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REVIEWS

Review

The Immune System in Cushing's Syndrome

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Cushing's syndrome (CS), or chronic hypercortisolism, induces a variety of alterations in the immune system, often leading to severe clinical complications such as sepsis and opportunistic infections. Prolonged exposure to high levels of glucocorticoids (GC), changes in the circadian rhythm, and the comorbidities associated therewith all combine to cause profound changes in the immune profile of affected patients. While traditionally associated with generalized immune suppression, such changes actually comprise a much more complex scenario, sharing traits with chronic inflammatory disorders. Persistently increased levels of interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNFα) and adipose tissue infiltration by immune cells lead to a chronic, nonresolving, inflammatory state. The combination of low-grade inflammation and selectively impaired immune response is thought to play a major role in the pathogenesis of clinical complications of CS, including diabetes, lipodystrophy, visceral adiposity, atherosclerosis, osteoporosis, and cognitive impairment. This dysregulation also explains rebound phenomena when CS is treated, involving new clinical complications sustained by an excessive immune response and autoimmunity. The aim of this review is to summarize the available evidence on the immune system in chronic hypercortisolism, while describing the main mechanisms of immune derangement and their role in the increased mortality and morbidity seen in this complex disease. A better understanding of immune system alterations in CS could help improve risk stratification, offer novel biomarkers, and provide the basis for more tailored therapies and post-remission follow-up.

Highlights

Increased GC exposure during the active phase of CS affects all immune cells, leading to overall immune suppression characterized by cell death, impaired immune regulation, and defective immune response.

CS-induced lymphopenia (mainly of the $CD4^+$ subset) with an altered CD4/CD8 ratio increases patients' susceptibility to viral infections.

GCs alter the ratio of Th1/Th2 subpopulations, leading to apoptosis of mature T lymphocytes.

The increased neutrophil count contributes to the establishment of a hypercoagulative state and the development of sepsis, both requiring active monitoring.

The remission phase of CS can be associated with the unmasking of autoimmune diseases elicited by reactivation of the previously suppressed immune system, occasionally leading to an excessive response.

Clinical Features of Cushing's Syndrome

Cushing's syndrome (CS) (see Glossary) is a rare and potentially fatal endocrine condition characterized by chronic **hypercortisolism**. Its main causes are pituitary neoplasms secreting **adrenocorticotropic hormone (ACTH)** (ACTH-dependent, 70%) or adrenal tumors, bilateral hypertrophy, or dysplasia (ACTH-independent, 20%) leading to excessive, chronic, and autonomous secretion of **cortisol** [1]. Less frequently, it can be caused by **ectopic ACTH secretion (EAS)** or **corticotropin-releasing hormone (CRH)** (10%) [2,3]. Pituitary CS can occur in children [4] or present with cyclical features that can substantially delay the time to diagnosis and cure [5]. Excessive and prolonged use of glucocorticoids (GCs) can also lead to exogenous CS, showing the same features and comorbidities as endogenous hypercortisolism [6]. The worldwide prevalence of CS is estimated at around 40 cases per million, with an incidence of 0·7 to 2·4 cases per million per year [7]. ACTH-dependent CS and ACTH-independent CS due to adrenal tumors or hyperplasia show a higher prevalence in women, with a 3.5–4:1 ratio [6].

Patients with chronic hypercortisolism show several characteristic clinical features: weight increase with visceral fat deposition, thin skin and tendency to bruises, *striae rubrae*, muscle atrophy, rounded face and facial plethora, along with hirsutism, reduced linear growth in children and decreased libido and menstrual irregularities in adults [6,8]. CS is also associated with numerous

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complications, including metabolic syndrome, cardiovascular diseases, hematological abnormalities [9], osteoporosis, bone fractures, and neuropsychiatric disorders [10]. Overall, patients with CS show a decreased quality of life and increased mortality, with an overall standardized mortality risk ranging from 0.95 to 9 in the active phase, while the mortality in CS patients cured by pituitary surgery within 1 year is not different from the general population [8,11–13]. The main causes of death in patients with active CS are cardiovascular complications and infectious diseases [8,14,15]. Recent data from a European database of CS patients show that infections are the second cause of death (31%) after disease progression and are the main cause of death (37%) in patients who died within 90 days of diagnosis [16].

