo* mast cell reactivity in patients with mastocytosis and related mast cell activation disorders.
(Manuscript)

Thesis Errata

Mast Cell Activation Disorders
A Liaison between Anaphylaxis and Mastocytosis

by Theo Gülen

Page 23, figure 8, last line	”cromolyn soodium” *should be* ”cromolyn sodium”
Page 28, first paragraph, line 9	“To date, over 150 of the...” *should be* ”To date, 145 of the...”
Page 39, first paragraph, line 5	“…patients with SM (28%)” *should be* “…patients with SM (22%)”.
Page 44, line 8	Reference lacking *should be* “ ...of intervention (320).”
Page 44, last paragraph, line 4	Following reference *should be included*: Ebrahim GJ, Sullivan KR. Mother and Child Health: Research Methods. London: Book Aid; 1995.
Page 45, paragraph 1, line 8	“…that have a sBT levels between...” *should be* “…that have sBT levels between...”
Page 49, line 7	“…significantly more prevalence...” *should be* “…significantly more prevalent...”
Page 52, line 9	“…before they to go...” *should be* “…before they go...”

CONTENTS

LIST OF ABBREVIATIONS

BM	Bone marrow
BAT	Basophil activation test
CM	Cutaneous mastocytosis
CMD	Clonal mast cell disorders
FENO	Fraction of exhaled nitric oxide
FEV_1	Forced expiratory flow in one second
GI	Gastrointestinal
HVA	Hymenoptera venom anaphylaxis
IA	Idiopathic anaphylaxis
ICT	Intracutaneous test
IgE	Immunoglobulin E
MC	Mast cell
MCAD	Mast cell activation disorders
MCAS	Mast cell activation syndromes
MCK	Mastocytosis Center Karolinska
MIS	Mastocytosis in the skin
MMAS	Monoclonal mast cell activation syndrome
nc-MCAS	Non-clonal mast cell activation syndrome
PGD_2	Prostaglandin D_2
sBT	Serum baseline tryptase
SM	Systemic mastocytosis
SPT	Skin prick test
UEA	Unexplained anaphylaxis
UP	Urticaria pigmentosa
WHO	World Health Organization

1 PROLOGUE

Fortunately, most people go through life without experiencing anaphylaxis, which is an exaggerated hypersensitivity response to a substance to which an individual has become sensitised. Ever since I started my training in the field of clinical allergy, I have been wondering why some people would react with anaphylaxis to some substances, whereas others do not. It usually happens to people who are known to be allergic; however, no one is really sure about the right answer as many allergic people react only with milder reactions. The more we study anaphylaxis the more we realize that the enigma of anaphylaxis persists.

In the autumn of 2005, I occasionally became familiar with this mysterious disease: mastocytosis. Sign and symptoms of mastocytosis resemble allergic reactions, but it is not an allergic disease indeed, as the triggers are often ambiguous. However, as a clinician, I have to admit that it is a fascinating disease and at the same time very convenient model to study mast cell activation processes and its consequences in the clinical settings. Observing clinical manifestations of these patients is like an amazing natural experiment; as the patients would experience recurrent anaphylaxis with or without known elicitors.

Because of the multi organ presentations of the symptoms, I also realized that mastocytosis has been a very challenging disorder for the clinicians as every specialist is used to look at a patient with mastocytosis from a different perspective, and seeing only the problems from their own scope. Thus, these patients typically are frustrated to run doctor-to-doctor without reaching a definite diagnosis. Probably, many patients never get a correct diagnosis and their quality of lives deeply perturbed in their search to find an explanation for their symptoms. Therefore, most of the mastocytosis patients believe in the importance of the early and accurate diagnosis, although mastocytosis is currently incurable.

So was the story behind this interesting project, which gradually became the basic concept for this doctoral thesis.

2 INTRODUCTION

In general, there is a broad spectrum of disorders that clinically manifest as a result of inappropriate mast cell activation. The responsible mediators often have overlapping functions, thereby providing unsuccessful intervention possibilities with mediator blocking agents to prevent clinical symptoms.

In following, the different aspects of mast cells and related diseases will be outlined. This discussion will focus primarily on the allergological aspects of mastocytosis and related mast cell activation disorders, with particular attention to mast cell mediator-related symptoms and anaphylaxis, which is the main theme of this thesis. Thus, it is beyond the scope of this dissertation to present an in depth description, for example, of hematological or bone metabolism issues related to these disorders.

2.1 MAST CELLS

Mast cells, also being referred to as mastocytes, are granulated, tissue-dwelling cells that are normally found in almost all tissues. Mast cells were first described by Paul Ehrlich in his doctoral thesis published in 1878 (1), and he coined the name of “Mastzellen”, meaning well-fed-cells (2). The original name was reflecting the fact that a mast cell contains large amount of cytoplasmic granules.

Mast cells are potentially long-lived and derived from pluripotent stem cells in the bone marrow and circulate in the blood as precursors (3) before they mature in tissues under the influence of stem cell factor (SCF) and other local cytokines such as interleukin (IL)-3, IL-4, IL-9, and IL-10 (4, 5). Circulating MC precursors express CD34, the tyrosine kinase receptor KIT (CD117), IgG-receptors (FcγRII), but not high-affinity IgE-receptors (6). Committed progenitors traverse the vascular space and complete their maturation into the diverse peripheral tissues (7). KIT (CD117) is expressed on haematopoietic stem cells and progenitor cells, and is down-regulated during their differentiation (8). However, KIT remains highly expressed on mast cells and is crucial for growth, differentiation, survival, and enhancement of signalling events during mast cell activation (8, 9).

Mast cells preferentially reside in tissues that have contact with external environment, such as skin, gastrointestinal and respiratory tracts, and localize close to blood vessels, nerves, and mucosal surfaces. Avascular tissues, including mineral bone, cartilage and cornea are absent from mast cells (10). Number of mast cells can be increased in various disorders, nevertheless, they are best known for their participation into the genesis of allergic inflammation as central effector cells (10). In addition, their function as gate keepers in innate and acquired immunity is also recognized, as mast cells can promote the host defense in some bacterial or parasite infections (11). Mature mast cells can be long-lived, and as an important feature, can reenter the cell cycle and proliferate and survive (12).

Mast cells are typically activated by cross-linking of their high-affinity receptors FcεRI by IgE/allergen complexes (13); but also other molecules may stimulate secretion including anaphylatoxins C3a and C5a (14, 15), immunoglobulin-free light chains (16), neuropeptides such as substance P (17), the human antibacterial peptides β-defensins (18) and by compounds acting on Toll-like receptors such as lipopolysaccharide (19).

Mast cells produce a large array of mediators and cell signaling molecules. Upon stimulation, MCs may secrete their mediators by following two different pathways. This may be either by a process called degranulation, which is also known as exocytosis, in which large amounts of pre-stored cytoplasmic granules will bind to each other and to the mast cell membrane (20, 21). This will, in turn, open up channels in the mast cell, allowing the quick release of the granule contents into the extracellular environment, thereby causing the symptoms of the acute allergic reactions and anaphylactic shock. It has also been suggested that mast cells can undergo so called piecemeal degranulation (22, 23). This process is much slower and more often seen in chronic inflammation.

Mast cells have the capacity to release a number of different mediators that are released through different pathways, i.e., degranulation, conversion of lipids to e.g., eicosanoids, and de novo protein synthesis of e.g., cytokines (24-26). It has been reported that selective release of serotonin, without histamine can occur (27, 28). Furthermore, eicosanoids (29, 30), and diverse cytokines/chemokines, such as IL-6 can be released without preceding degranulation (31-33).

In immediate hypersensitivity reactions, upon activation mast cells sequentially release numerous mediators either preformed in secretory granules or newly synthesized (24, 34, 35) as shown in Figure 1. Preformed mediators are released (degranulation) within seconds to minutes include histamine, proteases, serotonin, proteoglycans, and certain cytokines, e.g., tumor necrosis factor (TNF). Interestingly, mast cells are the only cells storing preformed TNF (36). Mast cells are also rapidly able to synthesize *de novo* and secrete lipid metabolites of arachidonic acid, such as cysteinyl leukotrienes (LT) LTC_4, LTD_4, LTE_4 and LTB_4, prostaglandins (PGDs) PGE_2 and PGD_2 (37, 38) and platelet-activating factor (PAF) (39). All these mediators are responsible for many of the acute signs and symptoms of mast cell mediated reactions and can exert profound effects on multiple tissues including respiratory, circulatory, skin, gastrointestinal, and central nervous systems (24-26). On activation, a specific program of gene expression is also activated, resulting in the *de novo* synthesis of several cytokines including IL-3, IL-4, IL-5, IL-6, IL-10, IL-13 and TNF-α, which can lead to e.g., development of late-phase responses of acute allergic reactions (10).

Cytokine and chemokine production by MCs is closely regulated and can occur independently from classical FcεRI receptor-mediated pathways, and interestingly a unique profile of cytokines is induced depending upon the nature of stimulus or type of infection (10). Thus, a broad array of such molecules may be secreted such as IL-1, IL-2, IL-5, IL-6, IL-8, IL-9, IL-10, IL-13, IL-16, IL-18, IL-25, IL-33 and interferon (IFN)-α, IFN-β and IFN-γ, and also growth factors including SCF, fibroblast growth factor (FGF), vascular endothelial

growth factor (VEGF), nerve growth factor (NGF), granulocyte-macrophage colony stimulating factor (GM-CSF) and platelet-derived growth factor (PDGF) (10, 40, 41).

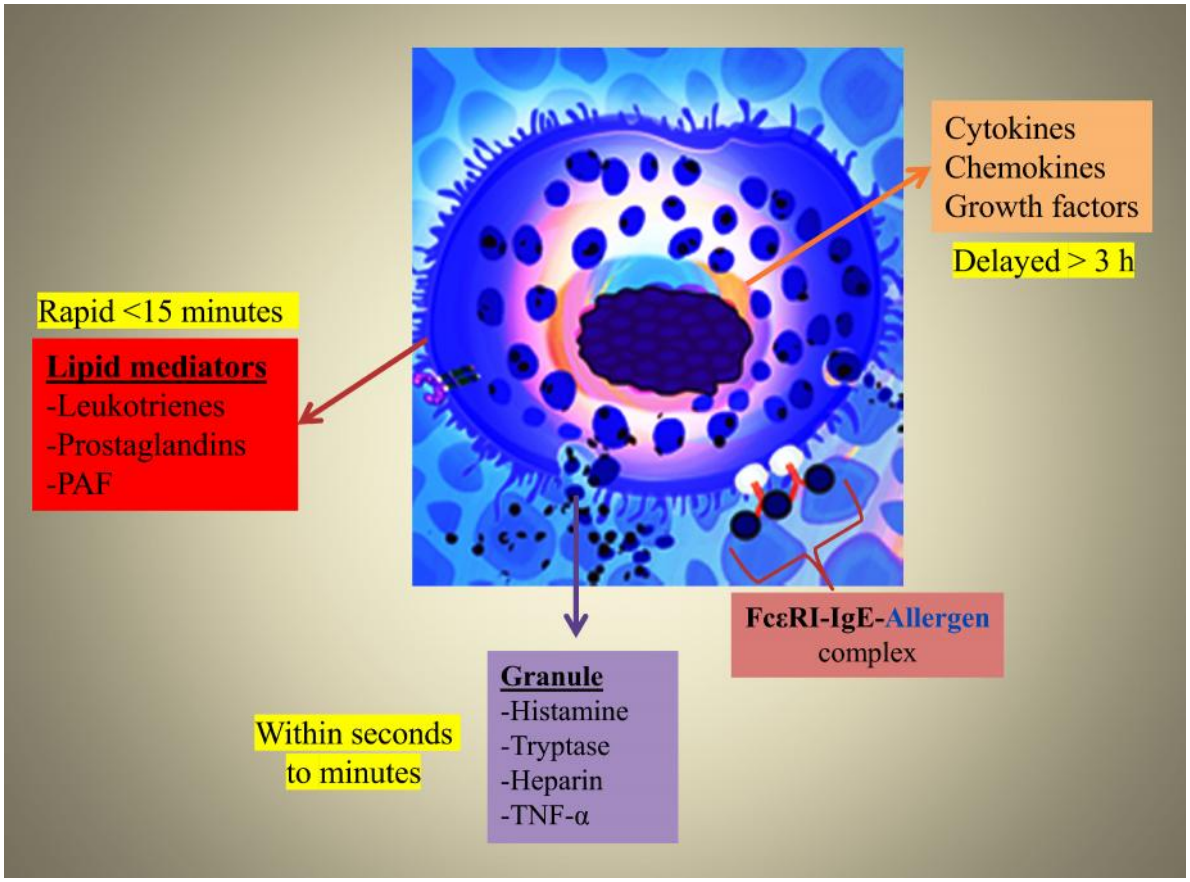

Figure 1. Schematic representation of sequential release of various mediators upon activation of mast cells in immediate hypersensitivity reactions.

The heterogeneity among human mast cells was determined at different tissue localizations and conventionally two distinct types of mast cells were described based on their protease content (42). One type consists of only tryptase, which is referred to as MC_T, and is predominantly found at the mucosal surfaces of intestinal and respiratory tract, where they located around T-cells. The other type is known as MC_{TC} and contains both tryptase and chymase along with other proteases such as carboxypeptidase A and cathepsin G, and is localized in connective tissue areas, such as skin, submucosa of stomach and intestine, myocardium, lymph nodes, synovium and conjunctiva. It has been reported that lung MC_{TC} express CD88 antigen (C5a receptor) and thereby can be distinguished from MC_T which do not express the antigen (43). Remarkably, under circumstances, heterogeneity of mast cells might be reversible (44). A third type of MC, MC_C, which contains only chymase without tryptase, has also been suggested (45). These cells reside mainly in the mucosa and submucosa of the stomach, colonic mucosa and intestinal submucosa (45).

Interestingly, mast cells can act as both positive and negative immunomodulators (9, 46). Mast cell heterogeneity, thus, appears not to be restricted to protease content, but might also include distinct functionality. One of the key features of the mast cell activation is their ability to selectively produce and secret mediators depending upon the triggering factors. A

certain stimuli might lead to mast cell degranulation, whereas another trigger releases only cytokines or chemokines. Selective release of specific mediators without degranulation has been purposed to explain the versatile roles of mast cells in different cellular processes (47). Whether this reflects mast cell plasticity, however, remains to be elucidated. Furthermore, mast cell, after their degranulation, can recover the original morphology and retain their ability to participate in multiple cycles of activation within few days (48).

2.2 ANAPHYLAXIS

Anaphylaxis is an old phenomenon and the first presumed cases of anaphylaxis was described in 2641 B.C., when pharaoh Menes died mysteriously following a wasp sting (49). However, the modern term of anaphylaxis was first coined by Richet and Portier in 1902 (50). They isolated the toxin produced by the jellyfish known as the Portuguese man-of-war, then attempted to immunize and protect dogs against it. During their experiments, they discovered something unexpected when the dog died dramatically after a second injection of the same amount of the toxin (51). They termed this as "anaphylaxis" or "lack of protection".

2.2.1 Definition

Today, anaphylaxis is defined as an acute, severe, potentially life-threatening systemic hypersensitivity reaction involving more than one organ system (52). It is one of the most alarming emergency conditions that presents with a broad array of symptoms and signs, many of which can mislead to other acute conditions including asthma attack, laryngeal edema, myocardial infarction, panic attack. Anaphylaxis is almost always unexpected and if not promptly treated may lead to death by airway obstruction or cardiovascular collapse or both. However, until recently, there has been no universally recognized definition of anaphylaxis, because anaphylaxis comprises a constellation of features. That has not only caused failure to diagnose and delayed treatment in patients but also hampered research facilities.

2.2.2 Epidemiology

The available epidemiological data about the exact prevalence and incidence of anaphylaxis are limited and often inconsistent. This is mainly due to diverse study designs among different populations, lack of globally accepted definition of anaphylaxis, and lack in reporting or misdiagnosing (53-55). In addition, most of the published data is based on hospital and emergency admissions, however, the International Classification Codes (ICD) recording anaphylaxis are insufficient and do not properly reflect the epidemiological needs.

With these limitations, it is, however, widely accepted that anaphylaxis is a relatively rare condition. Data from the USA on the epidemiology of anaphylaxis suggest an incidence of up to 40 to 50 people per 100 000 person- years (56), whereas the results of 10 European studies suggest an lower incidence of 1.5–7.9 per 100 000 person-years (57), with studies from the UK showing an increase in admissions with anaphylaxis over the last two decades (58). Recently, the incidence of anaphylaxis among Swedish children was reported to be 32 per 100 000 persons/year (59). The lifetime prevalence of anaphylaxis has been calculated to be approximately 0.05–2.0% (60). Although rare, deaths may also occur and suggested to be at a rate of 1 per 3 million population per year (61).

2.2.3 Etiology

Foods, insect venoms and drugs appear to trigger most cases of anaphylaxis. In emergency department studies, food is the most common cause in children corresponding to 80% to 92%

of the anaphylaxis, respectively (62, 63). Regarding adults, venom- or drug-induced anaphylaxis are more common followed by idiopathic (no apparent cause) anaphylaxis.

Interestingly, a large Central European cohort of 1985 patients involving 2012 anaphylactic episodes was recently published (64). In this study, age of patients ranged from 2 months to 87 years (median, 42.5) and insect sting was the most common elicitor (50%), followed by food (24%) and drugs (17%). The range of elicitors varies depending upon the geographical area. High percentage of venom-induced anaphylaxis in this cohort was striking as the corresponding numbers differed widely from the studies performed in the USA (19%) and Australia (30%) (56, 65). When data from the European cohort analyzed children (<18 years) separately, the most common trigger was food (58%), followed by insects (24%) and drugs (8%) (66).

Sometimes simultaneous occurrence of certain cofactors is needed in order to trigger anaphylaxis. This so-called “summation or augmentation anaphylaxis” may account for certain cases of unexplained anaphylaxis and can also explain why some patients experience only intermittent anaphylaxis (67, 68). Such cofactors include viral infections, stress, physical exercise, some drugs (β-blockers, angiotensin-converting enzyme inhibitors, non-steroidal anti-inflammatory drugs [NSAID]), alcohol or spicy food intake. This concept is, however, mainly based on clinical observations, as, to date, there have been no prospective studies with defined combinations to prove summation anaphylaxis apart from the combination of exercise and food.

2.2.4 Pathogenesis

There are different pathways in which mast cells can be activated leading to an anaphylactic reaction (Figure 2).

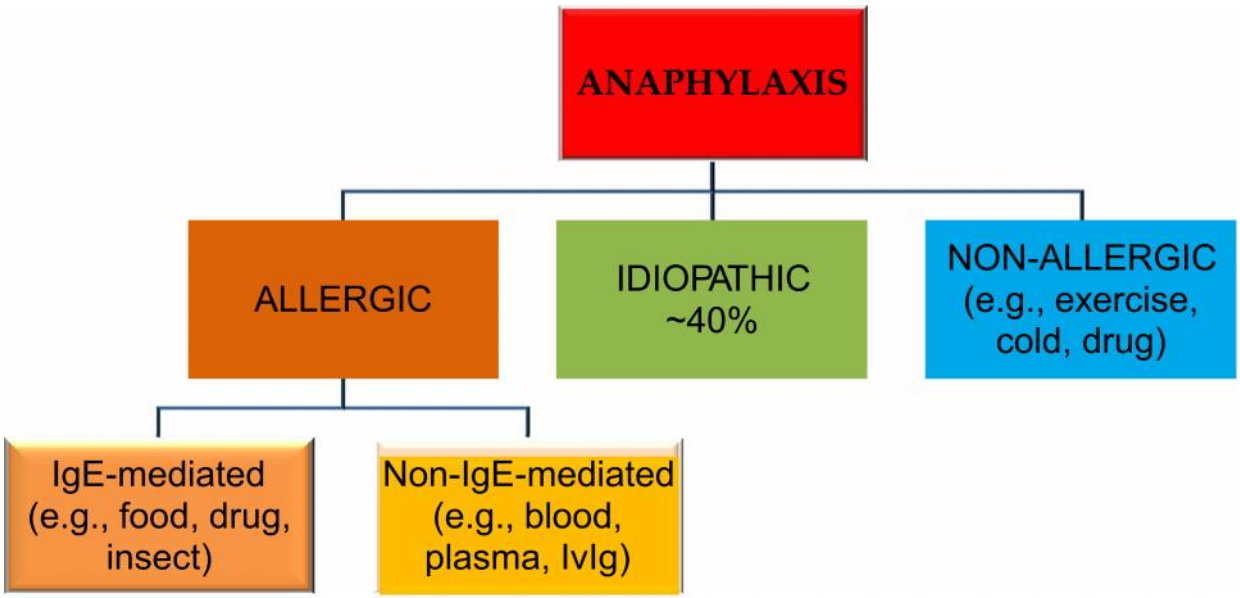

Figure 2. Mechanistic classification of anaphylactic reactions according to EAACI and WAO Nomenclature. Adapted from references (69, 70).
EAACI: European Academy of Allergy and Clinical Immunology; WAO: World Allergy Organization

Most episodes of anaphylaxis to foods, drugs and venoms are triggered via immunoglobulin E (IgE)-mediated immunological mechanism. After a sensitized person reexposes to the triggering allergen, crosslinking of two-specific IgE molecules to FcεRI on the surface of the mast cell leads to release of mediators and thereby related symptoms. However, not only IgE-mediated mechanisms cause anaphylactic reactions. In addition, activation of complement either via cytotoxic antibodies (e.g., IgG-mediated blood transfusion reactions) or by immune complexes (e.g., dextran infusions, complexes of gammaglobulin administered intramuscularly or intravenously), results in complement split products C3a and C5a, which can consequently trigger the release of mediators from mast cells directly. Apart from these immunological mediated reaction patterns, there are some poorly understood mechanisms causing direct mediator release, including histamine, through certain agents (e.g., hyperosmolar solutions including mannitol, radio contrast mediums, muscle relaxant drugs, opiates), direct activation of complement or other plasma protein systems (coagulation, kallikrein-kinin), or through the inhibition of cyclooxygenases (e.g., aspirin, NSAID).

When no cause can be identified, the term idiopathic anaphylaxis is used.

2.2.5 Symptom profile

Anaphylaxis represents a constellation of varied symptoms that generally are related to the cutaneous, gastrointestinal, respiratory, and cardiovascular systems. Distribution of different signs and symptoms were reported in a series of 601 patients (71) as follow: involvement of skin in 90%, respiratory symptoms in 59%, whereas 33% of patients were experienced syncope or lightheadedness and 29% abdominal cramps or diarrhea. Respiratory symptoms are more common in children, whereas cardiovascular symptoms appear to dominate in adults (62).

Skin manifestations including flushing, pruritus, erythema, urticaria, angioedema are the most common symptoms of anaphylactic episodes. Symptoms most commonly start with itching on palms, soles, palate or in genital areas. Skin symptoms are extremely beneficial to recognise anaphylactic reactions, however, they are absent in 10-20% of cases (65, 71, 72). The respiratory system is also commonly involved varying from upper airway obstructions due to oedema of the larynx, epiglottis to bronchoconstriction of the lower airways causing wheezing, dyspnoea, chest tightness and sometimes even hypoxia and respiratory arrest. In addition, cardiovascular symptoms can occur, including tachycardia, hypotension, presyncope/syncope, arrhythmias, myocardial ischemia/infarct, and as serious as cardiac arrest. Mechanisms behind the cardiovascular symptoms are thought to be peripheral vasodilatation, enhanced vascular permeability, leakage of plasma and intravascular volume depletion rather than direct effect on the myocardium (73). A wide variety of ECG-changes also have been observed (74-76). Confusion, collapse, unconsciousness and incontinence are strongly associated with hypotension and hypoxia (65). Gastrointestinal manifestations are also frequent and include nausea, vomiting, diarrhea and crampy abdominal pain.

The time of onset of symptoms, the sequence in which symptoms develop, and severity of symptoms frequently vary among individuals and may even vary in the same individual

during repeated episodes or in response to different exposures. However, sign and symptoms usually occur within 2 hours after exposure to the trigger (77). The more rapid the onset the more likely the reaction will be severe and life-threatening (78). Currently, there is no way to identify who will have a severe reaction or to predict when it will occur. The signs and symptoms can follow a uniphasic course resolving within hours or may follow a biphasic course in up to 20% of cases (79-83). The median time from symptoms to cardiac and/or respiratory arrest has been reported as 5, 15, and 30 min for parenteral medication, insect venom, and food, respectively (84).

2.2.6 Diagnosis

The medical history and physical examination is the most important tool to establish diagnosis of anaphylaxis as reactions are characterized with classical signs and symptoms in association with temporal relations to exposure. Recently, a consensus proposal for diagnostic criteria of clinical anaphylaxis was published by a multidisciplinary group of experts as shown in Figure 3 (52). Accordingly, diagnosis of anaphylaxis is highly likely when any one of the three criteria is fulfilled. A retrospective emergency department study demonstrated an excellent sensitivity (96.7%) and good specificity (82.4%) using these criteria for the diagnosis of anaphylaxis (85).

Acute onset of ilness with *unknown* trigger	**After exposure to a *likely allergen for that patient* and rapid occurrence of >2 of the following**	**After exposure to a *known allergen for that patient***
Involvement of the skin-mucosal tissue (e.g., generalized urticaria, itching-flushing, swollen lips-tongue-uvula)	Involvement of the skin-mucosal tissue (e.g., generalized urticaria, itching-flushing, swollen lips-tongue-uvula)	Reduced blood pressure (> 30% decrease from the person's baseline)
AND AT LEAST ONE OF THE FOLLOWING	Persistens gastrointestinal symptoms (e.g., crampy abdominal pain, diarrhea, vomitting)	
Respiratory comprimise (e.g., dyspnea, stridor, wheeze-bronchospasm, hypoxemia, reduced PEF)	Respiratory comprimise	
Reduced blood pressure or associated symptoms (e.g., hypotonia, collapse, syncope, incontinence)	Reduced blood pressure or associated symptoms	

Figure 3. Clinical criteria for diagnosis of anaphylaxis. Adapted from reference (52).
PEF: peak expiratory flow

The only immediate test that is useful at the time of reaction is measurement of serum tryptase levels (86). Tryptase is an indicator of mast cell activation but does not distinguish mechanisms or causes (87). It is almost always raised in insect- or drug-induced anaphylaxis. Serum tryptase levels peak 1 hour after the start of an episode and may persist for as long as 5 hours. A normal tryptase test result does not exclude the diagnosis of anaphylaxis, whereas an elevated tryptase level (>11.4 ng/ml) almost exclusively confirms occurrence of anaphylactic reaction.

2.2.7 Management

Anaphylaxis is a medical emergency and requires prompt recognition and treatment. Intramuscular adrenaline is the drug of choice for immediate episodes of anaphylaxis, even if the diagnosis is uncertain (88-92). This drug resolves the inappropriate effects of the mast cell mediators produced during anaphylaxis. Unfortunately, the usage of adrenaline is still underutilized, whereas steroids are widely used as first line therapy despite the lack of evidence (93, 94).

Prevention is the most important aspect of the anaphylaxis management. It is recommended that all patients who have had an anaphylactic reaction should be evaluated by an allergist to make a risk assessment. Identification of the culprit agent, if possible, and avoidance is the mainstay of the management. Specific immunotherapy is recommended for IgE-sensitized patients with hymenoptera venom anaphylaxis.

2.3 MAST CELL ACTIVATION DISORDERS

Mast cells are implicated in the pathogenesis of a broad spectrum of disorders that clinically manifest as a result of inappropriate mast cell activation and release of mediators and cytokines. The most dramatic and classic outcome of such clinical conditions is observed in anaphylaxis. In addition, many of the clinical features attributable to the systemic effects of mast cell mediators have become recognized in patients with what today is called systemic mast cell activation disorders, including mastocytosis.

2.3.1 Historical perspective

The first report describing the clinical features of mast cell disease goes back almost 150 years. British physicians Nettleship and Tay described in an article in 1869 (95), a rare form of urticaria in a 2-year-old girl, what is now considered to be urticaria pigmentosa (UP). The association of mast cells with UP was made in 1887 by Unna, when he demonstrated MCs in these patients' skin (96). However, the term *mastocytosis* was first applied by Sezary when in 1936 described individual lesions of UP (97). In 1949, Ellis was the first to recognize the systemic nature of mastocytosis in an autopsy finding of a 1-year-old child who died as a result of multi-organ infiltration by MCs (98), thereby defining systemic mastocytosis (SM).

There has been prominent progress in understanding the cellular and molecular aspects of this disease during the last decades. In 1987, Schwartz and colleagues defined tryptase as a sensitive biomarker of mast cells in mastocytosis (87). The molecular advances started with Furitsu in 1993 (99) by identification of the *KIT* D816V activating mutation in a human mast cell leukemia cell line, which was later proved to exist in most patients with mastocytosis (100). In the late 1990s, Escribano and Orfao showed aberrant immunophenotypes of MCs in the marrow of patients with mastocytosis and diagnostic implications of CD117/CD25 or CD117/CD2 expression (101). Mast cell accumulation in mastocytosis can be the result of an increase in proliferation (102), and due to KIT's anti-apoptotic effect (103, 104).

2.3.2 Classification and nomenclature

A parallel development emerged even from a clinical perspective; however, the complexity of classifying a heterogeneous group of disorders was apparent. The clinical conditions involved in uncontrolled growth of MCs were historically termed as urticaria pigmentosa (cutaneous mast cell disease) and/or systemic mast cell disease (systemic mastocytosis); and the terms mast cell disease, systemic mast cell disease, mastocytosis were used interchangeably in the medical literature since 1950s (105-121). Nevertheless, it was difficult to compare studies from different research groups as description of the mastocytosis was varied.

Certain classification proposals of mastocytosis were also presented through the years. Proposed classifications based on distribution of the lesions and involved organs, according to the nature of disease prognosis. A first comprehensive classification was introduced in 1979 by Lennert & Parwaresch (Kiel classification) (122). Independently, a new proposal

came from Mayo Clinic by Travis (123) and the term “indolent” mastocytosis was introduced for first time. Later, this classification was further refined and the first consensus classification of mastocytosis was proposed by Metcalfe in 1991 (124). Finally, in year 2000, in Vienna, Working Conference of Mastocytosis proposed new classification that resulted in the WHO official classification of mastocytosis (125, 126). The new classification introduced the concept of “minor” criteria thereby enabling the diagnosis of mastocytosis in the absence of bone marrow mast cell aggregates. This classification was reconfirmed in 2008 (127). In addition, Valent (Vienna) initiated the European Competence Network on Mastocytosis (ECNM) in 2002 to improve diagnostic and therapy in mastocytosis and also to provide most updated information in the field (128).

Intriguingly, already in the mid-1980s, Oates and Roberts hypothesized the existence of mast cell disease with little or no proliferation mainly characterized by mast cell activation resulting in mediator release and related symptoms (129-131). Interest in better understanding of such “mast cell activation disease” continuously expanded in the recent years and finally the term “monoclonal mast cell activation syndrome” was introduced in the literature in 2007 (132, 133). This was followed by a formal proposal for the diagnostic criteria (134).

Increasing pace of research in this area resulted in rapid evolving and currently, the spectrum of mast cell activation syndromes are extended including so-called non-clonal or idiopathic mast cell activation syndrome (135-152). Recently, Akin (Harvard) introduced a refined conceptualization of mast cell diseases by combining both proliferative and activating aspects of mast cells and proposed an umbrella term of “mast cell activation disorders” (153). Consequently, this comprises a broad spectrum of disorders characterized by inappropriate mast cell activation/accumulation, and presents with periodic or chronic symptoms that attributable to the local and systemic effects of mast cell mediators (153). Figure 4 illustrates these disorders (author’s own interpretation of the proposal).

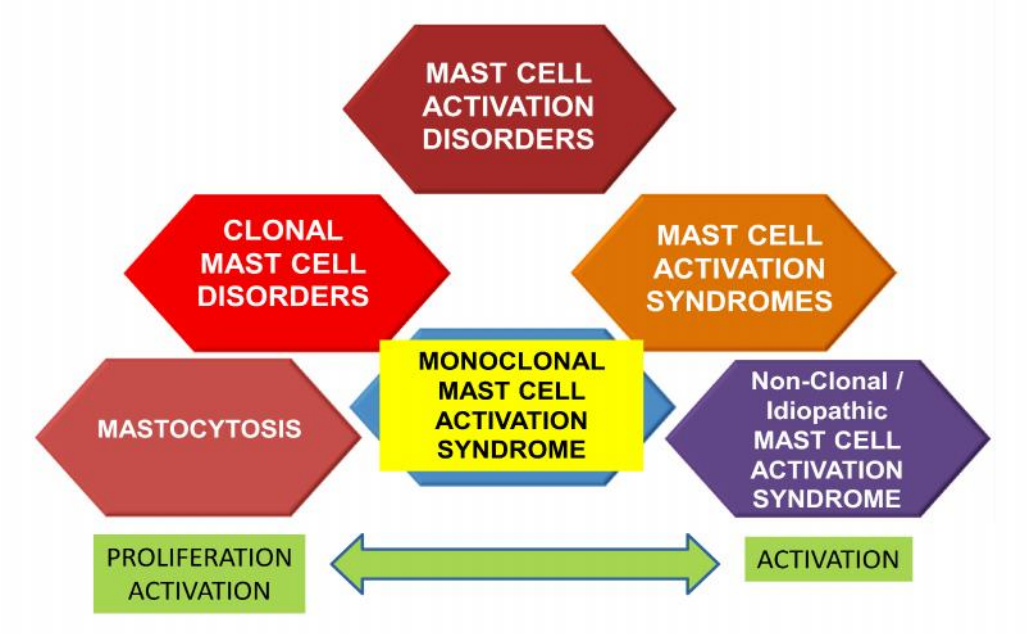

Figure 4. Schematic presentation of the main variants of systemic mast cell activation disorders.

In accordance with this proposal, patients with mast cell disorders can be broadly divided into three types: primary, secondary, and idiopathic (153). Severity grade of symptoms and involvement of various organs may vary from patient to patient due to the broad distribution of MCs and variable subset of their mediators. In secondary mast cell activation disorders, patients respond to an external stimulus as in hypersensitivity diseases; e.g., asthma, rhinoconjunctivitis, urticaria and angioedema, however, the quantity and function of mast cells are normal. For this doctoral thesis, however, my particular interest is primary and idiopathic conditions, where inappropriate systemic mast cell activation results in clinical signs and symptoms involving more than one organ system.

Primary mast cell disorders are associated with intrinsic defects in mast cells affecting proliferation and/or activation pathways (153). With this regard, it is tempting to hypothesize that hyperreactive mast cells causing diverse clinical signs and symptoms may emerge among these affected cells, although the underlying mechanism(s) that transforms cells into a hyper activated manner is presently not clearly understood (Figure 5).

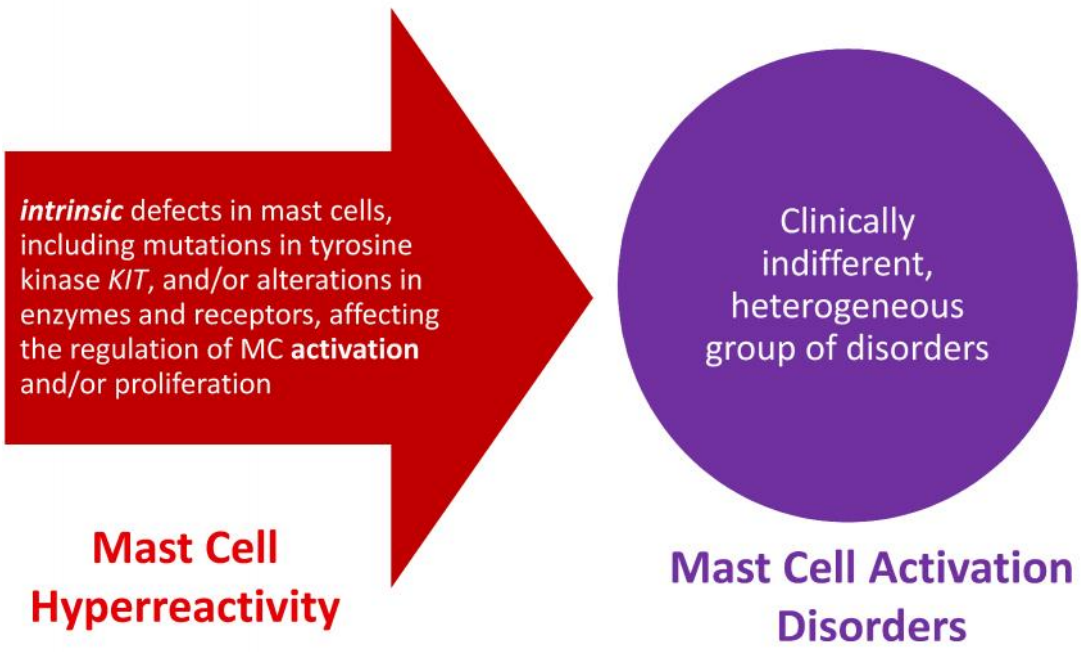

Figure 5. Mast cell hyperreactivity and its relation to mast cell activation disorders. Adapted from the references (144, 153)

The conditions including mastocytosis and monoclonal mast cell activation syndrome can be classified under primary mast cell disorders. What we currently know is that there are clonal populations of mast cells that arise from an affected progenitor and display abnormal genetic and surface markers involved in the regulation of mast cell activity (153), therefore, these conditions are also known as "**clonal mast cell disorders**" (154, 155). In this manner, activating mutations in the tyrosine kinase receptor *KIT* (100), such as D816V, are strongly associated with these disorders. In addition, the existence of an additional condition of hyperreactive mast cells, presently with undetermined clonality, called idiopathic mast cell activation syndrome (nc-MCAS), was adopted by an international working group in 2011 (136, 143).

2.3.3 Mastocytosis

Mastocytosis is a heterogeneous group of clinical disorders characterized by accumulation/activation of aberrant tissue mast cells in the skin and/or other visceral organs (125, 133, 156-171). The true incidence and prevalence of the disease is unknown, but the existing evidence suggests that it is a rare condition. One study estimated two new patients per year in a population of 300 000 corresponding to an incidence of 0.000667% (172). However, during the last decade, the number of patients substantially increased in the Western world, which is most probably due to increased awareness and better diagnostics. Thus, the general estimated prevalence is in Central Europe 0.5-1 in 10 000 (170) and in Denmark 1 in 10 000 (173). Gender distribution appears to be equal.

Mastocytosis occurs in both adults and children. Most of the patients are children, where the disease is congenital in up to 15% of cases (174), and the onset of mastocytosis appear before 2 years of age in a further 40% of the patients (175). In adult patients, the onset of the disease frequently occurs between the ages of 20-50 years, and usually detected at 40-60 years of age. Most cases of mastocytosis appear as spontaneous, although some rare familial cases have been reported (176-179).

The most commonly affected sites are the skin, bone marrow, lymph nodes, intestine, liver and spleen (123, 125, 134, 159, 161, 180), whereas the lungs and kidneys are virtually never involved (181). Mastocytosis can broadly be separated into cutaneous mastocytosis (CM) affecting only the skin and systemic mastocytosis (SM) involving at least one extra cutaneous organ. The WHO classification is based on specific criteria that differentiate between CM and SM, between SM and myelomastocytic disorders and between SM and a reactive increase in MCs (125, 126, 134). In addition, the rare and localized MC tumors MC sarcoma and extracutaneous mastocytoma are also included; thereby mastocytosis is classified into seven categories (125, 127) (Figure 6).

According to the WHO classification, there are different subvariants of CM and SM, and the clinical course and prognosis vary between these patients (Figure 6) (125). Cutaneous mastocytosis include most commonly maculopapular form of lesions (MPCM), better known as urticaria pigmentosa (UP), and followed by mastocytoma and diffuse cutaneous mastocytosis (DCM). Urticaria pigmentosa is characterized by symmetrically distributed 0.5 cm red-brown macules and papules, which induce erythema, wheal and pruritus, the so-called Darrier's sign, when rubbed or scratched. Children usually suffer from cutaneous disease and signs and symptoms resolve by adolescent (182). Most children present with UP (175, 183). however, another study stated mastocytomas as being the most common form in children (184). Prognosis of CM in children considered to be good with 67% complete resolution, 20% major regression and 13% partial regression in a study of 20 years follow-up (185).

In adults, skin lesions are commonly associated with systemic mastocytosis. The exact frequency of skin lesions in adults varies depending on the specialty of the physician that sees patients and therefore reported in 50-100% of patients with SM (181). The lesions are fixed at

their localizations and the sun-exposed areas, such as face and hands are usually spared from skin lesions in adults.

VARIANTS		SUBVARIANTS	PROGNOSIS
CUTANEOUS MASTOCYTOSIS (CM)		Urticaria pigmentosa (UP) = Maculopapular CM	Good
		Diffuse CM	Good
		Mastocytoma of skin	Good
SYSTEMIC MASTOCYTOSIS	Indolent SM (ISM)	Smouldering SM	Good
		Isolated Bone Marrow SM	Good
		Well-Differentiated SM	Good
	SM with an associated clonal hematologic non-MC lineage disease (SM-AHNMD)	SM-AML	Depending on the AHNMD
		SM-MDS	
		SM-MPN	
		SM-CEL /SM-HES	
		SM-CMML	
		SM-NHL	
		SM-myeloma	
	Aggressive SM (ASM)	Lymphadenopathic SM with eosinophilia	Poor
	Mast cell leukemia (MCL)	Aleukemic MCL	Very poor
MAST CELL SARKOMA			Very poor
EXTRACUTANEOUS MASTOCYTOMA			Good

Figure 6. Variants and subvariants of mastocytosis according to the WHO classification. Adapted from references (126, 135, 187).
CM: cutaneous mastocytosis; UP: urticaria pigmentosa; SM: systemic mastocytosis; ISM: indolent systemic mastocytosis; SM-AHNMD: systemic mastocytosis associated with hematological non MC lineage disease; AML: acute myeloid leukemia; MDS: myelodysplastic syndrome; MPN: myelproliferative neoplasm; CEL: chronic eosinophilic leukemia; HES: hypereosinophilic syndrome; CMML: chronic myelomonocytic leukemia; NHL: non-Hodgkin lymphoma; ASM: aggressive systemic mastocytosis; MCL: mast cell leukemia.

Patients with systemic mastocytosis (SM) can be categorized into four major variants and the vast majority (about 90%) of patients has indolent disease (ISM). In patients with ISM, the

percentage of mast cells in the bone marrow is usually below 5% (125), and the rate of mast cell proliferation is very low (187). There are special subvariants of ISM, for instance, *smouldering SM*, which can be differentiated from ISM by higher tryptase levels (>200 ng/ml) indicating excessive mast cell burden. However, there are no signs of bone marrow insufficiency in smouldering SM (important differentiation from aggressive SM) (188). Another rare subvariant of ISM is isolated bone marrow mastocytosis (127), where mast cell infiltration is only found in bone marrow, but not in the skin. Indolent SM appears to have a favorable prognosis without decreased life expectancy or organ damage.

SM-AHNMD is the second most common form of SM, in most centres being <10% of all mastocytosis cases. Its prevalence varies widely from centre to centre comprising up to 4% (173) to 40% (189). In these cases both WHO/FAB criteria for diagnosis of an AHNMD as well as the criteria for SM must be met (189). In this category, the prognosis is primarily depending on the associated haematological disorder.

Aggressive systemic mastocytosis (ASM) is a rare and rapidly progressive form of SM that presents with so-called C-findings, including organomegaly involving initially bone marrow and later gastrointestinal tract, liver, spleen, and lymph nodes that results with end-organ dysfunction (125, 134, 190). The serum tryptase level is usually >200 ng/ml. The survival of ASM patients is limited (189, 190). Mast cell leukemia (MCL) is a very rare aggressive MC neoplasm, which presents usually without skin lesions. In contrast to ASM, number of immature MCs found in bone marrow aspirates is ≥20% (108, 123, 189, 191, 192). Most patients survive less than one year.

2.3.4 Pathogenesis of mastocytosis

Mastocytosis is associated with an activating point mutation in the *c-kit* gene. This was first recognized in 1995, when Nagata *et al*. identified a point mutation consisting of a substitution of valine for aspartic acid in the catalytic domain of *KIT* (D816V) in the peripheral blood of four patients with mastocytosis (100). Further analysis of larger cohorts confirmed that *KIT* D816V mutation, regardless the variant of SM, is detected in up to 93% of adult patients with SM (193). By contrast, the frequency of this mutation in pediatric mastocytosis has been controversial; however, according to a recent study, *KIT* D816V mutation was found in skin biopsies of 44% of 50 children with mastocytosis (194). The mutation results in constitutive autophosphorylation of the molecule, independent from its ligand (SCF), thereby leading to an enhanced differentiation, survival and activation of MCs (163, 193, 195). KIT activation also augments the magnitude of IgE-mediated mast cell activation pathways through common signal transduction pathways, such as NTAL (196).

The *KIT* D816V is thought to play an important role in indolent SM that presents with MC-mediator related symptoms and have no signs of a substantial proliferation. In contrast to ISM, the *KIT* D816V mutation alone is not believed to be responsible for the manifestations of SM-AHNMD, ASM and MCL, where mast cells show increased proliferative capacity (197, 198). Indeed, in these patients, additional genetic defects have been identified including other *KIT* mutations apart from D816V (199-201), *RAS* mutations (202), *TET2* mutations

(203, 204), and mutations in *IgE receptor* genes (205). Interestingly, overall survival was found significantly shorter in patients with additional aberrations (206).