Patients with CS suffer from immune system alterations both during the active phase and while in remission. In the active phase, increased GC exposure directly causes immune suppression, influencing all parts of the immune response and exposing CS patients to an increased risk of systemic dissemination and complications from commonly acquired or opportunistic infections [8,17,18].

The prevalence of infections in CS is 21%–51% during the active phase [8], but few studies have described the epidemiology and they mainly focused on CS due to EAS [19–22]. Although malignancies and chemotherapy act as independent factors for the development of infections, a study comparing patients with EAS and nonsecreting malignancies, both receiving cytotoxic treatment, showed a significantly poorer prognosis in hypercortisolemic patients [23]. In a different cohort of 90 patients with EAS, infections were present in 51%, but the authors did not provide a risk stratification based on tumor site [20]. Fungal infections are more common in exogenous and ectopic CS, which are usually characterized by higher GC exposure, in part due to the direct effect of corticosteroids on fungal proliferation and translocation from the gastrointestinal tract via the bloodstream [17,24]. CS patients are also at a high risk of severe, prolonged, and disseminated viral infections [25]. Susceptibility to bacterial and opportunistic severe infections seems to be directly and positively correlated with cortisol levels and is more frequent in ectopic CS, suggesting that severely overt CS patients may benefit from routine screening for pathogens more often responsible for disseminated infections, like *Candida*, *Pneumocystis*, *Histoplasma*, *Cryptococcus*, *Pseudomonas aeruginosa*, *Nocardia*, and *Klebsiella pneumoniae* [21,24]. Prophylactic therapies have also been proposed, such as trimethoprim-sulfamethoxazole therapy, in preventing *Pneumocystis* pneumonia before [21] and after surgery for severe hypercortisolism, since the development of opportunistic infections has been often described after treatment, possibly due to a rebound increase in immune response to previous colonization [26]. Although cases of tuberculosis in CS patients are not frequent, given the described incidence of latent tuberculosis reactivation and *de novo* infection in patients undergoing GC therapies for rheumatic diseases [27], additional testing for latent tuberculosis seems a reasonable indication in severe hypercortisolism. Moreover, patients with CS may experience atypical presentations of infectious disease (fewer symptoms), probably due to the anti-inflammatory effects of hypercortisolism [21], leading to a delay in referral.

During remission, CS patients often experience rebound autoimmune diseases, probably due to overactivation of the previously suppressed immune function [8,28–30]. Thyroid autoimmune diseases, coeliac disease, rheumatoid arthritis, sarcoidosis, systemic lupus erythematosus, vitiligo, atopic dermatitis, and psoriasis are the most commonly described autoimmune conditions emerging after CS remission [8,31].

Patients who have underlying inflammatory or autoimmune disorders can experience worsening of their condition when the GC dose is decreased [32]. For example, rebound of autoimmune hepatitis during intravenous GC therapy for Graves' ophthalmopathy has been reported [33].