Another pathogenic aspect of mastocytosis is association of *KIT* D816V mutation with aberrant expression of CD25 molecule. Expression of CD25, α-chain of the interleukin-2 receptor, is usually limited to activated-T cells in healthy individuals, and mast cells normally do not express CD25. However, expression of CD25 on the mast cell surface is a hallmark of MCs in mastocytosis (101). The functional significance of CD25 molecule for the MCs is not known.

2.3.5 Clinical features

Mastocytosis is an intriguing disorder with protean clinical manifestations ranging from asymptomatic disease to a highly aggressive course with multi-system involvement. The most frequent clinical symptoms are related to the release of mast-cell mediators, and can be observed in all categories of mastocytosis.

In patients with indolent disease, symptoms are related to local or remote effects of excess mediator release from MCs, either spontaneously or in response to trigger stimuli (25, 207). Exogenous and endogenous triggers, such as cold, heat, physical exertion, consumption of alcohol, infections, nonsteroidal anti-inflammatory drugs, emotional stress vary greatly from patient to patient, and patients present a variable and often changing pattern of symptoms. The most presenting complaints include facial flushing, pruritus, palpitations, dizziness, hypotension, syncope, breathing difficulties, abdominal pain, nausea, vomiting, diarrhea, headache, sweating, lethargy, fatigue, arthralgia and myalgia, lack of concentration, irritability, anxiety, and depression. Not all patients experience all of the symptoms listed, and symptoms occur during the discrete episodes, which are usually referred to as attacks or spells by most patients (130). These attacks may be brief or prolonged, but the duration is usually in the range of 15 to 30 minutes (130). History of flushing or at least feeling hot during these episodes is a very important key factor. In addition, symptoms may either present isolated or in some patients a constellation of symptoms may resemble an anaphylactic reaction, which might be life-threatening as in the appearance of anaphylactic shock (159, 161, 180). Typically, the patient suddenly feels very warm and then experience palpitations, dizziness and fall in blood pressure due to systemic vasodilatation that often leads syncope (130). Following the attacks, these patients often experience severe fatigue lasting hours.

The results of previous studies and clinical observations of frequent episodes of hypotension in these patients led to the recognition of a strong association between anaphylaxis and mastocytosis (208-228). The prevalence of anaphylaxis has been reported to be 20% to 56% in adult patients with various forms of mastocytosis (218, 221, 222). This represents an approximately 1000-fold increase risk over general in population (229). Hymenoptera stings appear to be the most frequent causes of anaphylaxis, followed by unexplained, idiopathic reactions (155). The reason for this tight association is intriguing. Nevertheless, not all SM patients bearing *KIT* D816V mutation suffer from anaphylaxis. Therefore, there should be

additional genetic polymorphisms or mutations in MC signaling components, other than the activating *KIT* D816V mutation, contributing to dysregulation and predisposing to anaphylaxis (26, 230).

In addition, osteopenia/osteoporosis has long been recognized in SM patients (231, 232). Less commonly, osteosclerosis is also seen, and in occasional patients both osteoporosis and osteosclerosis may be observed in different sites (233).

By contrast to ISM, it may be difficult to attribute symptoms to mastocytosis in patients with SM-AHNMD, as the patients may have signs and symptoms related to the associated hematologic disorder. In addition, in advanced categories of disease, i.e., aggressive SM and mast cell leukemia, destructive infiltration of mast cells can cause symptoms related to end-organ dysfunction including malabsorption and weight loss, osteolyses, hypersplenism, hepatomegaly with impairment of liver function often with ascites, significant cytopenia(s) (125, 160, 180, 189, 190, 234). These symptoms represent so-called “C-findings” (125).

2.3.6 Monoclonal Mast Cell Activation Syndrome

In recent years, another condition with MC mediator-related symptoms have emerged, where one can detect a mast cell clonality by confirming the presence of an aberrant mast cell population in the bone marrow expressing $CD2^{+}$/$CD25^{+}$ and/or *KIT* mutation D816V (132, 133). However, these patients have neither an increased number of reactive mast cells nor an increased proliferation of mast cells, and they do not meet the criteria for SM. Such patients lack cutaneous mastocytosis and usually have normal or slightly elevated baseline serum tryptase. A typical patient presents with recurrent, severe anaphylaxis episodes of hypotension and syncope either associated with idiopathic attacks or after a reaction associated with an insect sting. Monoclonal mast cell activation syndrome (MMAS) was proposed as a new term to define this patient population and MMAS was recognized as a distinct primary mast cell disorder by an international consensus conference in 2007 (134).

2.3.7 Non-clonal/idiopathic mast cell activation syndrome

Currently, the further understanding of the mast cell activation mechanisms has generated a new clinical disorder in the literature, and this condition is now recognized as non-clonal or idiopathic mast cell activation syndrome (nc-MCAS) (136, 141-143, 145, 146, 149). The patients present all the signs and symptoms of mast cell activation in the absence of known mast cell clonality or allergy. Thus, nc-MCAS can only be diagnosed when primary and secondary disorders of mast cell have been excluded. Nevertheless, the mechanisms by which mast cells are activated in this disorder remains to be elucidated. In the future, using more sensitive techniques, it may ultimately be possible to identify new genetic defects and reclassify these disorders as primary mast cell disorders. Until then, nc-MCAS is best classified as an idiopathic disorder. In addition, it has also been suggested to distinguish these patients from idiopathic anaphylaxis; thus patients with nc-MCAS should not meet the clinical criteria for anaphylaxis (136, 153).

2.3.8 Diagnostic considerations in mastocytosis and related mast cell disorders

Clinical suspicion for mast cell activation disorders should reasonably start with presence of varied signs and symptoms that relate to mast cell mediator release. Most of these patients have seen several doctors before they have been correctly diagnosed. In addition, most patients are extensively investigated without proving any pathological findings. Although it is rare, suspicion for mastocytosis is somewhat easier than mast cell activation syndromes due to its typical cutaneous involvement in many cases. However, approximately 20-30% of cases lack skin manifestations, so it can be easy to overlook the diagnosis in these patients. Differential diagnosis may be difficult but the clues in the laboratory findings and the clinical picture may lead to the correct diagnosis.

Although typical cutaneous lesions leads to further investigation with bone marrow (BM) examination thereby establishing either SM or CM diagnosis, diagnosis of MCAD can be a real challenging in patients who lack cutaneous manifestations. Suspected mastocytosis patients may still have some specific manifestations, such as unexplained hematological abnormalities, unexplained osteoporosis, and most importantly a baseline (that is, when patients are in quiescent state) elevation in serum total tryptase (>20ng/ml; normal levels are between 1 and 11.4 ng/ml) that leads to BM-examination.

Most challenging patients, however, are those who only present with recurrent signs and symptoms of mast cell activation including venom-induced or unexplained anaphylaxis, lacks skin lesions and have normal baseline tryptase levels. Those patients can eventually suffer from indolent SM (without cutaneous lesions), or from clonal or non-clonal mast cell activation syndromes. The differential diagnosis in these three conditions, without BM-examination, is almost impossible, as all three can clinically mimic each other. Therefore, a bone marrow examination is necessary. Although there are different recommendations or scoring systems to assess risk for yet unrecognised clonal mast cell disease, there is presently no consensus. There is, of course, patients' aspect as well, since some patients are reluctant to undergo bone marrow biopsies. Thus, when to consider a BM-examination is not always an easy decision.

The REMA score, developed by the Spanish Network on Mastocytosis, is one of the most appropriate predictivity tools and includes assessment of clinical manifestations during a symptomatic episode and scores the presence/absence of anaphylactic reactions with documented hypotensive episodes or syncope; and also takes into consideration the presence/absence of urticaria and/or angioedema, gender and baseline tryptase levels (155). Another potential pre-screening method is to perform peripheral blood *KIT* D816V mutation analysis (235, 236). When decision is taken for bone marrow examination, the diagnosis is step-wise and in this manner, the patient would eventually be classified as one of these conditions. In the following, the diagnostic criteria for different MCAD variants will be discussed in details.

2.3.8.1 Diagnosis of mastocytosis

As mentioned previously, there are well-defined diagnostic criteria for systemic mastocytosis (125, 134). Diagnosis of SM is mainly based on histological, serological and molecular findings. As bone marrow is almost always involved, the histopathological evaluation of bone marrow biopsy specimens is crucial to establish the diagnosis of SM to assess tissue burden of MCs, and to rule out presence of other hematological disease (125, 134, 162, 237-239). In addition, biopsy of other tissues is not recommended to establish a diagnosis, as the histopathological aspects of mast cell disorders are not well studied in other tissues. According to the WHO diagnostic criteria, one major and four minor criteria have been defined for diagnosis of SM (Figure 7) and SM is diagnosed when the major and 1 minor, or when 3 minor diagnostic criteria are fulfilled (125).

The major diagnostic criterion is the demonstration of multifocal compact MC infiltrates in aggregates (at least 15 MCs/cluster). Immunohistochemical staining using antibodies against CD117 (KIT) and tryptase antibodies is strongly recommended since MC infiltrates may be small and scanty (237, 239, 240). Moreover, MC may be hypo- or even degranulated and can therefore escape detection when only Giemsa or toulidine blue dyes are applied (240). Histological discrimination of SM from other hematological malignancies with increase of mast cells may be difficult (241).

Minor criteria include various morphological, immunohistochemical, molecular and serological findings. Abnormal mast cell morphology (>25% of mast cells) including spindle-shaped MCs, abnormal granulation or cytoplasmic projections is one of the minor criteria. The aberrant expression of CD25 (minor criterion) is another important diagnostic marker since this antigen is expressed only on neoplastic MCs in SM, but not on normal/reactive MCs (101, 238, 242, 243). The diagnostic value of CD2 expression, on the other hand, is limited because of difficulties in interpretation, especially if MCs are surrounded by CD2-expressing T cells (244). Flow cytometry is also a sensitive and reliable method to identify expression of aberrant MC markers (101, 245, 246). Finally, an activating point mutation at codon 816, especially KIT^{D816V} (minor criterion) is detectable in the majority of patients with SM (125, 134, 247). The serological detection of a persistently raised serum tryptase level (minor criterion) may be helpful in SM cases lacking compact MC infiltrates as the major diagnostic criterion (125, 134). Tryptase has been found to be expressed in neoplastic MCs in all subvariants of SM (125, 134).

Once the diagnosis of systemic mastocytosis is made, the patient should be classified into a disease variant. By contrast to SM, the pathologic criteria for diagnosis of cutaneous mastocytosis are not well-defined. The CM, particularly in children, is usually diagnosed by a visual evaluation of typical skin lesions. However, the consensus group recommended a step-wise approach to diagnose mastocytosis in the skin (MIS) (134). Thus, in addition to have typical skin lesions (major MIS criterion), one minor criterion is required that can be either histology criterion (monomorphic MC infiltrate with aggregates > 15 MC/cluster or scattered MC >20 MC per microscopic high power field) or molecular criterion (detection of a *KIT*

mutation at codon 816 in affected skin). From the checkpoint of MIS, the algorithm leads to two final diagnoses, CM or SM. Therefore, in adults with MIS, a bone marrow examination should always be performed as CM is a diagnosis of exclusion.

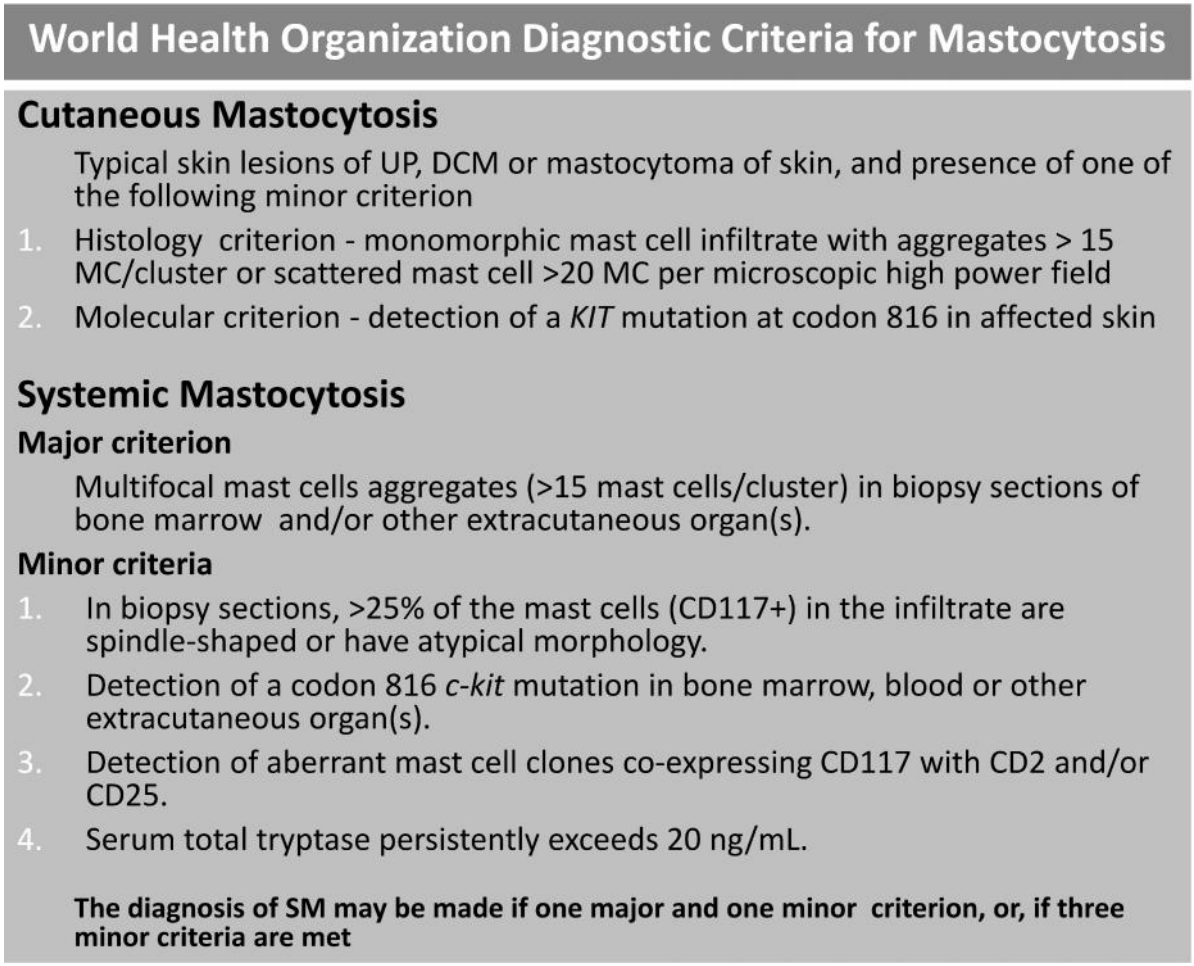

Figure 7. Diagnostic criteria for mastocytosis. Adapted from references (125, 134).
UP: Urticaria pigmentosa; DCM: diffuse cutaneous mastocytosis; MC: mast cell; SM: systemic mastocytosis; MIS: mastocytosis in the skin; MMAS: monoclonal mast cell activation syndrome

Diagnosis of **monoclonal mast cell activation syndrome** (MMAS) can be confirmed with bone marrow biopsy. These patients will often display aberrant mast cell morphology including spindling and hypogranulation and expression of CD25. The *KIT* D816V mutation may be detected as well. Bone marrow MCs may also form small aggregates comprising of < 15 mast cells. Ultimately, patients with MMAS will have 1 or 2 minor criteria fulfilled and will not meet the criteria for SM. In addition, MMAS patients virtually never have sBT levels >20 ng/ml and lack signs of mastocytosis in the skin.

2.3.8.2 Diagnosis of idiopathic mast cell activation syndrome

In contrast to MMAS, the diagnosis of **non-clonal or idiopathic mast cell activation syndrome** (nc-MCAS) cannot be established with bone marrow biopsy alone, as these patients display normal bone marrow findings. However, bone marrow examination is sooner or later necessary in order to rule out primary mast cell disorders despite that some patients

will be reluctant for this investigation. After ruling out presence of the clonal mast cell disorders through a BM-examination, diagnosis of nc-MCAS requires certain clinical and laboratory criteria to be fulfilled. This includes recurrent signs and symptoms of mast cell activation involving at least two organ systems, in combination with objective evidence of mast cell mediator release and response to mediator blockers as summarised in Figure 8. It has also been suggested that clinical diagnosis of anaphylaxis should be ruled out, that is idiopathic anaphylaxis, in order to establish a nc-MCAS diagnosis (136, 143, 153).

Some laboratory tests may serve as a pre-screening to assure that symptoms are mast cell-mediator related. Unlike SM, virtually all patients with MCAS have baseline serum tryptase levels <20 ng/ml. In this manner, a relative increase in serum total tryptase levels during a flare of symptoms is also indicative of mast cell activation. An increase greater than 1.2 x baseline value + 2 ng/ml are considered significant (143).

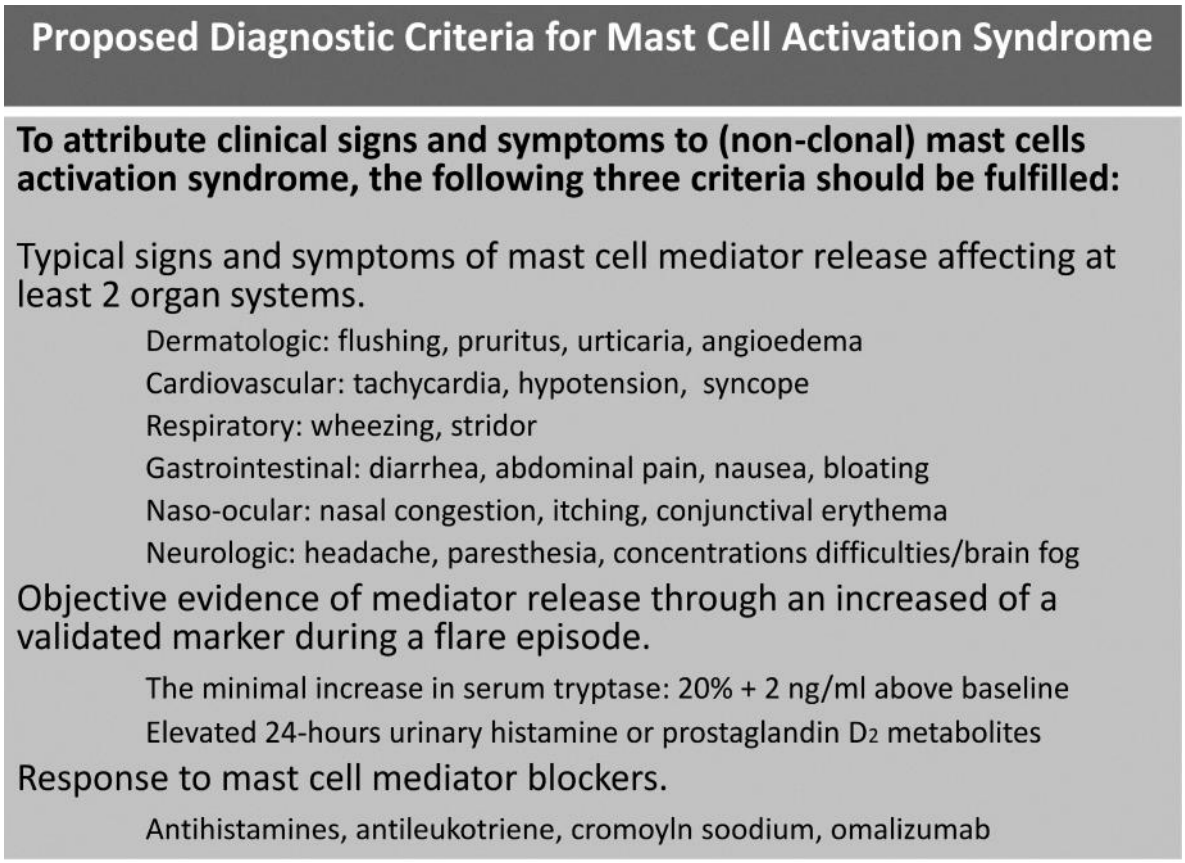

Figure 8. Proposed diagnostic criteria for mast cell activation syndrome. Adapted from references (136, 143, 153).

Additionally, analysis of other mediators including urine histamine metabolites 1-methylhistamine and 1-methylimidazoleacetic acid (248-252), and urinary PGD_2 metabolite 11β-$PGF_{2\alpha}$ (131, 253-255) can be useful. In particular, PGD_2 is predominantly produced by MCs, although other cell types including macrophages, Langerhans cells, platelets, Th2 helper cells, simulated osteoblasts can make minor amounts (256-258). It has been shown that in patients with MC activation the fold increase in urinary PGD_2 is greater than seen for the histamine metabolite (259). A major drawback though, there is few reference laboratories

for measurement. In addition, significant increases from baseline during a flare need to be defined for both histamine and PGD_2 metabolites.

In short, diagnosis of nc-MCAS is both challenging and time-consuming, thus, prospective larger cohort studies are needed to validate the proposed diagnostic criteria for these patients.

2.3.9 Mast cell hyperplasia

Mast cell hyperplasia is an important entity that should be in mind regarding differential diagnosis of mast cell activation disorders. This is strictly a histopathological diagnosis where local or systemic increases in tissue MCs are confirmed (134, 143, 260). By definition, no KIT-activating mutation or no other criterion for clonal mast cell disease including CD2 or CD25 expression in MCs, and no signs of myelogenous neoplasm are found. Mast cell hyperplasia is not an intrinsic MC disorder and no signs of mast cell activation related symptoms are detectable. In most cases, it is reactive and may be seen in wide variety of conditions, such as secondary to chronic infections, cancer, lymphoproliferative disorders, bone marrow suppression states, autoimmune disorders and other chronic inflammatory reactions. In some other cases, the clinical significance and mechanisms behind remain elusive.

2.3.10 Preventive and treatment strategies

Although lifespan for most patients appears unchanged, quality of life can be severely impaired as there presently is no cure for patients with systemic mast cell disorders of any form. The current pharmacological management is therefore aimed at controlling symptoms by reducing mediator production and release and blocking released mediators. In addition, there is, at the moment, no method of predicting best available approach to control the individual patient's disease. Patients need to find a tailor-made management strategy to cope with the consequences of acute- and chronic symptoms.

Avoidance of triggers is a prominent factor that may prevent systemic mediator release. Patients should therefore undergo thorough allergological evaluation including allergy tests for a number of known/possible triggers in order to assess their personal risk to develop mediator-induced symptoms, in particular anaphylaxis; and afterward, a trial-and-error approach usually succeeds in finding significantly helpful therapy. Most patients report on worsening of skin symptoms by sudden change of temperature, prolonged cold or heat, alcohol intake, exposing mechanical irritation, emotional stress, physical exercise. In addition, foods and most drugs appear only to play a minor role in inducing mediator release in most patients, therefore, for example, eliminations of histamine-rich diets are not routinely recommended. In contrast, hymenoptera stings appear to be the most frequent cause of anaphylaxis, followed by unexplained reactions. Those who are sensitized to hymenoptera venom should be recommended life-long venom immunotherapy, which has been shown to reduce recurrent anaphylaxis risk to 25% (261).