Glossary

Adaptive immunity: an antigen-specific immune response that takes several days to come into effect and continues developing throughout life. Antigens are transported to the lymphoid organs, where they are recognized by naive B- and T-lymphocytes. Once activated, the B- and T-lymphocytes proliferate and differentiate into effector cells. The adaptive immune system also has a 'memory', which guarantees long-term response.
Adrenocorticotropic hormone (ACTH): a tropic hormone produced and secreted by the anterior pituitary gland. It mainly regulates cortisol and androgen production in the cortex of the adrenal gland.
Corticotropin-releasing hormone (CRH): a peptide hormone that stimulates the synthesis and secretion of ACTH by the corticotropin-producing cells of the anterior pituitary gland.
Cortisol: a steroid hormone that regulates a wide range of vital processes, including metabolism and immune response. It is secreted by the adrenal gland with a circadian profile.
Cushing's syndrome (CS): an endocrine disorder sustained by an excessive production of endogenous cortisol, secondary to tumors secreting cortisol, ACTH, or CRH.
Cytokines: small, secreted proteins released by cells; they have a specific effect on cell-to-cell interactions and communications.
Ectopic ACTH secretion (EAS): a rare condition caused by the paraneoplastic release of ACTH, or very rarely CRH, from various tumors outside the pituitary gland and resulting in CS.
Hypercortisolism: excessive levels of cortisol in the blood. This can be caused by various endogenous conditions as well as by the exogenous administration of glucocorticoid drugs (exogenous CS).
Innate immunity: antigen-nonspecific defense mechanisms activated immediately or within hours after the host's exposure to almost any antigen appearing in the body. These mechanisms include physical barriers such as the skin, chemicals in the blood, and immune system cells that attack foreign pathogens in the body.
Leukocytosis: white blood cell count above the normal reference value. It is frequently a sign of an inflammatory response.

The effects of GCs on the innate and adaptive arms of the immune system are well known and have provided the rationale for their beneficial use in inflammatory, autoimmune, and neoplastic diseases. CS, however, is a distinct condition and has its own specific immune profile. The immune alterations observed in this condition could also contribute to other complications such as osteoporosis [34] and atherosclerosis [35,36]. The aim of this review is to summarize the available findings on the immune system in CS and to highlight the immunological features of this disease.

Lymphopenia: an abnormally low lymphocyte blood count.
Monocytopenia: a reduced monocyte blood count.

An Overview of Full Leukocyte Count

Leukocytosis is common in CS. Its mechanisms involve different lineages and functions. After remission, patients show a significant decrease in their white blood cell count [37], mainly due to a reduction in the percentage of neutrophils. Patients with chronic hypercortisolism also display significant **lymphopenia** and **monocytopenia** due to the effects of GCs on mononuclear leukocyte subsets, normalizing after successful treatment [37].

It is important to highlight that the increase in circulating neutrophils due to reduced adhesion and extravasation to potential infection sites, in addition to the decrease in circulating lymphocytes and monocytes, all contribute to the observed impaired immune defense against infectious diseases in active CS [17,25,37].

Innate Immune Components

While a distinct functional division between **innate** and **adaptive immunity** has been challenged by emerging evidence of their close interdependence, a systematic classification on an evolutionary basis is in common use. Innate immunity is an ancestral defense system responsible for first-line response to exogenous pathogens, as well as coordinating inflammation and antigens. Many of the elements involved in innate immunity are altered in CS [8,25] (Figure 1), as discussed in this section.

Neutrophils

In the healthy immune system, neutrophils are responsible for extravascular phagocytosis and amplify inflammation. However, their role in disease seems to be more complex, as demonstrated by emerging evidence describing many distinct functional subsets [38]. Patients with chronic hypercortisolism show a significantly increased polymorphonuclear leukocyte (PMN) count [37] (Table 1). In CS, the mechanisms involved in the increasing circulating neutrophil count can be imputed to several different effects: GCs promote release of PMNs from bone marrow and their recruitment from the marginal pool, and also prolong the PMN half-life in the bloodstream, by reducing apoptosis and extravasation [39,40]. In previous *in vivo* studies, dexamethasone (DEX) reduced L-selectin expression in PMNs [41]. The adhesion molecule L-selectin contributes to extravasation by binding to endothelial receptors. The GC-mediated shedding of surface L-selectin, an Annexin A1-mediated mechanism, significantly hinders the ability of neutrophils to extravasate to peripheral tissues [42–44]. The GC-mediated induction of Annexin A1 expression in peripheral blood mononuclear cells is mediated by the GC-induced leucine zipper gene [42]. In experimental models of GC treatment, Annexin A1 was found to be significantly increased in neutrophils, contributed to L-selectin shedding, and significantly reduced neutrophil extravasation [40,42].