All MCAD patients who have history of anaphylaxis should be prescribed self-injectable adrenaline after education on the appropriate use. A stepwise maintenance treatment should

be considered in all patients who present with recurrent or chronic mediator-related symptoms. The first step includes H1-histamine receptor antagonists which have been shown to control skin symptoms, tachycardia, and abdominal cramps in patients with SM (262, 263). Doses can be adjusted individually, and can be used up to 4 times higher doses of recommended doses similar to patients with chronic urticaria. In the same manner, H2-blockers can be added to relieve gastrointestinal (GI) symptoms (264, 265). If GI symptoms are persistent, oral cromolyn sodium can be useful to add (266-268).

Leukotriene antagonists can be beneficial in therapy refractory cases, as has been shown for a positive impact on wheezing, GI and skin symptoms in patients with pediatric mastocytosis (269-271). Contrary to what is thought by many, non-steroidal anti-inflammatory drug (NSAID) aspirin can lead to symptom improvement, especially for refractory flushing, by inhibiting PGD_2 production by MCs in some patients (135, 272). Higher doses, up to 650 mg twice daily, may be required to achieve clinical improvement (273). However, a subset of patients with MCAD experience hypersensitivity reaction to NSAID (274), therefore, a drug challenge with aspirin should be performed prior to recommendation.

In addition, some other drugs were reported to be effective in a subset of therapy-refractory MCAD patients. Ketotifen, an H1 antihistamine combined with MC stabilizing features (275), can also be used, particularly against skin symptoms (276, 277). Rupatadine, another recent drug, with H1 antihistamine and anti-platelet-activating properties may be tried (278). Systemic glucocorticoids may be considered in patients with severe symptoms (especially in the events of anaphylaxis), but should taper when patients achieve symptom control. Certain patients may need small doses of maintenance therapy.

Omalizumab, which is humanized monoclonal antibody that specifically binds to free human immunoglobulin E (IgE), has also been used with varying success in therapy refractory patients and reported to diminish in MC-mediator related symptoms and the frequency of anaphylactic episodes in anecdotal reports and case series (279-283). Omalizumab has also been shown beneficial in patients who receive venom-immunotherapy but have systemic reactions during updosing (284, 285). Nevertheless, there are presently no randomized, placebo-controlled studies to recommend omalizumab in routine use. Another factor to remember is that omalizumab, as all the others, is not a curative therapy.

Some specific agents may be considered in patients with indolent SM and MCAS after careful risk/benefit analysis, although these drugs are currently indicated in advanced/aggressive variant of SM. In this manner, cytoreductive therapies, interferon-alpha and cladribine are being the most commonly used (286-289), might be beneficial for example in patients with recurrent life-threating unexplained anaphylaxis (290). In addition, treatment with tyrosine-kinase inhibitors (TKIs), a promising group of drugs, are also indicated in advanced forms of SM patients as they target KIT (291). As imatinib might be of value only in patients who are not associated with *KIT* D816V mutation, it is possible that some other TKIs might be used for patients with therapy-refractory anaphylaxis in the future. For instance, both midostaurin (292) and dasatinib (293) inhibit FcεRI-mediated activation at

lower concentrations. However, these therapies can have major toxicity and may affect the natural course of the disease.

Finally, the natural flavonoids quercitin and luteolin have been shown in-vitro to inhibit release of MC mediators histamine, tryptase, leukotrienes, prostaglandins (294), and cytokines IL-6 and TNF-α (295). Although promising, these drugs need to be validated in patients with MCAD.

A final remark regarding management strategies is that the necessity of continuously monitoring response to therapy and reevaluating symptoms. It is important to consider discontinuing all therapies, if suspicion arises regarding false diagnosis, for example because of lacking objective evidence for MC degranulation.

2.3.11 Conclusion

Mast cell activation disorders serve as a unique disease model that enables us to study mast cell activation processes in a clinical setting. Presently, the definite diagnosis of these disorders can only be done after a bone-marrow biopsy and histopathological evaluation. Thus, there is an obvious risk that the diagnosis is delayed or missed, and patients with MCAD are underdiagnosed. Consequently, there is an apparent need for new diagnostic/predictive markers. In addition, there is currently a limited number of palliative treatment, but no curative potential to cure MCAD. To add further complexities, mast cell mediators, such as histamine, tryptase, leukotrienes or prostaglandins are not always increased in patients with MCAD, and patients do not always respond to mediator blocking drugs, such as anti-histamines or anti-leukotrienes.

In conclusion, despite increasing recognition, understanding of its pathophysiology, the etiology of systemic mast cell activation disorders (MCAD) remains largely unknown. All these conditions clinically mimic each other despite having distinct mast cell perturbations. There appears to be different opinions among experts in the field, for instance regarding spectrum and classification of these disorders, in particular mast cell activation syndromes (MCAS). Although there is a diagnostic proposal for MCAS, this is not yet globally accepted, as these recommendations are based on principally two retrospective studies (141, 155) with limited cohorts.

3 THE PRESENT STUDY

3.1 AIMS

The ultimate aim of this doctoral study was to evaluate various diagnostic and predictive parameters for identifying patients with distinct phenotypes of mast cell activation disorders (MCAD), and to explore the presence of hyperreactive mast cells, what we believe to be the main perturbation in these patients. The main focus was to explore the complex interaction between anaphylaxis and mast cell activation disorders, in particular mastocytosis.

More specifically, the following objectives were investigated:

- What are the problems encountered in diagnosis of mastocytosis? What is the impact of mastocytosis on quality of life of patients? (Paper I).
- To assess whether bone marrow examination provides more accurate diagnosis in patients with venom-induced anaphylaxis and elevated baseline tryptase levels (Paper II).
- To characterize patients with systemic mastocytosis and to investigate the prevalence and features of mast cell mediator-induced symptoms, triggers causing anaphylaxis in patients with SM; and to explore the existence of preceding risk factors in development of anaphylaxis in SM patients (Paper III).
- To examine the generalizability of the hypothesis whether the pathogenesis of unexplained anaphylaxis (UEA) reflects the presence of yet undetected aberrant mast cell populations; and if so, to what extent, and to explore the presence of potential predictive marker(s) that can differentiate different UEA phenotypes (Paper IV).
- To determine whether mast cells of patients with mastocytosis and related mast cell activation disorders express altered reactivity in the skin and lower airways compared to controls, and to compare release of mast cell mediators in these groups (Paper V).

3.2 METHODOLOGY

3.2.1 Study population

In order to study these hypotheses, it is apparently needed to have a large study base and comprehensive knowledge on mast cell activation disorders. Keeping in mind that we deal with rare conditions, we established the Mastocytosis Center Karolinska (MCK) in 2006 at the Karolinska University Hospital and Karolinska Institute, as a part of European Competence Network on Mastocytosis (ECNM). This is currently the only center in Sweden; and thus receives referrals from the whole country. In this manner, by the end of 2013, we have investigated over 200 patients (Table 1) by bone marrow biopsy, analysis of *c-kit* mutation, complete allergy work-up, and measurement of baseline serum tryptase levels in accordance with the WHO-criteria. To date, over 150 of the investigated cases have exhibited signs of clonal mast cell disorders as illustrated in Table 1.

Table 1. Distribution of the investigated patients due to suspected mast cell disorders at the Mastocytosis Center Karolinska.

Mastocytosis Center Karolinska 2006 - 2013						
	Cumulative number of patients investigated with BM-biopsy	Investigated patients presenting with **Anaphylaxis**	Patients with **SM**	Patients with **MMAS**	Patients with **CM** without SM	**SM patients** presenting with **Anaphylaxis**
2013	206	95/206 (46%)	120	25	8	52/120 (43%)

BM: bone marrow; SM: systemic mastocytosis; MMAS: monoclonal mast cell activation syndrome; CM: cutaneous mastocytosis

The center has clinical responsibilities in how these patients should be taken care of in the best possible way, and also conducts different research projects. In the beginning of 2012, we also established the Karolinska Mastocytosis-registry where we compile various data from investigated patients in order to use in basic and clinical research studies to explore epidemiologic and clinical aspects of these disorders. Thus, there have been good possibilities to implement research in regard to this dissertation, as subjects enrolled to the particular studies were mainly recruited from this database.

3.2.2 Study subjects and study design

As mentioned, all subjects involved in the studies were recruited from our MCAD-cohort at Karolinska University Hospital. Certain patients were involved in more than one study. Except for the study 5 (Paper V), which was a prospective study, all data were collected retrospectively through review of electronic patient records.

In Paper I, we conducted a descriptive case study to demonstrate difficulties both in recognition and diagnosis of mastocytosis and also present disease from a patient perspective. The reasons for choosing this subject was partly because of this case illustrates the classical clinical features of mastocytosis, but also the patient's own efforts to manage his symptoms. The patient himself was deeply engaged to find an explanation to his "awkward" symptoms since his quality of life was deeply perturbed. In addition, there was also a prospective aspect, as we evaluated disease activity with and without maintenance therapy through follow-up visits after patient's initial contact with us. The patient underwent comprehensive allergy work-up to evaluate his unexplained reactions and also a bone-marrow biopsy was performed to diagnose underlying mastocytosis.

In Paper II, we also applied a descriptive study design and evaluated diagnostic aspects of hymenoptera venom-induced severe anaphylactic reactions (HVA) in three middle-aged females in order to compare classical allergy work-up with bone marrow examination and its impact on accurate diagnosis. The reason for focusing only on these three patients was not due to lack of other patients with HVA in our cohort, but was rather because of great similarities in these subjects. The three subjects with the same sex and similar age included were previously healthy and had experienced more than one episode of HVA and all had an elevated baseline levels of tryptase (>11.4 ng/ml). The patients underwent comprehensive allergy work-up including skin prick testing, measurements of specific IgE for bee- and wasp venom and component-resolved diagnostic. Bone-marrow biopsies were also performed in all three patients to assess underlying clonal mast cell disease.

In Paper III, a cross-sectional study was conducted to estimate the prevalence of systemic mastocytosis and to characterize these patients, in particular, regarding absence/presence of MC mediator-induced symptoms. The study subjects were selected from among the 142 consecutive patients who were referred to the MCK between January 2006 and December 2011 due to suspected mastocytosis (Figure 9).

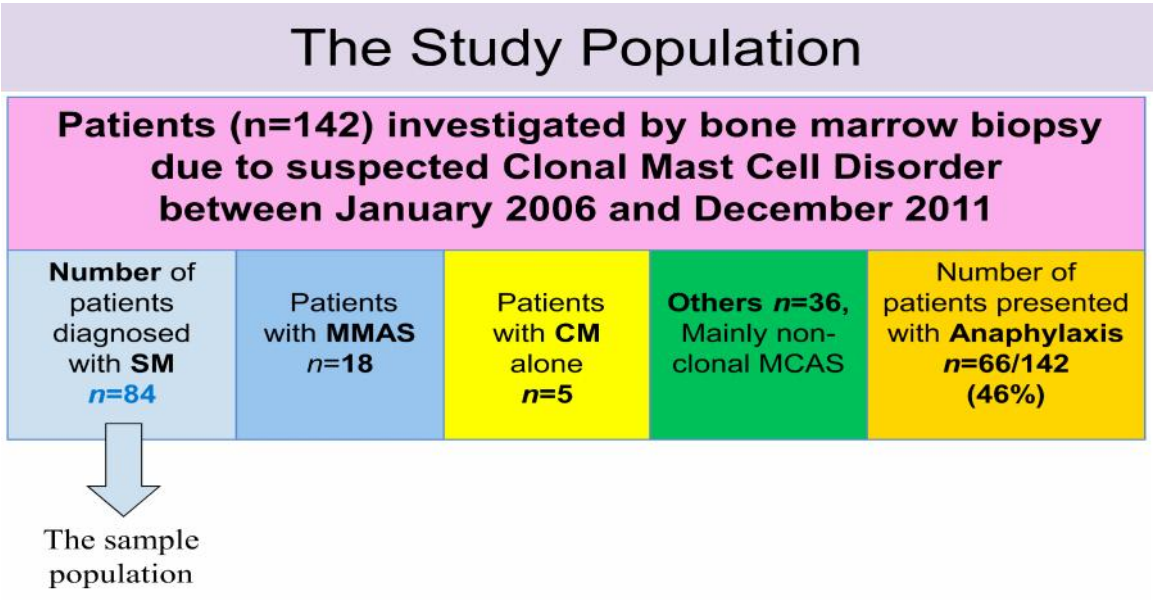

Figure 9. The study population and selection of the subjects into the study (Paper III).

All patients underwent medical evaluation including bone-marrow biopsy. We identified 84 consecutive SM patients (≥18 years) from our MCAD-cohort who were diagnosed using the WHO-criteria (125, 134), and these patients were enrolled in the study. In addition, 64 of 84 patients also underwent comprehensive allergy work-up including history taking (carried out by the allergist) and allergy tests (in 59 patients using skin prick test). The possible effect of non-specific triggers including heat, cold, exercise, stress, alcohol and histamine-containing food was evaluated. Further, the presence of signs and symptoms related to MC mediator release either isolated or constellated (representing anaphylaxis) was assessed. Anaphylactic reactions were diagnosed using NIH clinical criteria (52). In the remaining 20 patients, signs and symptoms were carefully assessed through their medical records.

In Paper IV, we conducted an observational study to examine the hypothesis that aberrant mast cell populations may underlie the pathogenesis of unexplained anaphylaxis (UEA). Of the 206 patients (≥18 years) investigated by December 31, 2013, 95 presented with anaphylaxis (Figure 10). Among these 95 patients, we have further identified 36 patients who were initially referred to allergy outpatient clinic at Karolinska University Hospital between February 2006 and November 2013 for one or more episodes of UEA. We excluded 6 of these 36 patients as they exhibited urticaria pigmentosa, the remaining 30 UEA patients were enrolled in the study.

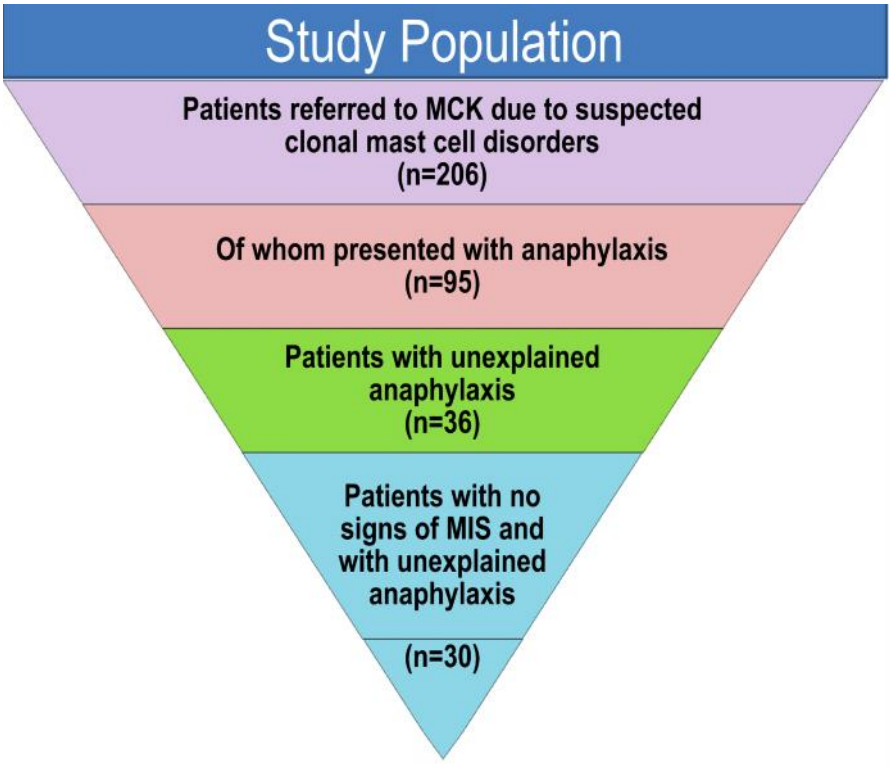

Figure 10. The study population and selection of the patients into the study (Paper IV). MCK: Mastocytosis Center Karolinska; MIS: mastocytosis in the skin.

Before being subjected to bone marrow examination, these 30 patients had initially undergone a comprehensive clinical work-up, including history, physical examination, laboratory tests and allergy work-up. In addition, we measured total serum IgE and baseline serum tryptase (sBT) levels in all patients. When no precipitating factors were detected and

other obvious causes eliminated, the exclusion diagnosis of UEA was made using NIH clinical criteria (52). Thereupon, bone marrow MCs of these patients were assessed to confirm/rule out underlying mast cell clonality according to current WHO-criteria (125, 134).

In addition, we also evaluated disease activity in the presence/absence of maintenance therapy with MC mediator-blockers through follow-up visits.

In paper V, a study was conducted to examine the hypothesis that mast cells of patients with MCAD present a hyperreactive phenotype. A total number of 52 consecutive subjects (≥18 years) were recruited from April 2010 to March 2013 from the outpatient clinic of Respiratory Medicine and Allergy at Karolinska University Hospital Huddinge and enrolled in this study. The subjects were categorized into two main groups: patients with suspected MCAD and control subjects (Figure 11).

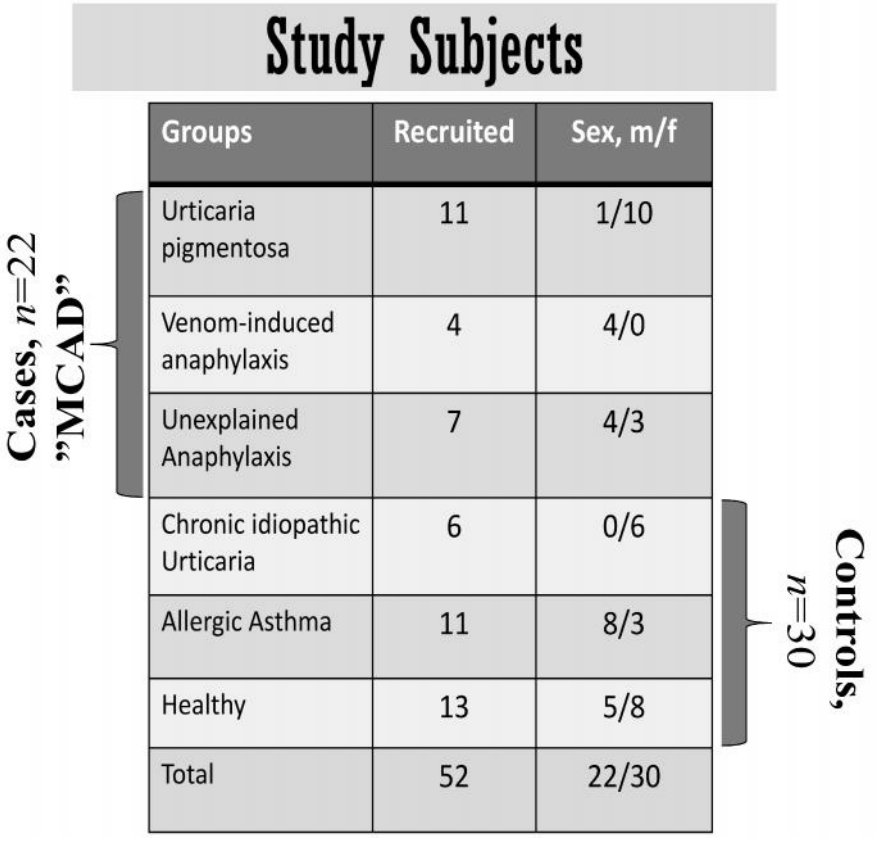

Figure 11. Distribution of different subjects included in the study (Paper V).

The study included a screening visit for all subjects and two more clinical visits for suspected MCAD patients. The screening visit comprised a complete clinical evaluation including physical examination, detailed history taking followed by routine biochemistry and allergy work-up, measurements of baseline pulmonary function test and exhaled nitric oxide (FeNO). In addition, bronchial challenge test with methacholine was performed in patients with suspected MCAD and with asthma (if it was not historically performed) to assess bronchial reactivity. Furthermore, serum and urine samples were collected and stored for analyses of baseline serum tryptase, and urinary metabolites of histamine *tele*-methyl-imidazoleacetic

acid (*tele*-MIAA) and PGD_2 urinary metabolite $11\beta - PGF_{2\alpha}$. All subjects were clinically in a stable period of their condition during the visit.

At the second visit a bone marrow aspiration and biopsy was taken (only patients with suspected MCAD who have not been already investigated) and patients were assessed by histology, cytology, flow cytometry and *KIT* D816V mutation analysis. On visit three (only patients with MCAD), bronchial challenge test with mannitol was performed to assess mast cell reactivity *in-vivo*.

3.2.3 Ethical aspects

Ethical approvals for the studies included in this thesis were obtained from the Regional Ethical review board, Stockholm, Sweden (with the approval numbers 2011/1750/31-3, and 2009/959-31-4 and 2009/1422-32) and all subjects enrolled were informed about the studies and thereafter provided their written consent to participate.

3.2.4 Bone marrow examination

After a comprehensive medical evaluation, all patients (except control subjects in Paper V) underwent bone marrow examination with biopsy and aspirate in order to determine whether they have underlying systemic mast cell disorders. Mast cells in bone marrow samples were evaluated following previously established methods and criteria for morphology (238), histology and immunohistochemistry (238, 240), flow cytometry (101, 245) and mutational analysis (247).

Diagnoses of systemic mastocytosis and monoclonal mast cell activation syndromes were made using current WHO-criteria (125, 134), and were based on bone marrow investigations to confirm the presence/absence of major criterion and/or minor criteria. Those patients who did not have any clonal markers of mast-cell disease such as immunophenotypic or morphologic abnormalities and the presence of D816V *KIT* mutation, were considered to have non-clonal/idiopathic MCAS.

Blood samples for baseline serum tryptase were drawn either on the day of bone marrow examination or the nearest possible day, but never at a symptomatic time. Further investigations included computerized tomography of the thorax and abdomen and the measurement of bone density to determine subvariant of systemic mastocytosis.

3.2.5 Allergy work-up

Allergy work-up was an important tool in all studies in order to provide a substantial risk assessment. All subjects underwent a thorough allergological evaluation (except 20 subjects in Paper III) at the Department of Respiratory Medicine and Allergy of Karolinska University Hospital Huddinge by an experienced allergist including investigation of their medical histories. The skin and/or in vitro allergy tests were performed, and when warranted, patients underwent drug or food challenges.