The anti-inflammatory effect of GCs on neutrophils is also mediated by a significant reduction in other endothelial adhesion molecules that promote detachment and impair peripheral migration and by a reduction in NADPH oxidase subunits, superoxide release and reactive oxygen species (ROS) levels [40]. Furthermore, GCs significantly dampen neutrophil response to inflammatory stimuli such as lipopolysaccharide, interleukin 8 (IL-8), tumor necrosis factor α (TNFα) and

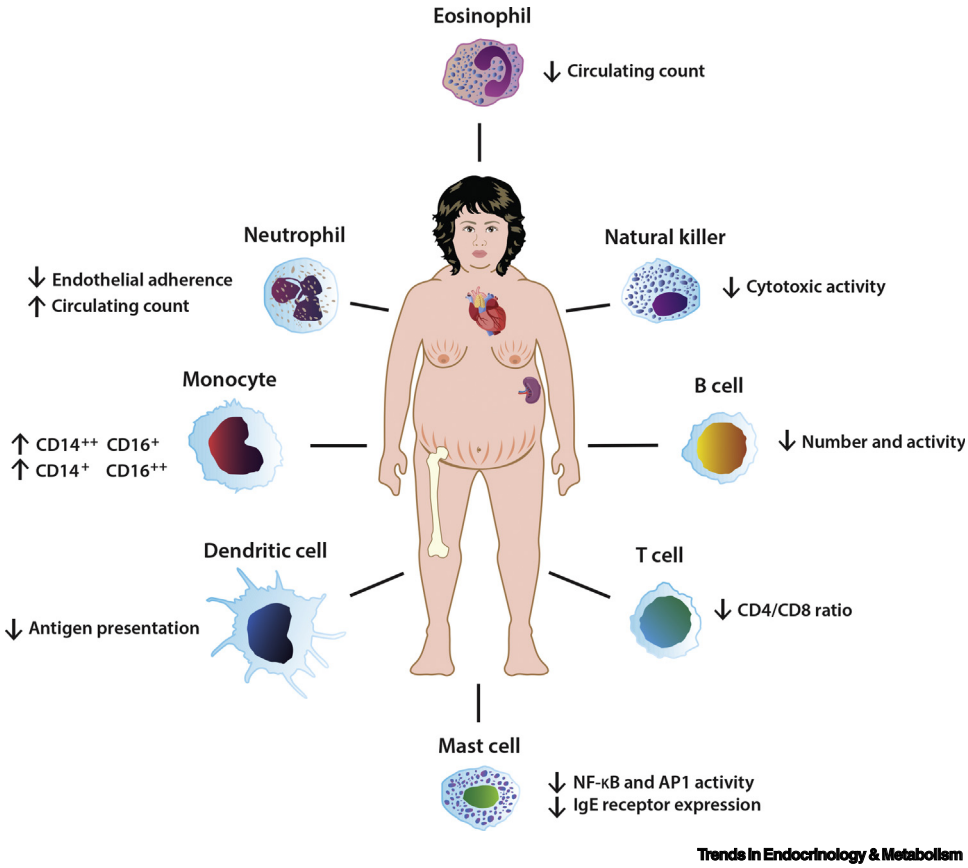

Figure 1. Changes in Immune Cells in Cushing's Syndrome.

interleukin 1β (IL-1β) and can also reduce mRNA levels of IL-1β, TNFα, and IL-8 (Figure 2). Data on the effects of GCs on chemotaxis and phagocytosis are still inconclusive [40].

GCs also increase glucose consumption in neutrophils by altering glycerol-6-phosphate dehydrogenase (G6PDH) activity and consequently impairing the NAPDH-dependent respiratory burst [40]. Interestingly, GCs seem to exert proinflammatory effects on neutrophils (Table 2) by increasing the expression of inflammatory **cytokines** and chemokine receptors, such as IL-1β receptor (IL-1βR) and leukotriene B4 receptor 1 (BLT-1), and by reducing the expression of anti-inflammatory molecules such as IL-1Ra, a soluble antagonist of IL-1 and IL-1βR. Accordingly, levels of IL-1βR were significantly reduced in patients with chronic obstructive pulmonary disease taking *inhaled* GCs [40]. GCs also reduce neutrophil apoptosis through different mechanisms and increase their survival and half-life in the bloodstream.

The balance of pro- and anti-inflammatory effects of GCs on neutrophils is closely regulated to stimulate and control the immune response during acute stress. Their twofold impact on this leukocyte subset explains why neutrophil-based inflammation cannot be efficiently counteracted with GCs alone.

The chronic and continuous hypercortisolism observed in CS provokes the alteration of all neutrophil functions, significantly hindering the immune response in these patients. In septic patients,

Table 1. Innate and Adaptive Immune Cells in CS and Associated Clinical Readout

Cell type	Effect of hypercortisolism	Refs	Type of variation	Refs	Clinical readout
Neutrophils	Polymorphonuclear leukocytosis	[37]	Reduced neutrophil apoptosis	[45]	Hypercoagulability Atherosclerosis
Monocytes and macrophages	Reduced circulating monocytes	[8,25]	Macrophage remodeling in visceral fat	[54]	Visceral obesity
	Increased intermediate ($CD14^{++}CD16^{+}$) and nonclassical ($CD14^{+}CD16^{++}$) monocytes	[8,25]	Weaker phagocytic capacity	[48]	
Natural killer cells	No significant alterations in NK cell count in peripheral blood	[61,62]	Suppressed cytotoxic activity in NK cells and IFNγ response	[57,58]	Susceptibility to infection
			Increased IL-2	[57]	
Cytokines and other circulating mediators	Inappropriately high circulating levels of TNFα	[74]	Low-grade inflammation profile	[73,74]	Visceral obesity
	Increased sTNF-R1 and sTNF-R2, IL-6 and C reactive protein	[73,74]	Lower levels of adiponectin and increase in proinflammatory adipokines	[73,74]	
T lymphocytes	Influence on Th1/Th2 balance	[82]	Production of IL-4 and Th2 polarization	[82]	Susceptibility to intracellular and opportunistic infections
	Shift towards Th2 differentiation	[82]	Enhanced lymphocyte proliferation by altering TCR-mediated apoptosis	[81]	
	Significant lymphopenia, reduction in $CD4^{+}$ subset and CD4/CD8 ratio	[25,37,61]			
B lymphocytes	Reduced B lymphocyte count	[89]	Impairment of activity may derive from altered T lymphocyte function	[89]	Increased susceptibility to microbial infections

Abbreviations: IFNγ, Interferon γ; IL-2/4/6, interleukin 2/4/6; NK, natural killer; sTNF-R1, soluble tumor necrosis factor receptor 1; sTNF-R2, soluble tumor necrosis factor receptor 2; TCR, T cell receptor; TNFα, tumor necrosis factor α.

several studies showed a significant increase in functionally impaired circulating neutrophils with a decrease in GC receptor (GR) expression and binding capacity; GC therapy seemed to enhance these alterations [45]. This acquired resistance could be at the basis of the inefficient response to GCs during sepsis in the general population and it is therefore interesting to speculate that this mechanism may contribute to the relatively frequent and often fatal development of sepsis in patients with chronic hypercortisolism.

Finally, an increased neutrophil count also seems to contribute to the hypercoagulative state in CS. Patients with hypercortisolism show increased levels of neutrophil extracellular traps: extracellular chromatin and cytosolic granular proteins on the activated neutrophil surface that act as prothrombotic agents [46]. Some of the immune changes induced by GC resemble, with the given proportion, what occurs in sepsis (Box 1).