Skin prick test (SPT) was performed in Paper I-V. In Paper V, SPT was an aiding tool to recruit controls, but also utilised to assess skin reactivity to histamine and morphine. For screening purpose, we used commercial extracts (ALK Allergologisk Laboratorium A/S, Horsholm, Denmark) of standard aeroallergens (including birch, timothy grass and mugwort pollens, cat, dog and horse dander, house dust mites, moulds) and food allergens (milk, egg, nuts, cereals, fish, shrimp), and hymenoptera venom. All subjects had abstained from taking antihistamines at least 72 hours before skin testing. Controls used were histamine dihydrochloride 10mg/ml and saline (NaCl 0.9%). In accordance with the EAACI guidelines (296, 297) a skin test panel was considered positive if the wheal diameter was at least 3 mm larger than that elicited by the saline control. The wheal size was measured after 15 minutes and recorded as the mean diameter (adding the longest diameter to the orthogonal diameter and dividing it by 2).

The specific IgE antibody test (ImmunoCAP Phadiatop®, ThermoFisher, Uppsala, Sweden) was also applied as a complementary tool, and additionally, component-resolved diagnostic of serum specific IgE antibodies with purified and recombinant species-specific allergens (ThermoFisher, Uppsala, Sweden) was utilised, particularly of hymenoptera venom, when appropriate. Those tests were considered positive for values >0.35 kU/L. Moreover, total serum IgE was controlled in all subjects in Paper IV-V.

In Paper II, we have also performed basophil allergen threshold sensitivity assay (298), CD-sens, in a patient who were negatively tested for hymenoptera venom with conventional allergy diagnostic. In the very same patient, **intracutaneous test** (ICT) (299) was performed by using commercial honey bee and vespula extracts (ALK-Abelló A/S, Horsholm, Denmark).

3.2.6 Definitions of terms and diagnostic criteria

Atopic status was defined as one with at least one positive reaction to SPT and/or CAP against aeroallergens. Atopic subjects who had history of rhinitis or conjunctivitis (i.e., rhinorrhea, sneezing, congestion of the nose, red/itchy eyes) and/or had attacks of dyspnea or wheezing when they came into contact with a particular allergen were considered as having an **atopic disease**. Subjects were fulfilled asthma diagnosis according to GINA criteria, i.e., typical clinical symptoms accompanied with documented airway reversibility (>12% and 200 ml) or had increased airway reactivity to a bronchial challenge test.

Anaphylactic reactions were diagnosed according to the NIH clinical criteria (52), when either reduced blood pressure or associated-symptoms like syncope/pre-syncope; and/or respiratory compromise or a laryngeal oedema were present accompanied by the involvement of the skin-mucosal tissue or gastrointestinal symptoms. In cases, where assessments were difficult as a result of insufficient documentation, only patients who had syncope episodes after exposure to a likely or known trigger, with or without other accompanying symptoms, were assessed as having an anaphylactic reaction. The conditions such as hereditary angioedema, carcinoid tumors and pheochromocytoma were considered in differential diagnosis and were ruled out with appropriate investigations. Furthermore, patients with

recurrent, unexplained anaphylaxis underwent an extensive work-up to exclude other causes of anaphylaxis before diagnosed as idiopathic anaphylaxis.

Chronic idiopathic (spontaneous) urticaria (CIU) was diagnosed according to the current guidelines (300).

3.2.7 Measurement of pulmonary function

Measuring pulmonary function, using a spirometer, has been a key part of allergological evaluation that we routinely use in all investigated patients in our clinic. The test provides objective, reproducible and reliable information depending upon height, age, gender and ethnic originity of the subjects. In Paper V, a dynamic spirometry was performed (Jaeger MasterScope, software version 5.31; Intramedic AB, Sollentuna, Sweden) to screen and assess baseline pulmonary function in subjects in accordance with published recommendations (301, 302), and using the reference values reported by Zapletal (303**).** The baseline defined as the best of 3 recordings.

3.2.8 Measurement of exhaled nitric oxide

In Paper V, using a chemiluminescence analyser (NIOX, Aerocrine AB, Solna, Sweden), the fraction of exhaled nitric oxide (FENO) was measured prior to spirometry according to the guidelines of American and European Thoracic Societies (304). The subjects inhaled to their total lung capacity and immediately exhale at a constant flow rate of 50 mL/s until a 3-second NO plateau was reached. The cut-off value for a positive response was defined as ≥20 part per billion (ppb).

3.2.9 Methacholine provocation test

In Paper V, using a dosimeter-controlled jet nebulizer (Spira Electro 2; Respiratory Care Center, Hämeenlinna, Finland), methacholine (MCh) inhalation challenges were performed in subjects with mastocytosis and other related MCAD as previously described (305). Provided inhalation of saline (diluent) did not produce a change in FEV_1 by more than 10%, doubling doses of methacholine was administered every third minute (14.2 – 7256 μg). The results were positive if the provocative dose of methacholine causing a 20% fall in FEV1 (PD_{20}) was ≤894 μg.

3.2.10 Mannitol provocation test

In Paper V, mannitol (Aridol, Pharmaxis, Frenchs forest, NSW, Australia) capsules were inhaled using a dry powder inhaler (Plastiape, Osnago, Italy) according to the manufacturers protocol. The challenge was initiated with an empty capsule and FEV_1 measured in duplicates 60s later. If the change in FEV1 was <10% from baseline value, incremental doses of mannitol (5 mg – 160) were inhaled until FEV_1, measured at 60s after each dose, dropped by more than 15% from baseline A positive response was defined as the provocative dose causing 15% fall in FEV1 (PD_{15}) being 635 mg or less (306).

3.2.11 Measurement of mast cell mediators

Baseline serum tryptase levels (normal reference values 1 to 11.4 ng/ml) were measured routinely in all patients in Paper I-IV according to the ImmunoCAP tryptase assay (ThermoFisher Scientific, Uppsala, Sweden). Additionally, in Paper V, subjects provided both blood and urine samples at the same occasion (during an asymptomatic episode). Furthermore, an additional urine sample was collected 1 to 2 hours after the preceding one. The samples were stored at −70°C until analysed. Serum tryptase levels were measured as mentioned above. The major urinary histamine metabolite *tele*-MIAA was measured by LC/MS (307) and values were expressed as micromoles per milimole creatinine. The early prostaglandin D_2 metabolite, 11β-$PGF_{2\alpha}$, was measured using a commercial enzyme immunoassay kit (11β-Prostaglandin $F_{2\alpha}$ EIA Kit, Cayman Chemical Co., Inc., Ann Arbor, MI). Absolute values were expressed as micromoles per milimole creatinine. Urinary creatinine concentrations were measured by automated colorimetric Jaffe method.

3.2.12 Basophil histamine release assay

The histamine release test (HRT) is performed in microtiter plates coated with glass fibers providing a solid phase for histamine binding (308-310). The glass fibers bind histamine with high affinity and selectivity.

In Paper V, we performed HRT in accordance with the manufacturer's instructions (RefLab Aps, Copenhagen, Denmark). Accordingly, five ml of heparinized blood were collected from subjects to determine histamine released from whole blood cells, presumable basophils. The blood was washed with PIPES buffer and incubated on the assay plate for 60 minutes at 37°C with various concentration of anti-human IgE. Histamine is determined by the fluorometric o-Phthal-di-aldehyde method. The net histamine release was calculated by subtracting the value of the negative control (308).

3.2.13 Determination of plasma cytokines SCF, IL-31 and IL-33

In Paper V, plasma levels of SCF, interleukins IL-31 and IL-33 were determined by commercially available magnetic beads-based assays (Bio-Plex-Pro Assays, Bio-Rad Laboratories, Inc.) as described by the manufacturer. The assay sensitivity, defined as limit of detection, was 0.1 pg/ml, 0.49 pg/ml and 0.58 pg/ml for SCF, IL-31 and IL-33, respectively.

3.2.14 Statistical methods (Paper III-V)

The statistical analyses were performed using the statistical software SPSS version 20.0 (Paper III) and version 21.0 (Paper IV) for Windows (SPSS Inc., Chicago, IL, USA), and GraphPad Prism version 5 (GraphPad Software Inc., San Diego, CA, USA) in Paper V.

In general, a *P*-value of <0.05 was considered to be statistically significant. Categorical variables were presented as numbers and percentages and were compared with the chi-square test or Fisher’s exact test, when appropriate. Continuous variables are presented as median values and ranges, and because the distribution of this data was not normal, group differences were analyzed using the Mann-Whitney *U*-test.

Spearman's rank correlation coefficients and linear regression were used to assess correlation between sBT and serum total IgE levels in Paper IV, and to assess interrelations between sBT, *tele*-MIAA and 11β-$PGF_{2\alpha}$ values in Paper V**.**

In addition, in Paper IV, diagnostic tests sensitivity, specificity, as well as positive and negative predictive values was used for comparison of four different pre-screening tools before making a decision for further investigation with bone-marrow examination.

In Paper V, Methacholine and mannitol responses were log-transformed and were presented as geometric means, since the dose-response curves to these agents are exponential and need to be normalized before statistical analyses. Principal component analysis (PCA) was carried out using SIMCA-P+11 (Umetrics AB, Umeå, Sweden) to interpret the trends between three different mediators in the context of different diagnosis.

3.3 RESULTS

"The long and winding road to diagnosis"

In Paper I, the case presented pedagogically illustrated the many faces of mastocytosis both from patients' and doctors' perspective. We gave a descriptive picture of the patient's signs and symptoms that continued almost 2 decades and despite that he had consulted several doctors and underwent extensive medical assessment, no one could provide him an accurate diagnosis. The symptoms occurred with no known trigger with varying severity; thereby patient's quality of life had been tremendously perturbed.

From the clinical perspective, the differential diagnosis of spells is challenging as these short, frequent and stereotyped symptoms can be observed in various clinical conditions, including endocrinologic, psychological and neurological diseases. This case apparently illustrated that there is a lack of recognition of mastocytosis symptoms among physician due to its protean and paroxysmal nature. Symptoms in mastocytosis were typically periodic rather than continuous, and attacks usually lasted for a short time and varied from mild to severe in intensity some of them represent in full blown anaphylactic reactions. From the onset in 1988, this patient experienced 97 such attacks. We confirmed through elevated baseline levels of serum tryptase (160 ng/ml) that these symptoms were associated with the release of mast cell mediators. This was the turning point of making a decision for further examination with bone marrow biopsy as the patient did not exhibit signs of mastocytosis in the skin.

After evaluation of his bone marrow (BM) MCs by pathology, flow cytometry and detection of *KIT* D816V mutation, the patient fulfilled both major and all minor criteria and received the diagnosis of SM using WHO-criteria. We could additionally confirm that this patient had elevated levels of urinary PGD_2 metabolite 11β-$PGF_{2\alpha}$ and leukotriene E_4.

Another important aspect of this case was that we had some evidence of the beneficial effect of maintenance therapy. At first, the reaction frequency and intensity was decreased as the patient regularly used H_1- and H_2 antihistamine combined with leukotriene-receptor blocker since June 2009. Later, he responded even better since he did not experience the recurrence of reactions during the last 3½ years.

"The wasp ladies"

In Paper II, we illustrated three puzzling cases with hymenoptera venom-induced anaphylaxis (HVA). Interestingly all three patients had sBT levels > 11.4 ng/ml, and one of the patients also had negative venom tests. Notably, none of the patients presented with signs of mastocytosis in the skin.

Both SPT and specific IgE antibody test by ImmunoCAP were positive in Case 1 and 2 confirming an IgE-mediated HVA. By contrast, in Case 3, despite clear temporality, allergy diagnostic with SPT, ImmunoCAP, component-resolved analysis of hymenoptera venom r Ves v1, r Ves v5 and r Api m1 IgE (<0.10 kE/L), and also basophil activation test CD-sens for hymenoptera venom were all negative (<0.10 kE/L). However, further investigation

with intracutaneous test (ICT) gave a positive reaction for wasp venom at concentration of 10^{-4} µg/ml, thereby confirming an IgE-mediated HVA.

We analysed characteristics of BM MCs by morphology, histology, immunohistochemistry, flow cytometry and detection of *KIT* D816V mutation and evaluated underlying clonal mast cell disease (CMD) by using established WHO-criteria (125, 134). Three distinct diagnostic outcomes were associated with the HVA. The monoclonal mast cell activation syndrome (MMAS) diagnosis was made in Case 1 since only 2 minor criteria was fulfilled (aberrant phenotype and KIT D816V mutation); whereas Case 2 received diagnosis of SM by fulfillment of 3 minor criteria (atypical mast cell morphology, aberrant phenotype and *KIT* D816V mutation). However, there were no signs of mast cell clonality in Case 3 (Table 2).

Table 2. Demographic and clinical characteristics of cases and final diagnosis.

CASE SERIES							
Cases	**Age**	**Gender**	**Elicitor**	**Prominent reaction-pattern**	**sBT ng/mL**	**Standard Diagnosis**	**Diagnosis after BM-biopsi**
Case 1	63	Female	Wasp	Syncope	12	HVA	MMAS
Case 2	60	Female	Wasp	Syncope	14	HVA	SM
Case 3	67	Female	Wasp	Syncope	23	HVA	Non-clonal HVA

sBT: serum baseline tryptase; HVA: hymenoptera venom anaphylaxis; MMAS: monoclonal mast cell activation disorder; SM: systemic mastocytosis.

"A quasi-experimental disease"

In Paper 3, we characterized our relative large cohort of 84 SM patients. The median age of patients was 56 years (range, 21-78) and regarding gender patients were equally distributed.

We evaluated characteristics of BM MCs by using established WHO-criteria (125, 134), and detected mast cell aggregates in 60 (71%) of patients, i.e., major criterion, and in the remaining 24 patients SM diagnosis was based on at least 3 minor criteria. An atypical morphology comprising >25% of MCs was detected in 81 SM patients (96%). The *KIT* D816V mutation was analysed in 59 patients and detected in 53 (90%). In addition, all 84 patients expressed aberrant marker CD25, whereas 88% of patients expressed both CD2 and CD25. The median sBT levels were 52 ng/ml (range, 4.3-710) and 88% of the patients had a sBT level > 20 ng/ml. Furthermore, patients were classified into different SM variants and/or

subvariants according to WHO-criteria. Seventy-six of the SM patients (91%) had indolent variant of SM (including one with smouldering SM). Of the remaining patients, six had SM-AHNMD (7%) and two had ASM variant of SM. Among 84 SM patients, 59 exhibited with CM (70%).

The overall rate of atopy among the 64 SM patients that underwent allergy work-up was 30% (19/64), which was similar to sensitization rate of 39% in adult subjects in the general population in Sweden (311). The most common allergens in SPT were found to be pollens (67%), followed by animal dander (47%). Furthermore, the presence of an atopic disease was also determined in patients with SM (28%), including asthma (8%), rhinoconjunctivitis (17%) and concomitant presence of asthma and rhinoconjunctivitis (3%). The overall prevalence of rhinoconjunctivitis and asthma was comparable to the general Swedish population (312, 313).

We have also evaluated presence of mast cell mediator-induced symptoms and found that 90% of SM patients suffered from either isolated or constellated such symptoms. In overall, symptoms related to gastrointestinal (GI) system including nausea, diarrhea and/or abdominal cramps were dominating (63%), followed by dermatological signs and symptoms such as pruritus (53%) and flushing episodes (48%). About one third (31%) of the patients in this cohort experienced cardiovascular syncope.

In overall, 36 patients (43%) had had at least one episode of an anaphylactic reaction in all times, that is, not only during the time of data collection (2006-2011). In addition, we identified 77 episodes of anaphylaxis in these 36 patients, of whom 14 experienced 55 episodes. The clinical courses of the anaphylactic reactions were usually severe and included dominantly cardiovascular symptoms, mainly syncope attacks (72%). Of 36 anaphylaxis patients, 53% (19/36) reacted after hymenoptera venom stings, thereby giving an overall prevalence of 23% (19/84) in the whole cohort. Interestingly, in 39%) (14/36) of the anaphylaxis patients, a clear aetiology could not be determined.

Anaphylactic reactions occurred more frequently in patients without cutaneous engagement, $P = 0.02$ (44% vs. 19%). The rate of atopic sensitization and presence of atopic diseases was significantly higher in SM patients with anaphylaxis as compared with SM patients without anaphylaxis, 42% vs. 16% ($P < 0.03$) and 33% vs. 10% ($P = 0.033$), respectively. In regard to sBT levels, we found that levels were lower in SM patients with anaphylaxis reaching a marginal significance ($P = 0.042$). While males and females were equally frequent among the overall SM patients, anaphylaxis patients with SM showed a male predominance (61%), although that did not reach statistical significance.

“The harder you look the more you find”

In Paper IV, we comprehensively analysed a cohort of 30 patients with unexplained anaphylaxis (UEA). The median age was 53 years (range, 25-75), and gender distributions were 47% men and 53% women. The overall median sBT levels were 14 ng/ml (range, 1-160). The overall atopy rate was 43%, and most UEA patients exhibited multi sensitizations

as pollens being the most common allergen. Furthermore, atopic diseases were identified in 30%. The median level of total IgE was 46.5 kU/L (4.4-1600).

As previously described, the characteristics of BM MCs were evaluated by using WHO-criteria (125, 134). Ten (33%) of the patients received diagnosis of SM, and four of these patients fulfilled the major criterion as they had mast cell aggregates, while the remaining six patients had at least three minor criteria thereby obtaining the diagnosis of SM. The *KIT* D816V mutation was detected among the four of six analysed SM patients. Regarding aberrant MC markers, all 10 SM patients had CD25 expression, and additionally, eight of ten SM patients expressed CD2. All SM patients had sBT levels >11.4 ng/ml. Furthermore, four UEA patients obtained the diagnosis of MMAS as they expressed aberrant CD25 MCs. None of these patients carried *KIT* D816V mutation or the other minor mastocytosis criteria. Two of MMAS patients had elevated sBT levels. In overall, 14 (47%) of investigated UEA patients were diagnosed with clonal mast cell disorders (CMD). In the remaining 16 UEA patients, we were not able to detect any sign of clonality in BM MCs supporting clonal mast cell disease, although four of those exhibited elevated sBT levels (>11.4 ng/ml). Thus, we classified them as idiopathic anaphylaxis (IA).

The most common clinical manifestations in episodes were related to cardiovascular system, such as syncope, presenting in more than 90% of patients. This was followed by GI symptoms and cutaneous symptoms including flushing, urticaria and angioedema. In overall, the subjects experienced 148 episodes of UEA, and 119 of these episodes occurred before they visited us. Twenty-three patients were referred to us after they had experienced ≥3 episodes of UEA, whereas the remaining seven patients all had elevated sBT and had experienced either single or two episodes.

This study also had a prospective follow-up aspect with a median follow-up period of 31 months (range, 2-94). During the follow-up, seven patients had experienced a total of 29 *de novo* episodes. Although all patients were instructed, only 71% of them used intramuscular adrenaline upon commencing of acute symptoms. With respect to maintenance therapy, 25 patients received mediator-blockers, such as anti-histamines (mainly H_1-blockers), on a regular base. Seven patients also received anti-leukotrienes and 4 of these 7 received triple combination therapy with H_1- and H_2-histamine blockers and anti-leukotrienes. In addition, three of our cases were treated with the anti-IgE, omalizumab, injections starting with the standard dose of 300 mg, given twice monthly because severe nature of their reactions. At the time of last follow-up, in overall 70% of UEA patients were in remission.

When we compared CMD patients with IA patients, we observed differences in both clinical and laboratory findings between the two groups. In regard to clinical manifestations, urticaria/angioedema was significantly more common in patients with IA (P=0.001), whereas flushing occurred more frequently in the CMD group (P <0.04). By contrast, there were no significant differences between groups concerning cardiovascular, GI or respiratory symptoms. However, in regard to remission rates, we observed significantly higher remission rate in patients with CMD (P < 0.02).

Serum baseline tryptase levels were significantly higher in patients with CMD compared to patients with IA *(P*< 0.001, whereas total IgE levels were significantly higher in the IA group *(P* < 0.03), resulting in an inverse correlation (*r* = - 0.44; *P* = 0.016). Otherwise, there were no significant differences between the groups in regard to age, sex, presence/absence of atopic sensitization and atopic disease, or number of anaphylactic episodes.

"The silence of the cells"

In Paper V, we evaluated 22 patients with suspected systemic mast cell disorders and 30 controls to test our hypothesis that patients with mast cell activation disorders display signs of increased mast cell reactivity.

The characteristics of BM MCs were evaluated in 22 patients with suspected MCAD using current WHO diagnostic-criteria; thereby 15 patients were diagnosed with systemic mastocytosis (SM) (eleven patients with UP, two patients with recurrent, unexplained anaphylaxis [UEA] and two patients with severe hymenoptera venom anaphylaxis [HVA]). Among the remaining patients, four obtained the diagnosis monoclonal mast cell activation syndrome (MMAS) and three had diagnosis of idiopathic anaphylaxis. With regard to 11 UP patients, seven never experienced anaphylaxis, whereas two of remaining patients had HVA, one had UEA and the fourth one reacted to shrimp. Among SM patients, 60% (9/15) had mast cell aggregates in BM and the *KIT* D816V mutation detected in 14 SM patients (in one patient not analyzed). All SM patients in this study had indolent variant of the disease.

Patients and control groups were comparable, with the exception that the age of patients with SM was higher compared to healthy controls. Baseline levels of all three MC mediators, tryptase and urinary metabolites of histamine and PGD_2, were significantly higher in SM patients compared to other subjects with MCAD and compared to controls. In addition, when we correlated mediators levels in SM patients, we found that serum tryptase levels were significantly correlated both with urinary *tele*-MIAA (r_s = 0.62, *p* = 0.013) and with urinary 11β-$PGF_{2\alpha}$ (r_s = 0.53, *p* = 0.042). There were no significant correlation between urinary *tele*-MIAA and 11β-$PGF_{2\alpha}$ (r_s = 0.26, *p* = 0.36) levels.

There were no difference between the groups in skin reactivity to histamine or morphine, airway reactivity to methacholine and mannitol, basophil histamine release tests or the serum levels of SCF and IL-31/33.

3.4 GENERAL DISCUSSION

When we started the Mastocytosis Center Karolinska in 2006, epidemiological data was sparse and there were no specific estimates on the number of existing potential patients with mast cell disorders in Sweden. We were though aware of that these conditions were rare, however, we had no idea to what extent these disorders might cause problem in patients. Therefore, the basic aim of the initial studies (in Paper I-IV) was to characterize different features of patients with MCAD by providing demographic and epidemiological data, in particular prevalence of these disorders, evaluating laboratory abnormalities and clinical signs and symptoms in regard to differential diagnosis. In addition, these clinical studies would be useful in generating hypotheses. Paper V, on the other hand, was the first analytic study where we sought to explore the presence of hyperreactive mast cells. In the following, I will give more insight to the individual papers to start with an epidemiological background.

3.4.1 An epidemiological approach

When planning a study, the important question to ask is whether the aim of the study to describe or to compare and whether it will be observational or an intervention planned. Another aspect to consider is to choose a study design that provides strong level of evidence as the traditional evidence-based medicine classifies different types of studies on the basis of research design as the criterion for hierarchical rankings (314). Randomized controlled trails (RCT) or systematic reviews and meta-analysis of RCTs are at the top of the pyramid, while anecdotal evidence is at the bottom (315) (Figure 12).