Monocytes and Macrophages

Macrophages and their circulating precursors, monocytes, are differentiated mononuclear myeloid cells involved in phagocytosis and surveillance in peripheral tissues. They play a role in the activation and modulation of inflammation, tissue repair, and innate and adaptive immunity. They also participate in the intricate crosstalk between the neuroendocrine and immune systems, as recently reviewed by Jurberg *et al.* [47].

CS patients can show a reduction in circulating monocytes [8,25] and an imbalance in subset percentages, with an increase in intermediate ($CD14^{++}CD16^{+}$) and nonclassical ($CD14^{+}CD16^{++}$) monocytes compared with controls (Table 1). Nonclassical and intermediate monocytes have a

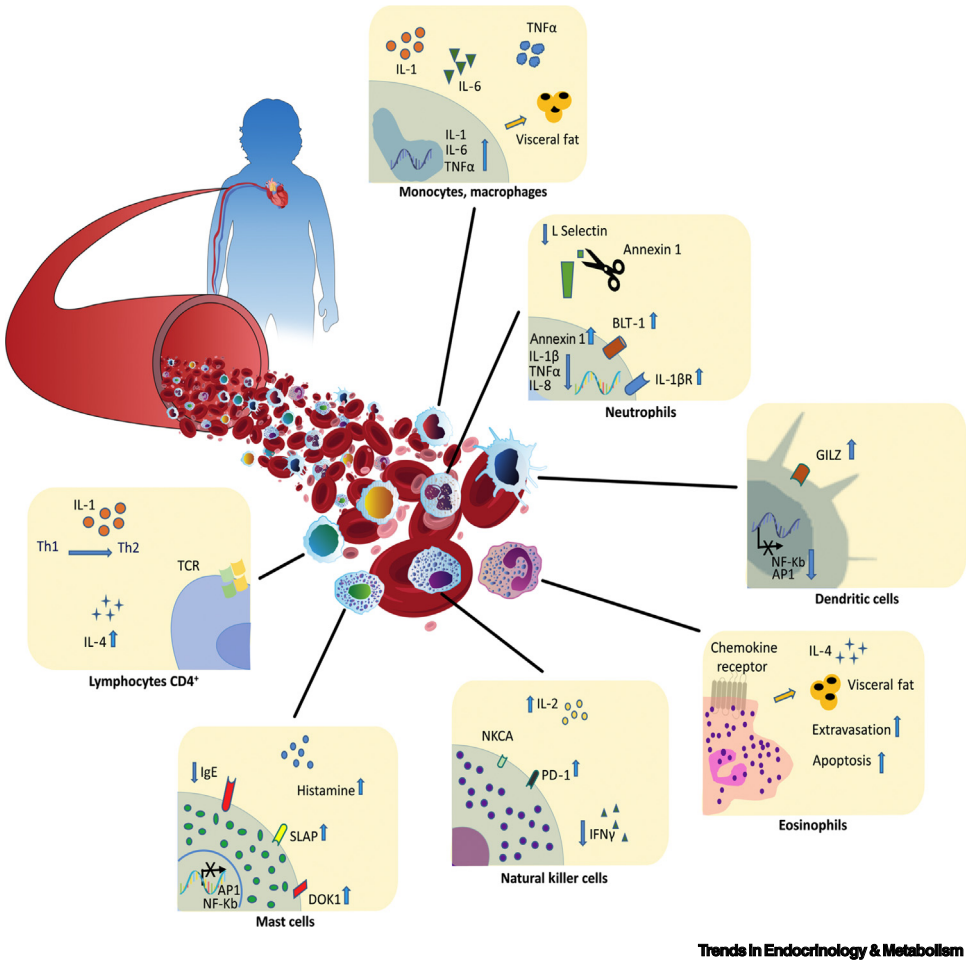

Figure 2. Main Molecular Mechanisms Involved in Immune Cell Dysfunction Associated with Cushing's Syndrome. Abbreviations: BLT-1, Leukotriene B4 receptor 1; DOK1, Doking protein 1; GILZ, GC-induced leucine zipper protein; IFNγ, interferon γ; IL, interleukin; NKCA, cytotoxic activity in NK cells; PD-1, programmed cell death protein 1; SLAP, Src-like adaptor protein; TCR, T cell receptor; TNFα, tumor necrosis factor α.

weaker phagocytic capacity compared with the classical $CD14^{++}CD16^{-}$ subpopulation and do not generate ROS. They are involved in autoimmune and inflammatory diseases and in conditions characterized by an increased cardiovascular risk, such as obesity and insulin resistance [48]. Interestingly, these changes were more prominent in ACTH-independent CS, possibly due to the absence of the modulatory effects of ACTH and other melanocortin-related peptides on immune function [48]. In fact, several immune cell lines such as monocytes [49] and lymphocytes [50] express melanocortin receptors (MCRs), with anti-inflammatory and immune modulating effects that are independent from the stimulation of GC secretion [49]. While MCR-2 is expressed in the adrenal gland and selectively binds ACTH, MCR3-5 can bind all melanocortin-related peptides, reducing monocyte influx to inflammatory sites and subsequent cytokine production [49]. The targeting of MCR in inflammatory diseases (such as experimental lung inflammation) has shown significant efficacy in modulating macrophage and eosinophil response, achieving clinical improvement [51], and the absence of MCR-3 caused an enhanced and imbalanced inflammatory response in experimental models [52]. In addition, ACTH and alpha-MSH exert inhibitory effects

Table 2. Effects of GC Treatment on Different Cell Types

Cells type	Effects of GCs	Refs
Neutrophils	↑ Annexin 1 expression, contributing to L-selectin shedding, ↓ extravasation	[42,43,46]
	↓ G6PDH activity, impairing the NAPDH-dependent respiratory burst	[40]
	↓ mRNA levels of IL-1β, TNFα, and IL-8	[40]
	↓ Peripheral migration	[40]
	↓ Superoxide release and ROS levels	[40]
	↑ IL-1βR and BLT-1 expression	[40]
Natural killer cells	↑ Secondary response by NK memory cells	[59]
Eosinophils	↑ Proapoptotic factors and chemokine receptors	[67,68]
	↓ Macrophage polarization towards an alternatively activated phenotype	[69]
Mast cells	Inhibition of transcription factors such as NF-κB and AP1	[71]
	↓ Degranulation and calcium release after IgE-mediated activation	[71]
	↓ IgE receptor expression	[71]
	↑ SLAP and Doking protein 1 (Dok1) expression	[71]
	Inhibition of MAP kinase signaling cascade	[71]
Dendritic cells	↓ The ability to activate Th1 response	[72]
T lymphocytes	↑ Proliferation by altering TCR-mediated apoptosis	[81]
B lymphocytes	↓ Proliferation and cytokine production	[88]
	↑ Antiproliferative effects in early stage of B cell development	[89]

on leptin secretion that, in turn, enhances cortisol release; thus, the milder effects observed in ACTH-dependent CS might be related to an involvement of adipose tissue [53].

Macrophages are terminally differentiated immune cells resident in the peripheral tissue. They can modulate inflammatory response and activation by secreting cytokines and modulating chemokine secretion in host tissues. In active CS, adipocyte hypertrophy was correlated with an increase in macrophages and in local leptin levels in visceral fat, but not in subcutaneous fat [54]. The increased presence of inflammatory macrophages in visceral fat in chronic hypercortisolism suggests a possible mechanism of escape from the anti-inflammatory effects of GCs and from the increased proinflammatory chemokines that are observed in CS and that significantly contribute to the increased cardiovascular risk [55]. Finally, the trafficking of monocytes in CS could also depend on macrophage migration inhibitory factor (MIF). A critical mediator of septic shock, MIF is released by many cell types in response to stress, including the pituitary, where is secreted in parallel with ACTH. MIF can regulate GC-mediated immunosuppression, acting as a counter-regulatory hormone-modulating host response. We demonstrated that in pituitary CS, MIF levels are not increased nor respond to pharmacological stimulation [56], excluding a major role in the active phase; however, given the emerging role of MIF in autoimmunity, its dysregulation could play a role in the rebound diseases after CS cure.