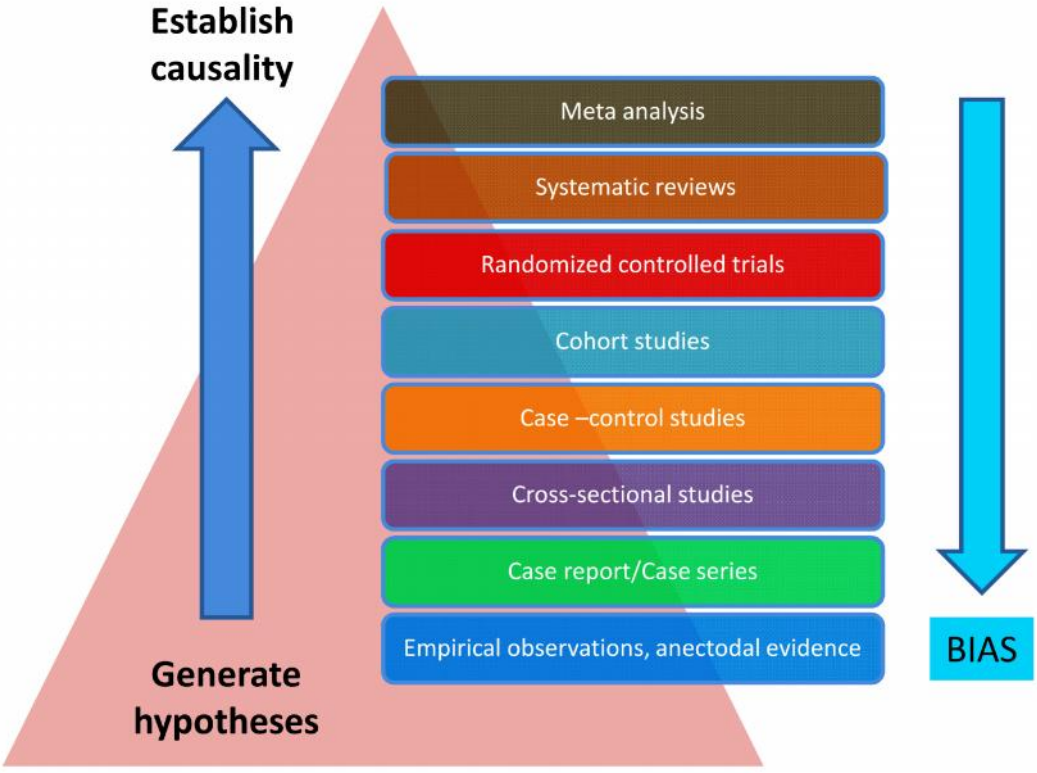

Figure 12. Hierarchy of evidence pyramid. Adapted from references (316, 317).

However, the rules of evidence vary with circumstances (318). There are other factors to consider and the study design should not only reflect the level of evidence. The choice of study design is largely influenced by specific features including logistics of time and available resources to conduct research, results from previous studies and gaps in the knowledge to be filled. We also have to make sure that the study design is appropriate for what we would like to study, and the "rareness" issue here has prominent impacts on various epidemiological aspects, including limitations of choosing suitable study designs.

In the following, I will summarize some important aspects of different study designs; however, to supply a comprehensive overview on the broad characteristics of different varieties of study designs is beyond the scope of this chapter.

Thus, scientists generally apply a sequential use of different research designs in studying a special topic. If the knowledge is limited about a subject, a general sequence of research designs will be proceeding from descriptive to analytical design to build up reliable information (Figure 13).

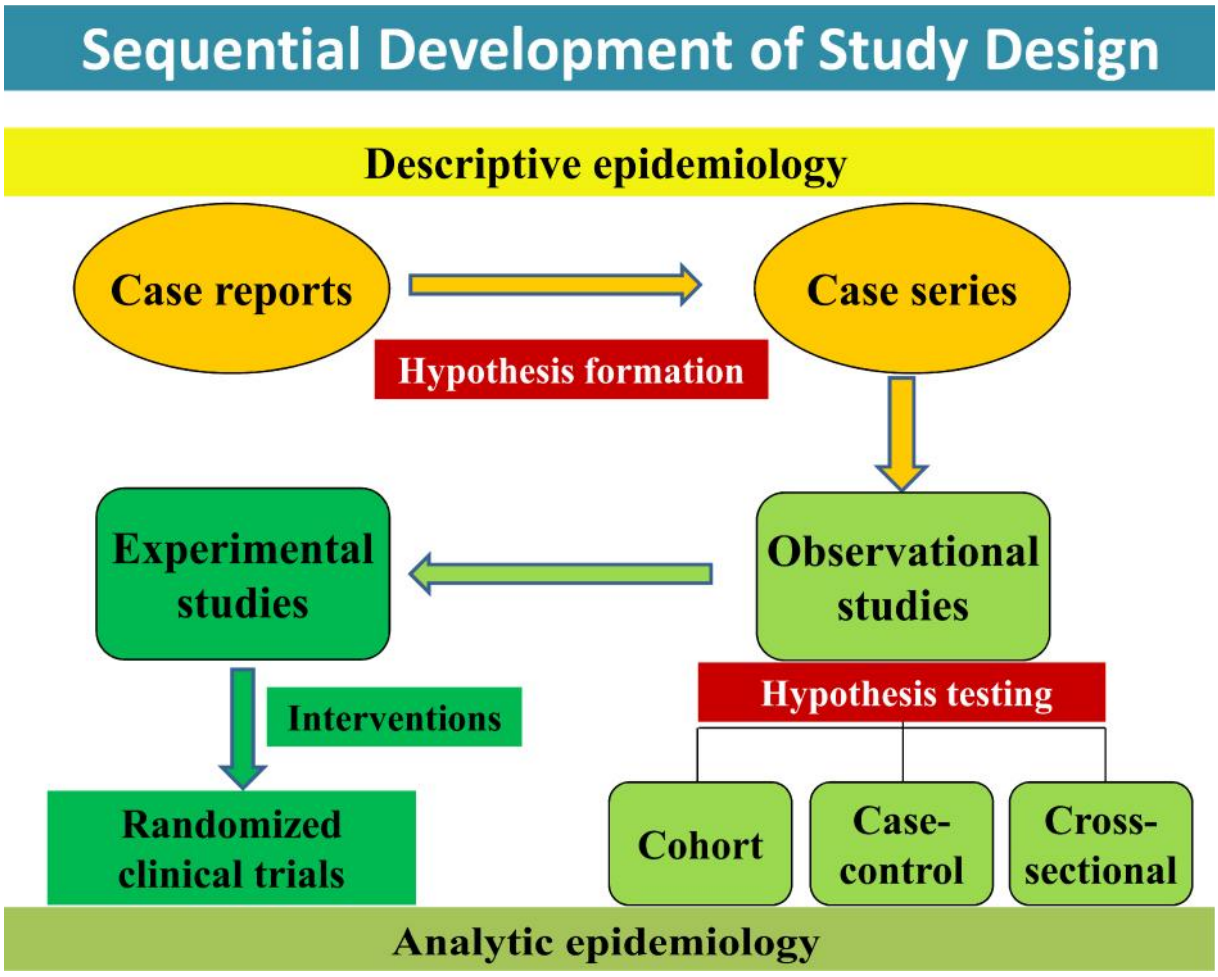

Figure 13. Sequential development of different study designs in context of knowledge.

Descriptive studies document facts of clinical or theoretical significance and describe patterns of disease occurrence in a group of subjects. There is no testing of a causal

hypothesis or any comparisons with other groups; however, descriptive studies can help to generate/support a hypothesis by demonstrating a phenomenon that the hypothesis state (319, 320). The simplest forms of descriptive studies are Case Reports and Case Series. This was the approach we used in Paper I-II.

The distinguishing feature between **observational** and **experimental** studies is that in the case of the former the intervention has not been applied by the researcher. In experimental studies, which are a form of prospective cohort, interventions are deliberately applied, and other circumstances altered in a way as to clarify the impact of intervention. In addition, there are **quasi-experimental** studies, which may be described as own experiments of the nature. For instance, mastocytosis patients that experience recurrent, unprovoked anaphylaxis would provide us an "experimental design" without provocation.

Cross-sectional studies form a bridge between simple descriptive studies like case reports and case series and those that can be used to test hypotheses. Most published cross-sectional studies describe the prevalence of a condition in a population (321). They can suggest the presence of relationships but it is not possible to demonstrate a temporal sequence in terms of causality. The major drawback, which limits cross-sectional studies, is if subjects are of different ages or in different stages of advancement of a disease false conclusions may be derived.

Comparative studies draw contrasts between two or more groups with the intent that the comparison will test a particular hypothesis (320). They document processes without the researcher's intervention. Cohort and Case-control studies are the two basic types of observational analytic studies. In theory, it is possible to test a hypothesis using either design; however, each design offers certain unique advantages and disadvantages. Cohort studies are usually seen as the strongest type of observational study; nevertheless, they are not the design of choice for studying rare diseases. Case-control studies are especially useful for outcomes that are rare or that take long time to develop (320). These studies require less time, effort and money compared to cohort studies. However, the problem with case-controls is selecting the appropriate controls. Furthermore, experimental studies include randomized control trials, which are considered to be the gold standard. They are suited best to assess treatment effects of novel drugs; however, it is impossible to conduct such studies in most clinical settings (321).

Consequently, each study design has its specific advantages and disadvantages, as a given research question may be addressed using different approaches. The decision to use a particular design is based on features of exposure and disease, current state of knowledge, and the logistics of time and resources. In reality, there is no clear-cut distinction between the descriptive and analytic studies; rather there is invariably an overlap. All studies involve some description, even those which are undertaken primarily to compare two groups of subjects. Similarly, most analytical studies provide descriptions of the attributes of the subjects. A relevant example of this issue is that the study designs were used in Paper III-IV.

3.4.2 Clinical perspective

In Paper I, a descriptive approach was an appropriate design of introducing the problems of mast cell mediator-induced symptoms, in particular presence of anaphylaxis in patients with mastocytosis. The main massage provided was how essential it is to check baseline levels of serum tryptase in patients presenting with recurrent flushing and hypotensive chock with no known triggers. It is proposed by consensus group that sBT levels of >20 mg/ml is an indication for bone marrow examination in similar patients without presence of other signs and symptoms of mastocytosis. Nevertheless, this recommendation does not look firm enough as there are patients with clonal mast cell disease that have a sBT levels between 11.4 – 20 ng/ml.

The study also provided information, although limited, about impact of regular mediator-blocking therapy, as the recurrence of reactions could be prevented by combination of H_1- and H_2 antihistamine with leukotriene-receptor antagonist in the case presented during the follow-up period of 3½ years. However, considering the natural course of disease that spontaneous remissions may occur, this has to be confirmed with controlled trials.

Paper II provides interesting aspects to discuss**.** Assessment of these challenging cases exemplifies the high stakes diagnostic encountered by the allergist and lend further support to the hypothesis of a clear-cut association between severe HVA and clonal mast cell diseases as previously reported (154). As illustrated here, investigation of bone marrow MCs may lead to changes in the final diagnosis compared to the assessments done by classical allergy work-up and measurements of serum baseline tryptase. Interestingly, none of the patients presented with urticaria pigmentosa (UP) like skin lesions. These patients presented with demographically (age and gender), clinically and somewhat serologically similar data, yet all three ended up with three distinct diagnoses including systemic mastocytosis, monoclonal mast cell activation syndrome and non-clonal HVA.

As diagnosis of anaphylaxis could be confirmed clinically in all cases by using NIH criteria (52) in the presence of a clear temporality, other differential diagnosis seemed unlikely in the clinical context. In addition, there were no alternative explanations of elevated sBT levels in any of the patients since none of them suffered from associated diseases, such as renal failure or hematological neoplasms. Unfortunately, tryptase levels were not determined during acute episodes in any of the cases. This is a well-known global phenomenon that emergency physicians do not consider tryptase analyses as an emergency test. The issue is something that allergy/immunology societies should act and handle; however, it is still matter of prioritization from emergency perspective.

The absence of venom sensitization by routine allergy diagnostic including SPT and/or ImmunoCap despite a convincing history has been a well-known phenomenon in mastocytosis patients with HVA (217, 322, 323). Interestingly, a recent study (324) reported that even performing a basophil activation test (BAT) did not provide any further support for wasp or bee sensitization as in the Case 3. Nevertheless, when we performed intracutaneous test (ICT) in this patient, we could finally prove a wasp sensitization.

However, ICT is not an easy test to interpret since false positive results may interfere. The relevant question in this context appears to be how far one should search evidence for an IgE-mediated etiology.

Personal reflection: The interesting and remaining question is whether there are patients with true negative venom tests, if so, what is the likely pathological mechanism(s) that would provoke an anaphylactic reaction?

During the last decade, allergists became more and more aware of increased sBT levels (>11.4 μg/L) and their potential impact of providing a greater risk to develop more severe anaphylactic reactions to hymenoptera stings compared to those with normal values (325, 326). In addition, elevated sBT levels are also thought to indicate an increased risk for the appearance of side-effects during venom immunotherapy (228). Fortunately, two cases treated with specific venom immunotherapies tolerated both updosing and maintenance stages without any side effects (follow-up of eight years and four years). Furthermore, the management of HVA patients with CMD involves unusual problems including severe, potentially fatal anaphylactic reactions after new stings, even if patients undergo venom specific immunotherapy (213, 223). Effectiveness of immunotherapy in HVA patients with CMD has been questioned through the years; however, this issue has been recently reevaluated (261, 327, 328). The immunotherapy provided protection from anaphylactic reactions in 86% of the patients who had been re-stung (328). The current guidelines, therefore, recommend life-long immunotherapy for patients with CMD, whereas 3–5 years venom immunotherapy induces long-term protection in patients with HVA alone. Thus, it is of immense importance to identify risk-prone patients by investigating a possible underlying CMD in patients with severe HVA.

Personal reflection: Yet an unanswered question here is why elevated sBT levels cause a severe anaphylaxis episode. We need a clear-cut answer whether elevated tryptase *per se* is an independent risk factor or it is because of their contribution to yet unrecognized CMD.

Without any doubt, measurement of sBT levels in patients with severe HVA provides a possibility to screen for patients with CMD; however, as demonstrated in this study, sBT *per se* is not a sufficient marker to distinguish different anaphylaxis phenotypes. Currently, the definite diagnosis in such patients can only be established after a bone marrow biopsy. Nevertheless, the eligibility criteria for bone marrow examination remain a subject of discussion. According to the current guidelines, a bone marrow biopsy in patients with HVA is recommended if sBT is higher than 20 ng/ml, or when signs and symptoms of mastocytosis, such as urticaria pigmentosa, are present (134). Yet, 34% (and 2 of our 3 cases) of the patients with HVA and CMD have a sBT lower than 20 μg/L (154). Therefore it has been proposed that the sBT cut-off should be lowered to 11.4 μg/L to assess mast cell clonality including histopathological evaluation of bone marrow (228, 329). However, this practice has not yet come under consideration as a standard routine. Interestingly, the Spanish Network on Mastocytosis, REMA, has recently proposed a scoring tool to prescreen HVA patients before considering possible further investigation with bone marrow examination (155). All three cases in this study scored enough to qualify bone marrow

examination supporting that REMA-score could be a beneficial pre-screening tool in similar cases. In addition, other approaches in the field being made, a sensitive assay for analyzing peripheral blood *KIT* D816V mutation has been proposed (236, 330, 331), however, this assay remains to be validated in these kind of patients.

In conclusion, the accurate diagnosis provides both therapeutic and preventive implications for management strategies in these patients. Hence, patients with cardiovascular collapse following an insect sting and have elevated sBT (>11.4 μg/L) should be strongly considered for bone-marrow investigation in order to obtain a better risk assessment regardless of other signs or symptoms of clonal mast cell disorders.

Paper III provides a comprehensive insight into patients with SM in regard to allergological aspects of the disease, in particular, prevalence and impact of anaphylaxis. The ultimate aim of this study was to develop a predictive model for a potential anaphylaxis phenotype in SM patients by using a deductive approach; nevertheless, the current study has only indicated certain risk factors in SM patients who develop anaphylaxis.

When this study was performed, there were only two systematic studies investigating an association between anaphylaxis and mastocytosis (221, 222). The reported prevalence of anaphylaxis in adult mastocytosis patients varied widely from 20% to 56% in the literature (218, 222). Discrepancies may result from a number of reasons, including heterogeneity of the patients in investigated cohorts, lack of a uniform definition of diagnostic criteria for anaphylaxis and varying recruitment strategies in different centers causing a selection bias. In addition, SM-investigation routines is unfortunately not standardized in different centers, for instance, allergy work-up is not routinely performed in all centers. In some studies, since not all patients were investigated by bone-marrow biopsy, diagnosis of mastocytosis and systemic mastocytosis was not differentiated thereby contributing a more heterogeneous cohort. In our study, all patients underwent BM-biopsy before receiving a diagnosis of SM (those who received diagnosis of CM alone were excluded) and patients were almost equally recruited by allergology, hematology and dermatology referrals.

In line with a previous study (155), we found hymenoptera stings as the main elicitor, accounting for 53%, as the wasp sting being the exclusive trigger. Remarkably, we could only confirm an actual venom sensitization in only 63% (12/19) of patients via traditional allergy diagnostic by SPT and/or ImmunoCAP or component-resolved diagnostic; although a clear temporal association was established in all cases. This phenomenon can be explained by different approaches. Firstly, this may refer loss of sensitivity - if reactions occurred more than a decade earlier (299). Secondly, these reactions may indeed occur by non-IgE-mediated mechanism(s) (299, 322). A third simple explanation could be due to lack of diagnostic accuracy, as discussed for Paper II, that is, further investigation by basophil activation test or intracutaneous test. However, most probably, all these reasons have been contributed to these controversial results.

Patients with unexplained triggers despite an extensive search, i.e., those with idiopathic anaphylaxis, were the second most common group. By contrast, elicitors such as drugs and

food seem not to be a major cause in this study, as only three patients showed likely reactions with these triggers. Although these elicitors were reported in the context (221, 222), reactions often remain patient-reported, since it is difficult to verify as a result of insufficient data, lack of reliable in-vitro tests and lack of provocation tests. Our results are consistent with those in another study that also demonstrated a weak association between food- or drug-induced anaphylaxis and mastocytosis (332). Table 3 supplies a comparative overview regarding clinical and demographical characteristics and presents somewhat differing elicitor patterns in different studies in the context of anaphylaxis and systemic mastocytosis.

Evaluation of non-specific triggers such as heat, cold, friction, stress, ingestion of histamine-containing food demonstrated that these triggers were only associated with the isolated organs, for example aggravation of the skin reactions, but never caused a systemic reaction themselves. Another interesting point to note was to observe that there were no elicitor switches in individual subjects since all patients maintained their elicitor profiles in consequent reactions by the time of this study.

Table 3. Comparison of different studies in the context of anaphylaxis in systemic mastocytosis

		Gülen et al. 2014, CEA	Brockow et al. 2008, Allergy (222)	Gonzales et al. 2007, CEA (221)
Number of patients with SM		84	61	155
Patients with anaphlaxis, n (%)		36 (43%)	34 (56%)	36 (23%)
Gender, male %		61%	n/a	72%
Reaction pattern (syncope%)		71%	53%	n/a
CM/UP involvement		71%	n/a	n/a
TRIGGER	Insect Venom	53%	27%	25%
	Idiopathic	39%	20%	42%
	Food+Drug	9% (3%+6%)	42% (24%+18%)	28% (3%+25%)

SM: systemic mastocytosis; CM: cutaneous mastocytosis; UP: urticaria pigmentosa

Consistent with another study (221), we confirmed the feasibility and safety of the skin prick test in patients with SM, since none of the tested patients showed any kind of adverse reactions. In accordance with earlier reports (221, 333), the overall prevalence of atopic sensitization (30%) in SM patients does not differ from the general population in Sweden. In addition, the overall prevalence of atopic diseases in SM patients was found to be 22%, which was also comparable to previous studies that observed a prevalence of atopic diseases in 21%

of urticaria pigmentosa patients (333), and 28% in mastocytosis patients (222). However, SM patients with anaphylaxis had significantly higher prevalence of atopic sensitization and atopic disease ($P< 0.03$ and $P= 0.033$, respectively) when compared SM patients without anaphylactic reactions. Thus, the atopic predisposition appears to be a risk factor to develop anaphylaxis in SM patients. Nevertheless, we did not measure total IgE levels in this study to strengthen this hypothesis.

Remarkably, we also observed that anaphylactic reactions were significantly more prevalence in SM patients without cutaneous engagement ($P<0.02$). In addition, the study provided a gender perspective as we had exactly equal number of males and females in our SM cohort. For instance, although it did not reach statistical significance, we observed a clear male predominance among anaphylaxis patients. Interestingly, the male predominance was obvious even in different elicitor profiles, such as patients with hymenoptera-induced anaphylaxis or unexplained anaphylaxis with SM.

There appears to be a complex relation between baseline tryptase levels, mast cell burden and the presence/absence of anaphylactic reactions in SM patients. Previously, there was speculation that higher mast cell burden may potentially lead to spontaneous mast cell degranulation causing anaphylactic reactions; thereby SM patients with anaphylaxis should have higher sBT levels. This notion was supported by findings in a previous report (222). In contrast, we detected significantly lower levels of serum baseline tryptase in SM patients with anaphylaxis (P=0.042). Interestingly, two recent studies (334, 335) with venom-induced anaphylaxis in SM patients supported our findings, as they did not show a correlation between mast cell burden and the presence/absence of anaphylaxis. Additionally, since in these studies bone marrow mast cell numbers were quite low in most cases, anaphylaxis in these patients thought to be related to pathological alterations in mast cell activation processes rather than mast cell numbers *per se*. Nevertheless, this issue still remains to be proven, i.e., whether these observations are only limited to SM patients with venom-induced anaphylaxis or if it also can be applied to overall anaphylaxis patients with SM.

From an epidemiological perspective, the primary strength of this study arises from the fact that we provided complete and detailed characteristics of patients with systemic mastocytosis and all patients were diagnosed using WHO-criteria. In addition, we measured a reliable estimate of the point prevalence of anaphylaxis in our study population. However, our cross-sectional approach was a limitation and therefore, the temporal relationship between SM and presence/absence of anaphylaxis could not be assessed. Furthermore, we were dependent on complete and accurate recording of relevant information in the medical records as a result of lack of an allergy work-up in 20 cases in the study, thereby providing a potential risk for information bias.

In conclusion, this study indicates an increased risk for developing anaphylactic reactions in patients with systemic mastocytosis. Therefore, considering the broad heterogeneity of the elicitor patterns and clinical symptoms, a comprehensive risk assessment based on allergy work-up should be mandatory for individual SM patients in order to provide a tailor-made

treatment and preventive strategies. At present, there is no global agreement on whether or not all SM patients should be equipped with pre-loaded adrenaline injectors for self-treatment, since some SM patients would most probably never experience an anaphylactic reaction. However, all anaphylaxis-prone patients must be equipped with adrenaline injectors after careful self-training. In addition, all IgE-sensitized hymenoptera venom-anaphylaxis patients should be given life-long venom specific immunotherapy. Moreover, patients who are refractory to mediator blockers and experience frequent, severe episodes of anaphylactic reactions should be considered for omalizumab treatment (279, 280).

Personal reflection: Is there a distinct SM-anaphylaxis phenotype?

Paper IV provides an inductive approach to anaphylaxis-mastocytosis relation, taking unexplained anaphylaxis as a starting point. The study aimed to identify characteristics of bone marrow mast cells in patients with unexplained anaphylaxis and to determine whether clonal mast disease might be the underlying cause in some patients with this condition. As investigating 30 UEA patients in depth, this study represents one of the most comprehensive data in the field of idiopathic anaphylaxis.

When we started to conduct this study, one of the most crucial question was (as still is) when to send a UEA patient for bone marrow examination, as there is no universally accepted consensus. Although the study was not prospectively designed, after patients obtained their final diagnosis with bone marrow examination, we retrospectively tested our original hypothesis of patients with either ≥ 3 UEA episodes regardless sBT levels or elevated sBT levels and at least 1 UEA episodes would predict an as-yet-unrevealed clonal mast cell disease. This study proved that the number of UEA episodes *per se* did not appear to be a reliable screening marker. Using this tool, we could only predict 1 in 7 UEA patients. By contrast, a screening tool that utilizes elevated sBT levels (≥ 11.4 ng/ml) seemed to be more reliable, demonstrating 86% sensitivity and 75% specificity in this study. We also applied an existing prescreening tool, the REMA-score (155, 336), and this method exhibited greater specificity (94%) but similar sensitivity compared to tool of sBT levels alone. As we observe through our daily clinical practice that there are certain anaphylaxis patients with critical sBT levels (11.4-15 ng/ml) and have indeed underlying clonal mast cell disorder, we applied a **modified REMA-score** by adjusting cut-off values for sBT. Therefore, the lower sBT levels, i.e., 11.4-20 ng/ml instead of 15-25 ng/ml as in used in the classic REMA-score were taken into consideration. The modified version of REMA-score gave the best sensitivity (93%) and specificity (94%) in our UEA cohort (Table 4). Nevertheless, the modified REMA-score needs to be validated in other cohorts. In addition, an alternative or complementary tool appeared recently. To analyze D816V *KIT* mutation in peripheral blood mononuclear cells seems to be a promising screening tool (236, 330, 331); however, the assay also remains to be evaluated in anaphylaxis patients. Besides, it is not currently available in all mastocytosis centers yet.

Another aspect that previous studies have reported was that UEA develop predominantly among atopic subjects (337, 338). In this study, the overall atopy rate was 43%; nevertheless,

it is difficult to discern whether this may support previous hypothesis as the prevalence of atopy varies from 27% (339) to 39% (311) among the adult Swedish population. Interestingly however, although patients with IA had significantly higher levels of total IgE compared to patients with CMD, the groups were not different in regard to prevalence of atopic sensitization or atopic diseases. As expected, sBT levels were significantly higher in patients with CMD, thereby total IgE levels and sBT levels were inversely correlated. We also observed an interesting gender aspect as patients with CMD were predominantly men, as in Paper 3.

Table 4. Comparison of different prescreening tools for the diagnosis of CMD and their sensitivity/specificity compared to diagnosis after BM-biopsy.

Predictivity indicators	Sensitivity (95% CI)	Specificity (95% CI)
Patients with normal sBT (<11.4 ng/ml) and with ≥ 3 episodes of UEA	14% (2.2%-42.8%)	25% (7.4%-52.4%)
Patients with elevated sBT (≥11.4 ng/ml) and with ≥ 1 episodes of UEA	86% (57.2%-97.8%)	75% (47.6%-92.6%)
*REMA-score (≥ 2) with a sBT cut-off 15-25 ng/ml	86% (57.2%-97.8%)	94% (69.7%-99.0%)
#Modified REMA-score (≥ 2) with a sBT cut-off 11.4-20 ng/ml	93% (66.1%-98.8%)	94% (69.7%-99.0%)

CMD: clonal mast cell disorders; sBT: serum baseline tryptase; UEA: unexplained anaphylaxis; CI: confidence interval.
*REMA-score (155) is the sum of positive and negative points as follows: male (+1), female (-1), sBT <15 ng/ml (-1) or >25 ng/ml (+2), presence (-2) or absence (+1) of pruritus, hives or angioedema and presence (+3) of pre-syncope or syncope. A score of ≥2 is considered to be positive and indicate a high probability for CMD, thereby warranting a bone marrow examination. #Modified REMA-score applies the sum of positive and negative points as follows: male (+1), female (-1), sBT <11.4 ng/ml (-1) or >20 ng/ml (+2), presence (-2) or absence (+1) of pruritus, hives or angioedema and presence (+3) of presyncope or syncope.

Regarding clinical aspects of episodes, the most frequent manifestation observed in the entire cohort was syncope (93%), which differed widely from previous studies that reported 23% (337) and 9% (338). By contrast, the incidence of urticaria and/or angioedema were previously reported to be 100% (337) and 91% (338), whereas it occurred in 50% in our study. Apparently, these clinical distinctions cause difficulties in comparing different studies. This most probably depends on the differing anaphylaxis criteria in inclusion, as both studies mentioned were performed before the NIH clinical criteria for anaphylaxis (52) were

available. Another aspect that might be of interest, in comparing patients with CMD and IA, is our observation that episodes of syncope was preceded by urticaria with /without angioedema in patients with true IA, whereas in CMD, it was preceded by flushing.

After the initial visit, 25 patients received regular maintenance therapy with mediator-blockers, mainly H_1-antihistamines. Doses were adjusted on the basis of the frequency and severity of their previous reactions and follow-up visits were scheduled at different intervals on a case-by-case basis for the individual patient's needs, but at least one visit per annum for all patients. All patients were equipped with adrenaline auto-injector and advised to self-administer intramuscularly upon commencement of acute symptoms, before they to go to the emergency ward for objective assessment. During follow-up, seven patients (23%) had experienced 29 subsequent episodes of UEA, while five (71%) of these seven patients were on regular maintenance therapy with H_1-antihistamines. Remarkably, six (86%) of these patients belonged to the IA group, and more interestingly one of these patients accounted for 16 (55%) of the episodes. Although all instructed, only 5 (71%) patients used adrenaline, and 2 (29%) of the 7 also sought emergency care.

Although none of our patients took oral steroids on regular basis, three of the cases were treated with omalizumab, a monoclonal anti-IgE antibody. The first patient, who suffered from MMAS with a total of eight UEA episodes, was given 300 mg and twice monthly gained no benefit despite 6 months trial. The other two patients, both with SM and suffered from severe UEA episodes, did not experience any new episodes during 32 (as of now 42) months of treatment using same dosage and interval as above. Nevertheless, as both patients had earlier suffered infrequent episodes (i.e., once every two-three years), it is difficult to assess whether their remission was spontaneous (340) or due to omalizumab treatment and/or their regular usage of mediator-blockers. As the mechanism(s) of action of omalizumab in patients with SM is unknown, and the drug is currently expensive, controlled studies are apparently required. Therefore, at present, omalizumab treatment should be considered only in those patients with severe and frequent (at least 3/year) episodes, who are refractory to standard treatments.

The prognosis of UEA appears to be good, as most patients being in remission (defined as no new episodes for at least one year) according to long-term follow-up studies (340-342). Although relative short follow-up period (median 31 months, range 2-94), 70% of our UEA patients were in remission at the time of last follow-up. Interestingly, the number of patients with resolved disease was significantly higher in the CMD group ($P < 0.02$). This might be due to their more extensive usage of mediator-blockers and also a gender aspect might contribute since patients were predominantly men in this group. Although spontaneous recovery of patients has been reported previously (340), we suggest that maintenance therapy with mediator-blockers was beneficial for our patients.

The strength of the study is its comprehensive data on characteristics of patients with unexplained anaphylaxis. Although the diagnosis of UEA was highly reliable here, the findings are from a single mastocytosis center and may therefore not be generalizable to all

patients with UEA. Another concern is potential recall bias, at least to a certain extent, since a number of the UEA episodes were based on patient-reported data and not documented by a physician. The relative paucity of the study subjects investigated is an additional limitation highlighting the pressing need for further research in this area.

In conclusion, unexplained anaphylaxis appears to be a heterogeneous and complex entity. As the term "IA" was coined before the CMD nomenclature had been introduced into practice, the interchangeable use of the terms "IA" and "UEA" appears in current literature. A definitive diagnosis of true IA requires careful elimination of all potential triggers of anaphylaxis, as well as other conditions with similar symptoms. In this study, 47% of 30 subjects who were previously given a diagnosis of UEA had evidence of clonal mast cells in the bone marrow. Thus, a bone marrow investigation in UEA patients appears essential in certain circumstances, particularly in patients who experience hypotensive syncope during an anaphylactic episode and exhibit an elevated level of sBT (≥11.4 ng/ml). Until the presence/absence of CMD is established, using the term unexplained anaphylaxis (UEA) instead of "IA" is therefore more appropriate.

3.4.3 An explanatory approach

In Paper V, we sought to examine the hypothesis that mast cells of patients with mast cell activation disorders express hyperreactivity in the skin and/or lower airways compared to control subjects. The whole idea is based on the clinical and laboratory observations indicating that these patients suffer from excessive MC-mediator release and related clinical symptoms, thereby suggesting signs of "clinical state of mast cell hyperreactivity".

All three mediators analyzed were significantly higher in patients with SM compared to healthy controls. In addition, sBT levels in patients with SM were significantly correlated with urinary *tele*-MIAA and with urinary 11β-$PGF_{2\alpha}$ excretions. Associations of sBT and urinary *tele*-MIAA in SM patients has previously been reported (343); however, correlation between sBT and 11β-$PGF_{2\alpha}$ was not reported. By contrast, we did not observe any significant correlation between urinary *tele*-MIAA and urinary excretion of 11β-$PGF_{2\alpha}$. Moreover, we did not find significant differences between MMAS or IA patients compared to healthy controls. This may be due to the sample size because number of patients in these groups were sparse (four vs. three, respectively). When we clustered the MCAD group (patients combined from SM, MMAS and IA), results were still significant from healthy controls. From PCA analysis using serum tryptase and averaged urinary concentration of *tele*-MIAA and 11β-$PGF_{2\alpha}$, we could show that the SM patients clustered together and separated from the healthy controls. Also inter individual spread within the SM group perhaps reflects the complex clinical diversity of symptoms within this group.

In order to assess skin reactivity, we analyzed wheal-sizes induced by histamine and morphine prick tests. As known, histamine skin reactivity is non-specific and independent from MC activation and reflects increased vascular permeability and axon reflex, whereas MC secretagogues morphine directly activates skin MC resulting in a wheal and flare response caused by the release of MC mediators, including histamine (344). Remarkably,

when we compared the median wheal-sizes induced by morphine and histamine, the results were not significantly different between healthy controls and patients with SM/MCAD. These findings, are consistent with a previous investigation (345), and suggest that MCs from MCAD patients do not have altered threshold for release to secretagogues, at least not to morphine.

Another aspect of this study was the evaluation of airway reactivity in these patient groups. Although both histamine and leukotrienes are potent airway smooth muscle contractors, baseline pulmonary function and airway reactivity have previously not been extensively explored in patients with SM/MCAD. This is, to our knowledge, the first study that investigated this issue in depth. The only study addressing this question was a case report (346) that suggested bronchial hyperreactivity assessed by histamine challenge in a patient with systemic mastocytosis. However, methacholine induced airway reactivity is independent from mast cell mediator release; by contrast, mannitol causes airway bronchoconstriction by activating mast cells. In this study, five patients (two with SM, two with MMAS and one with IA), without a history of asthma or other obstructive pulmonary diseases, reacted with methacholine challenge. Nevertheless, none of these five patients or any other MCAD patients reacted to mannitol challenge. Consequently, we could not demonstrate any sign of mast cell hyperreactivity in the lower airways of MCAD patients.

As we did not find any support for hyperreactive MCs in skin or bronchial airways, we analyzed basophil histamine release by hypothesizing that histamine could be released by basophils. This was because these two cells are both granulated, and express complementary and partially overlapping functions, although they display distinct features. However, the mechanisms engaging interaction between MCs and basophils in the context of mast cell activation disorders have not yet been investigated. Nevertheless, our evaluation could not support this notion as no altered basophil reactivity in patients with MCAD was observed either.

Further, we thought that the answer might be in the interaction molecules, such as cytokines, by inducing a hyporesponsive state in MCs. That would also be an explanatory model, as the patients; despite constantly elevated mediator levels have only symptoms under certain periods. In that manner, IL-33 appeared to be potential candidate as a recent study (347) reported that prolonged exposure of human MCs to IL-33 downregulated FcεRI-mediated and KIT-enhanced degranulation of MCs in culture, and thereby induced a hyporesponsive MC phenotype. We therefore attempted to measure levels of IL-33 in patients with MCAD to study their potential role. Nevertheless, we could not consistently detect plasma levels of IL-33 in our study, as there were only four SM patients and one healthy control with detectable levels. This suggests that studies of IL-33 should be made in other samples than plasma. In a manner similar, a prolonged exposure to SCF has also been shown to induce a hyporesponsive MC phenotype in the mouse model (348). We thus measured plasma SCF levels, and, consistent with a previous report (349), found that plasma concentrations were comparable between patients and healthy controls. By contrast, another study reported

significantly higher SCF plasma levels in mastocytosis patients (350). The reason behind these discrepancies might be due to heterogeneity of the investigated cohorts.

As plasma IL-31 levels has also been reported to be elevated in patients with in subsets of mastocytosis, particularly in more aggressive variant of the disease (351), we also detected IL-31 levels. Nonetheless, our study could not show any significant difference between patient groups and healthy controls. The reason for contradictory results could be due to fact that patients in our study suffered merely from indolent variant of SM. It remains, however, elusive whether IL-31 levels are primary event or are secondary to the disease process. Although we did not measure IL-6 in this study, this cytokine has been shown to be elevated in patients with systemic mastocytosis (349), thereby its potential contribution to the biologic consequences of the disease was suggested. However, it is fair to assume that contribution of different cytokines to pathogenesis of SM appears not to be on the mediator level but rather at the inflammatory level, i.e., in patients with aggressive disease rather than indolent patients. This notion has been supported by a previous study (352), where an association between elevated IL-6 levels and presence of organomegaly and hematologic disease has been shown.

Accordingly, these results suggest that patients with MCAD, including SM, are not more sensitive to histamine or morphine compared to healthy controls. One possible explanation of this phenomenon is that patients with mastocytosis become relatively insensitive to histamine, perhaps through induction of receptor tachyphylaxis or a down-regulation of the number of histamine receptors. However, it is more difficult to speculate what makes these cell normoreactive to morphine. In similar manner, airway challenges with the methacholine occurs independent from mast cells; however, why they do not react to mannitol remains elusive.

Another important aspect of this study was to show that patients with systemic mastocytosis express distinct cellular features compare to other patients with MCAD including monoclonal mast cell activation syndrome and idiopathic anaphylaxis. Although all MCAD patients have indifferent clinical manifestations, the levels of mediator release differed between SM vs. MMAS/IA patients. Therefore, in patients with MMAS/IA, there should be distinct intrinsic defects other than known clonal markers of MCs that enable these cells to behave in a clinically hyperreactive manner. Nevertheless, we are also aware of a major drawback in our study, namely paucity of the study subjects, in patients with MMAS and IA.

Taken together, although this study confirmed elevated levels of varied mast cell mediators in SM/MCAD patients, we found no evidence to support the hypothesis that a hyperreactive mast cell phenotype would exist in the skin or bronchial airways of these patients. However, one may also speculate that mediator releasing hyperreactive mast cells can do exist in other organs than skin/airways, for instance, in the gastrointestinal tract. The source of mast cell mediator excess, thus, remains still ambiguous.

Personal reflection: Is there a hyperreactive mast cell phenotype?

4 CONCLUSIONS

The term mast cell activation disorders (MCAD) is characterized by inappropriate mast cell activation and may be used for a heterogeneous collection of conditions when specific criteria are fulfilled (Figure 14). All of these disorders present with recurrent signs and symptoms of mast cell activation, so-called mast cell mediator-induced symptoms, and differ in severity and involvement of various organ systems.

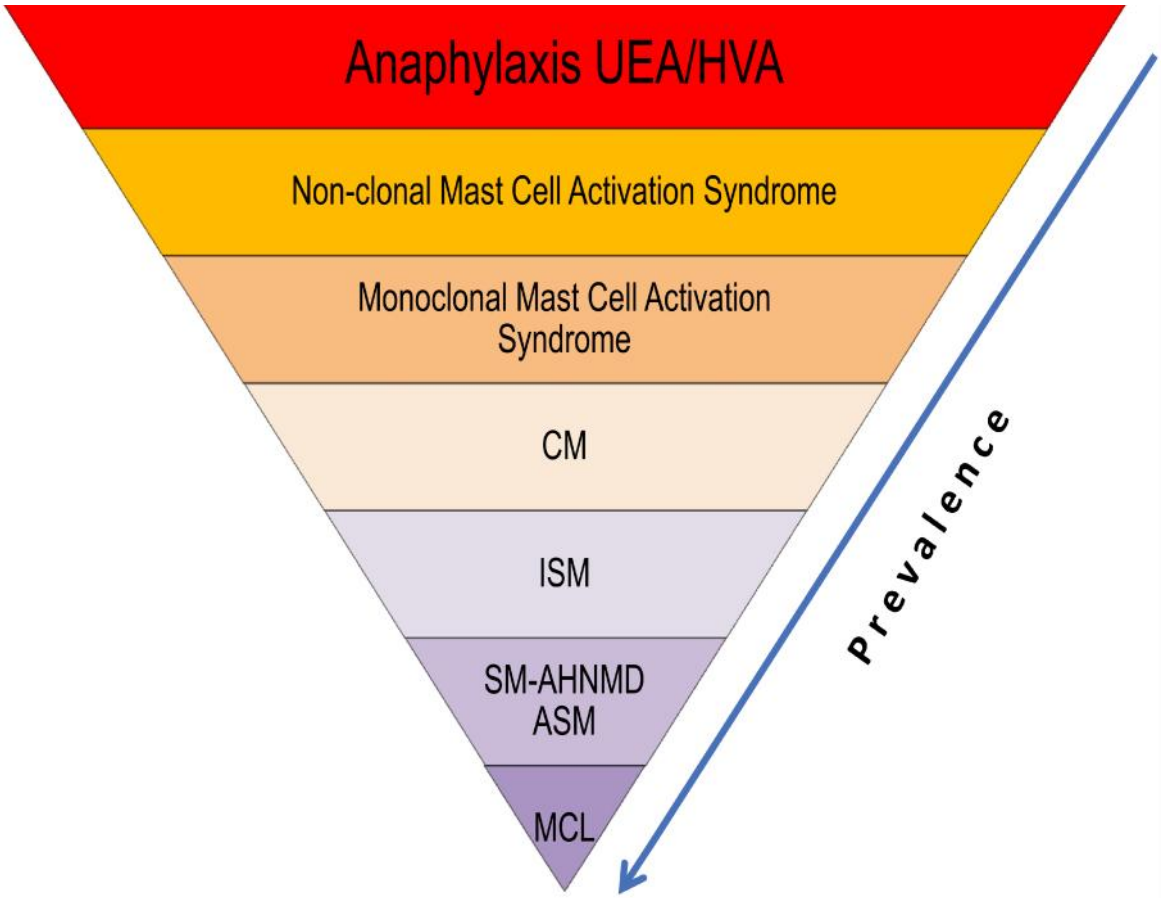

Figure 14. Illustration of broad spectrum of systemic mast cell activation disorders. Note that prevalence of disorders getting rare down to the pyramid. Adapted from reference (152) UEA: unexplained anaphylaxis; HVA: hymenoptera venom anaphylaxis; CM: cutaneous mastocytosis; ISM: indolent systemic mastocytosis; SM-AHNMD: systemic mastocytosis associated with hematological non MC lineage disease; ASM: aggressive systemic mastocytosis; MCL: mast cell leukemia.

Although our understanding has developed gradually since its discovery at the turn of the 19th century, anaphylaxis still generates lots of excitement and at the same time a sensation of dismay. The findings from studies presented in this thesis imply a *de facto* association between anaphylaxis and mast cell activation disorders, where the prevalence of anaphylaxis is clearly increased. Consequently, this also infers one of the predominating clinical problems that these patients encounter. However, the mechanism(s) behind these complex interactions between anaphylaxis and mastocytosis including related mast cell disorders are obscure.

Through this dissertation project, we attempted to explore the complex relationship between anaphylaxis and mastocytosis using two different approaches (Figure 15). On the one hand, we sought to analyze patients with anaphylaxis who eventually received the diagnosis of systemic mastocytosis; on the other hand, we started our survey from patients with systemic mastocytosis and evaluated presence/absence of anaphylaxis in these patients. By doing so, we examined the existence of a possible SM-anaphylaxis phenotype.

Although the presence of some risk factors to predict this phenotype has been indicated, the current studies could not otherwise provide any evidence for such an absolute SM-phenotype.

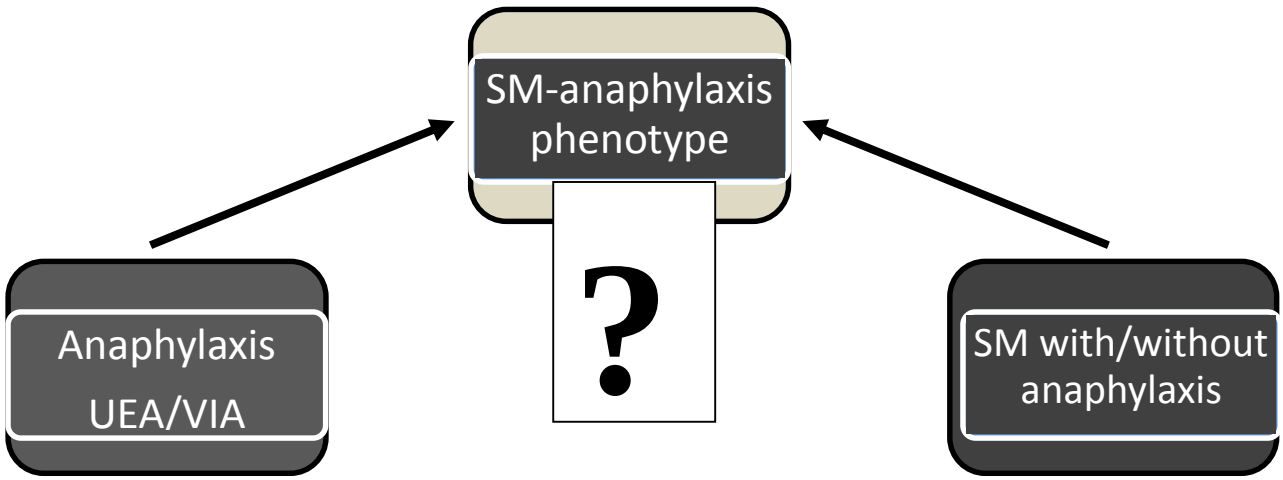

Figure 15: Approaches used to explore a possible SM-anaphylaxis phenotype.

What these studies can support on the other hand is that there are certain predictivity tools, including modified REMA-score, to predict underlying clonal mast cell disorders in patients with certain forms of anaphylaxis (for instance patients with unexplained anaphylaxis and patients with severe venom-induced anaphylaxis) and have no sign of mastocytosis in skin. A bone marrow examination is essential in these patients to search for markers of clonal mast cell populations; however, the hardest question yet to answer is when a bone marrow examination should be applied. It is proposed by consensus group that sBT levels of >20 mg/ml is an indication for a bone marrow examination. Nevertheless, this remains an eminence based recommendation, since there are certain patients with unexposed clonal mast cell disease who have a sBT levels between 11.4 – 20 ng/ml.

The author's personal approach (Figure 16) is, based on the studies included here; a bone marrow investigation is justified if the following circumstance(s) exist:

- Patients obtain ≥2 points in modified REMA-score; including involvement of cardiovascular syncope during an anaphylactic episode and presence of elevated baseline serum tryptase levels (>11.4 ng/ml). There is also a gender aspect, as males dominate in clonal mast cell disorders.
- Presence of D816V *KIT* mutation in peripheral blood samples, though at present as a complementary tool (not validated in the included patient groups).

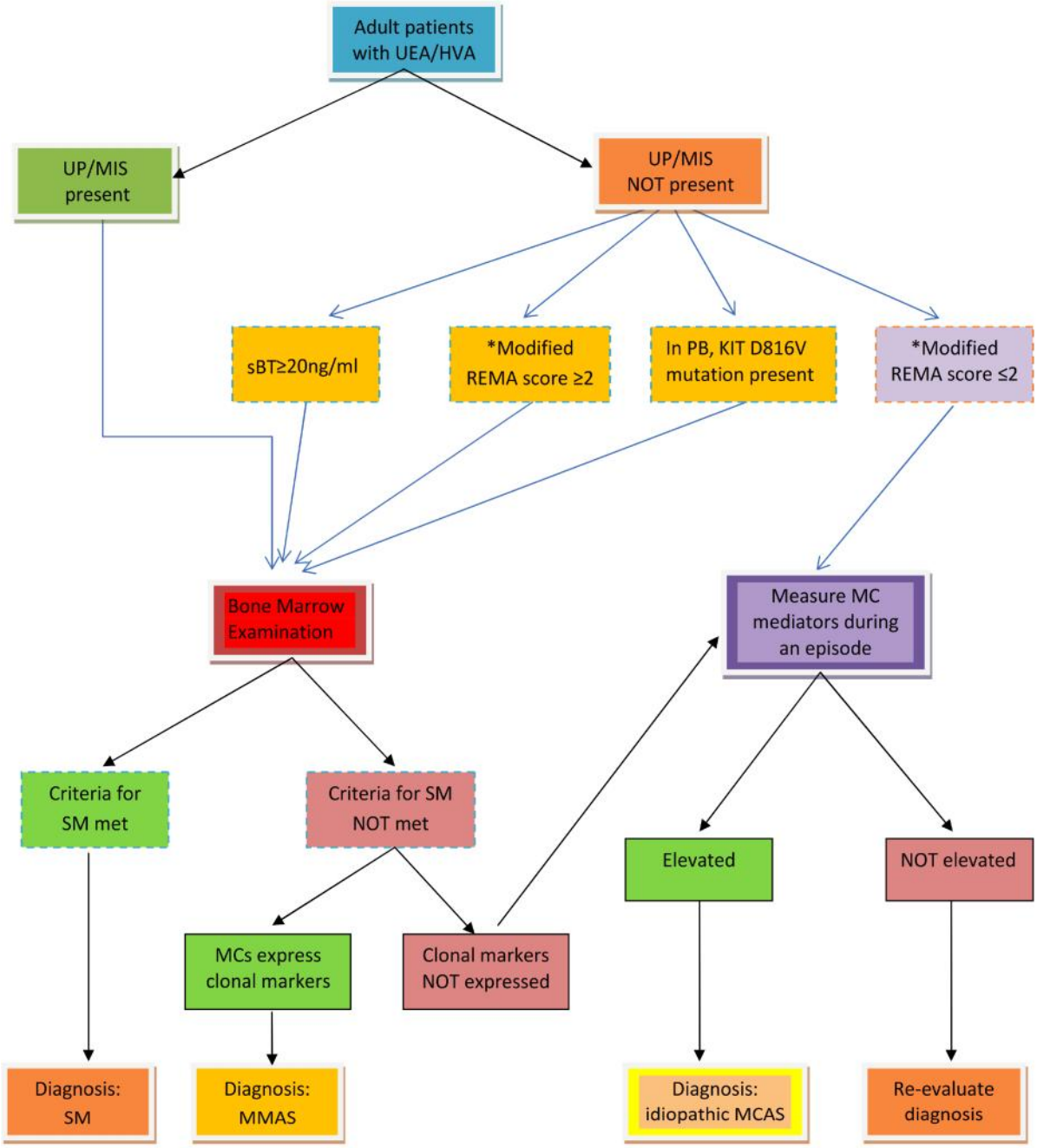

Figure 16: Proposed algorithm for sequential investigations of adult patients with unexplained and venom-induced anaphylaxis.
UEA: unexplained anaphylaxis; HVA: hymenoptera venom anaphylaxis; UP: urticaria pigmentosa; MIS: mastocytosis in the skin; PB: peripheral blood; SM: systemic mastocytosis; MMAS: monoclonal mast cell activation syndrome; MCAS: mast cell activation syndrome.
*Modified REMA-score: please see paragraph 2 on page 50 and Table 4 on page 51 for information.

Allergists are the primary specialists who care for mast cell mediator-induced symptoms. When physicians encounter patients who present with mast cell activation symptoms, a standardized clinical evaluation including comprehensive medical history, physical examination, allergy work-up and specific laboratory tests, and most probably a bone marrow examination should be applied.

Furthermore, in the light of studies included in this thesis, and the clinical experience gained through these years, the following list demonstrates some of the important remarks to be considered:

- Mast cell activation disorders can be classified into three categories: primary, secondary and idiopathic.
- Tryptase is a rather specific mediator for MCs and it is widely available. It is therefore, recommended to be the biomarker of choice to screen patients with suspected MCAD. Urinary metabolites of histamine and PGD_2 are other feasible mediators to evaluate involvement of mast cells in these conditions.
- The establishment of SM diagnosis requires one major and one minor or three minor criteria using the WHO-criteria. Screening tissue sections (most preferably bone marrow) for mast cells should be performed with antibodies against tryptase, CD25 and CD117. In certain cases, bilateral BM biopsies might provide higher yield (353).
- If the patient receives diagnosis of systemic mastocytosis, due to its clinical heterogeneity, an interdisciplinary approach is essential. In patients with SM, different staging investigations should be performed including bone mineral density measurements and skeletal x-rays, computed tomography scans of thorax and abdomen at the time of diagnosis.
- Patients with secondary MCAD are triggered by allergic or non-specific stimuli, nevertheless, there are no signs of intrinsic mast cell defects since the quantity and function of mast cells are normal (to best of our current knowledge).
- Idiopathic MCAS should fulfill both the clinical criteria, and also requires a BM-biopsy that shows no detectable clonal mast cells and no underlying reactive disease.
- As there is no curative treatment for mast cell activation disorders at the present time, *sine quo non* for all MCAD patients is to undergo a comprehensive risk assessment made by an experienced allergist, thereby tailor-made preventive measures for the known or potential mast cell triggers can be provided.
- The first aim of the maintenance therapy is to provide a good control of symptoms with combination of different mediator blocking agents for improved quality of life.
- All patients and relatives should be given comprehensive information on the disease and its related concerns including prognosis.
- All anaphylaxis-prone MCAD patients should be equipped with adrenaline auto-injectors after proper self-training.
- MCAD patients, who are IgE-sensitized for venom and have a history of venom-induced anaphylaxis, should undergo lifelong specific venom immunotherapy.

- Before the planned surgical interventions, the patient, anesthesiologist and surgeon should discuss about possible perioperative risks. The risk assessment for individual patients may be facilitated if the patient has already undergone comprehensive allergy work-up at an allergist. Measures should be taken case-by-case basis as there are no observational or controlled studies supporting for qualified guidelines. There is no evidence that preventive drugs such as antihistamines and/or steroids given before the procedure will be effective. Hence, such procedures in these patients should be performed in a hospital with emergency care facility and intensive care units. Day case surgery is probably not appropriate with respect to possible delayed reactions. Likewise, certain x-ray investigations with contrast media may also convey risks. Therefore, a similar approach can be useful.

- Follow-up visits can be scheduled at different intervals on a case-by-case basis accordingly individual patient's need; however, at least one annual follow-up visit for all MCAD patients should be arranged with the control of baseline tryptase, routine blood chemistry and complete blood count including differential counts. In addition, bone density measurements may be performed every second or third year.

5 FUTURE PERSPECTIVES

The work presented in this thesis contributes a better understanding of mast cell activation disorders, in particular, its relation to anaphylactic reactions. There is, however, an obvious need for further research to characterize the features of these conditions in-depth and also perform studies to explore mechanisms behind.

In addition, the following issues should be taken into consideration to improve patient care:

- Investigation of patients with suspected mast cell activation disorders should be standardized through ECNM Centers. This would enable us to perform comparative studies and also conduct multi-center studies.
- The introduction of the global clinical criteria for diagnosis of anaphylaxis in medicine, being one of the greatest achievements in the field of allergy, helps tremendously in emergency settings; however, a mechanistic *de-novo* classification of anaphylaxis with implementation of the current knowledge appears to be essential. The unanswered question is whether it could be the same disease that causes IgE-mediated food reactions in children on the one hand, and venom-induced or unexplained hypotensive shocks in adults on the other hand. A clonality aspect should be taken into consideration (Figure 17).

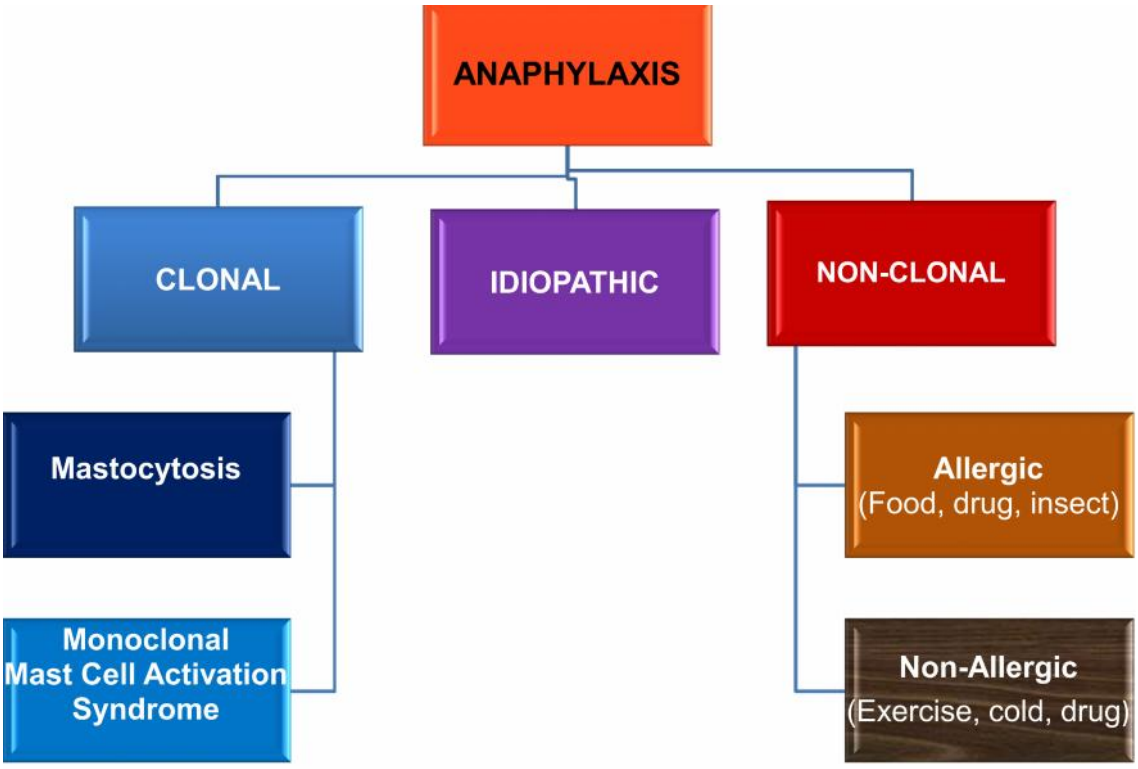

Figure 17. Proposed mechanistic classification of anaphylactic reactions.

- With reference to non-clonal/idiopathic mast cell activation syndrome (nc-MCAS), there are still big gaps in our knowledge. Current diagnostic criteria for nc-MCAS appear to be incomplete and somewhat cumbersome. The proposal in the consensus

paper (143) is mainly based on empirical observations, as the diagnostic criteria are not validated by clinical studies. Clinical features of the syndrome are categorized arbitrarily. For instance, which two organ systems should be included (or more appropriate to say not included) is ambiguous. Furthermore, why idiopathic anaphylaxis should be excluded from nc-MCAS is not convincingly discussed. Consequently, larger cohort studies with nc-MCAS patients are required to supply comprehensive data to develop more robust proposals.

- ❖ An adapted clinical-practice classification of mast cell activation disorders (MCAD) should be implemented.

  Because:

  - It is difficult to apply the complex terminology into clinical practice
  - It is confusing to use very similar, though distinct entities: MCAD-MMAS-MCAS or even, mast cell disease, clonal mast cell disorders etc.
  - Not all entities globally recognized (MMAS, MCAS)
  - International Classification of disease (ICD) diagnostic codes do not exist for all categories
  - Current classification causes trouble for patients to explain their health condition and trouble to understand for care takers due to unawareness of scientific nomenclature. Thus, there is an apparent need to make a distinction between pathologic versus clinical classification of mast cell activation disorders (MCAD).

Accordingly, a simplified classification may serve recognition of these conditions. For instance, with a pragmatic approach, it appears tempting to classify all these disorders/syndromes under the umbrella term of “mastocytosis”, since the term “mastocytosis” is somewhat better recognized compared to other entities. On the one hand, this umbrella ”definition” may, from a histopathological perspective, be inappropriate, as traditional definition of mastocytosis emphasizes its accumulative features due to pathological findings. On the other hand, mast cell activation related clinical symptoms are the predominating problem in most SM patients. Consequently, the definition of mastocytosis could be broadened to “inappropriate mast cell activation disorders (with or without mast cell accumulation) caused by intrinsic defects in mast cells”.

This would ease the situation, at least, for the patients.

6 SUMMARY IN SWEDISH (POPULÄRTVETENSKAPLIG SAMMANFATTNING)

Uttrycket mastcellsaktiveringssjukdomar innefattar ett brett spektrum av heterogena tillstånd och inkluderar bland annat den sällsynta sjukdomen mastocytos. Problemet hos dessa patienter är att det finns störningar i mastceller, en celltyp som vanligtvis utför uppgifter i kroppens immunförsvar. Dessa tillstånd kännetecknas av olämplig mastcellaktivering och/eller ackumulering. Diagnosen av dessa sjukdomar kräver idag benmärgsundersökning av mastceller. Patienter presenteras med varierande kliniska symptom och svårighetsgraden av symptom kan variera från patient till patient. Symptomen, som kan vara periodiska eller kroniska i sin natur, kan hänföras till de lokala och/eller systemiska effekter av mastcellsförmedlare. Sjukdomen kan i vissa fall uppträda med enstaka, isolerade symtom, till exempel rodnad, klåda, huvudvärk, magkramp, diarré, illamående, hjärtklappning, blodtrycksfall eller medvetslöshet. Vid andra tillfällen kan den yttra sig som en kombination av olika symtom som ger en klinisk bild av anafylaktiska reaktioner, dvs. allergisk chock, som medför en känsla av rädsla i de flesta drabbade patienter. Patienternas livskvalitet kan allvarligt försämras eftersom det för närvarande inte finns något botemedel för dessa tillstånd och nuvarande farmakologiska behandlingar syftar därför till att kontrollera symtomen.

Det övergripande syftet med denna avhandling var att öka våra kunskaper om patienter med mastcellsaktiveringssjukdomar genom att skaffa nya fakta och rön om de demografiska, epidemiologiska och kliniska kännetecken, samt att undersöka förekomst och olika sorters mastcellsförmedlade/inducerade symtom, i synnerhet anafylaktiska reaktioner. Dessutom ville vi studera det komplexa samspelet mellan anafylaxi och mastcellsaktiveringssjukdomar genom att identifiera riskfaktorer.

Mastocytosis Center Karolinska etablerades under 2006, och i slutet av 2013 har vi undersökt över 200 patienter. Således var förutsättningar för att driva dessa forskningsstudier goda och patienter rekryterades från utredda patienter.

I det första delarbetet redovisade vi ett väldigt illustrativ patientfall som pedagogiskt belyser problematiken kringsjukdomen mastocytos. Denna studie visade också att det fanns en klar brist på erkännande av mastocytos-symtom bland läkare. Att ställa en korrekt diagnos krävde i det fallet nästan 20 år, trots att patienten hade samrått med flera läkare och genomgick omfattande medicinska undersökningar. Fram till att diagnosen ställdes upplevde patienten 88 attacker och hans livskvalitet var starkt påverkad. När vi fick veta att han hade förhöjd basalt (dvs. ett prov som kontrolleras under ett icke-symtomatiskt skede) serum tryptas (ett ämne som frisätts från aktiverade mastceller), var det nästan klart för oss att denna patient var drabbad av mastocytos.

I den andra studien har vi presenterat tre förbryllande fall av anafylaktisk reaktion utlöst efter getingstick. Alla tre patienterna var också lika gamla och av samma kön. Efter traditionell allergiutredning fick de diagnosen anafylaxi utlöst av getinggift. Eftersom samtliga patienter också hade förhöjda nivåer av basalt serumtryptas, vilket indikerade bakomliggande störningar i mastcellen, utreddes de vidare med benmärgsbiopsi. Undersökning av benmärgsmastceller ledde till förändringar i slutlig diagnos och därmed fick två av de tre

patienterna diagnosen mastcellsaktiveringssjukdom och erbjöds livslång allergivaccinering med getinggift.

I det tredje delarbetet ville vi inskaffa en omfattande inblick i patienter med systemisk mastocytos (SM), framför allt kring sjukdomens allergologiska aspekter. Vi rapporterade förekomst av mastcells – förmedlade/inducerade symtom i 90 % av samtliga SM patienter och 63 % av dessa patienter hade symtom som relaterade till mag-tarmkanalen. Dessutom har förekomsten av anafylaktiska reaktioner i denna patient grupp befunnits vara kraftigt ökade (43%). Huvudorsaken till anafylaktiska reaktioner var i 53 % av fallen getinggift, följt av oförklarlig anafylaktiska reaktioner (39 %). Vidare förekom anafylaktiska reaktioner oftare hos SM patienter med atopiskt anlag (dvs. hos patienter som har en ärftlig benägenhet att bilda allergiska antikroppar) och bland SM patienter som inte har mastocytos i huden. Även nivån av basalt tryptas var något lägre bland SM-patienter med anafylaxi.

I det fjärde delarbetet bidrog vi med fullständiga uppgifter om egenskaper hos patienter med oförklarad anafylaktiska reaktioner genom att undersöka dessa patienters benmärgsmastceller. Vi visade att 47 % av utredda patienter hade avvikande markörer på sina mastceller och därmed fick diagnosen mastcellsaktiveringssjukdom. Basala nivåer av serum-tryptas var signifikant högre (≥ 11,4 ng /ml) hos dessa patienter, däremot hade de låga värden av totalt immunglobulin E (IgE) (dvs. allergiska antikroppar).

I det femte delarbetet ville vi testa hypotesen att mastceller från patienter med mastcellsaktiveringssjukdomar uttrycker tecken till hypereaktivitet i huden och nedre luftvägarna jämfört med kontrollpersoner. Vi analyserade också olika ämnen som släpps ut från mastceller (tryptas, histamin och prostaglandin). Även om vi fann förhöjda halter av samtliga testade mastcells-mediatorer hos patienter med mastcellsaktiveringssjukdomar, hittade vi inga bevis till stöd för hypotesen att hyperreaktiva mastceller skulle existera i huden eller nedre luftvägarna hos dessa patienter.

Sammanfattningsvis, det arbete som presenteras i denna avhandling ger en bättre förståelse för olika kategorier av patienter med mastcellssjukdomar. Våra resultat stödjer att alla patienter med mastcellsaktiveringssjukdom bör genomgå gängse allergologisk utredning för att man ska kunna göra en individuell riskbedömning och kartlägga eventuell allergi mot födoämnen, läkemedel och insekter samt övriga utlösande faktorer, samt för att kunna ge en skräddarsydd profylax innan man sätter in förebyggande behandling och åtgärder. Patienter som har haft anafylaktisk reaktion ska utrustas med adrenalinpenna och träna injektionsteknik under tillsyn. Våra data indikerar också att mastcellsaktiveringssjukdomar förekommer i en betydande undergrupp av patienter med oförklarlig anafylaktiska reaktioner. Prov på basalt serum-tryptas har stor betydelse för att göra en preliminär riskbedömning för oupptäckt mastcellsaktiveringssjukdom. Dock utgör än så länge benmärgsundersökning den enda säkra utredningsmetoden.

För närvarande finns det inget botemedel och framtida terapeutiska framsteg beror på en bättre förståelse av de grundläggande mekanismerna som reglerar frisättning av mastcellernas mediatorer.

7 EPILOGUE

Through this work, I attempted to provide some sense of our current knowledge in this complex field of medicine. It is, however, important to realize that ideas and concepts framed here were based on limited data; mainly our own experience with 250 patients investigated at Karolinska University Hospital, to date, and experience gained by exchanging ideas with other colleagues in the field through the annual ECNM meetings. Although recommendations provided express our best and up to date knowledge, the possibility cannot be ruled out that the ideas might differ in the course of time with growing experience and knowledge. Hence, there is an obvious need for further research in the field to establish firmer advices.

Finally, as patients with mastocytosis and related mast cell activation disorders can present to a wide variety of physicians, I hope ideas presented here would be valuable not only for allergist but also for all physicians encounter these patients in their practice, and perhaps patients themselves who would like to gain more information on these complex conditions.

September 2014, Stockholm

8 ACKNOWLEDGEMENTS

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