Natural Killer (NK) Cells

NK cells are a lymphocyte subpopulation involved in innate immunity. They show a strong antiviral and antitumoral effect and have been used for cancer immunotherapy.

NK cells can be divided into different subpopulations with specific functions according to their surface markers. $CD3^{-}CD56^{++}CD16^{-}$ cells are known as the cytokine-secreting subset, involved

Box 1. Sepsis and Glucocorticoids

Sepsis is a life-threatening condition caused by a dysregulated host response to infection, when the body's response injures its own tissues [91]. Patients with CS are at increased risk of sepsis [21,24]. This is not surprising, given that the immune changes observed in CS resemble some of the features observed in sepsis. Lymphopenia, common in CS, has been associated with an increased risk of sepsis in large cohorts [92], as well as obesity, diabetes, and cardiovascular diseases [93]. In CS, we observe an increased neutrophil count due to the impaired extravasation and functional capacity; similarly immature granulocytes are seen in sepsis and correlated with a poorer prognosis [94]. The increased neutrophil extracellular traps, endothelial damage, and hypercoagulative state are common features of both CS and sepsis [95]. The molecular mechanisms described earlier found a correlate in the clinical trials evaluating the effects of GC on the incidence and prognosis of sepsis. The REGARDS study, one of the largest prospective cohort studies in the United States, showed that baseline steroid therapy significantly increased the risk of infections and subsequent sepsis in the long-term follow-up, independently of other comorbidities [96]. In patients with septic shock, steroids have been used at relatively high dosages. The recent ADRENAL trial compared continuous administration of 200 mg hydrocortisone daily with placebo and showed no beneficial effects on the 90-day mortality [97]. Almost simultaneously, another controlled trial, APROCCHS, showed a significant reduction in mortality of septic patients receiving hydrocortisone plus fludrocortisone therapy [98]. The conflicting data could be explained by baseline differences: the APROCCHS patients were sicker, the timing of GC administration from sepsis shorter (<24 hours), and hydrocortisone was administered intravenously by 50 mg boluses, reaching more rapidly higher therapeutic peaks compared with the ADRENAL study. A recent meta-analysis of all randomized controlled trials on steroid therapy in sepsis highlighted a beneficial effect of GC on 28-day mortality, with no increase in superinfections, gastroduodenal bleedings, or any severe adverse events [99]. Post hoc analysis on ADRENAL selecting patients who met the APROCCHS Sepsis-3 inclusion criteria confirmed no benefit in 90-day mortality, but the short term 28-day mortality was lower in the hydrocortisone group [100]. However, findings on GC therapy in intensive care units are not comparable with chronic endogenous hypercortisolism. In conclusion, the short duration of therapy (in both the APROCCHS and ADRENAL trials hydrocortisone was administered for few days) and the high pulsed doses rise interesting hypotheses, but do not allow us to draw conclusions immediately transferable to CS.

in immune regulation with little to no cytotoxic activity, while $CD3^{-}CD56^{+}CD16^{++}$ cells are the effector subset, expressing high levels of activating receptors such as natural cytotoxicity receptors (NCR) and FcRIII (CD16) on their surface, which mediate direct and antibody-dependent cellular cytotoxicity, respectively.

GCs have been reported to suppress cytotoxic activity in NK cells (NKCA) by epigenetic interactions, but this effect seems to be dichotomous and time-dependent. In fact, chronic treatment with GCs seemed to blunt NKCA [57,58] and to prime the production of inflammatory cytokines such as IL-2 [57], while suppressing IFNγ secretion [58]. This twofold effect also seemed to be influenced by previous NK activation, since DEX increased secondary response of NK memory cells [59] (Table 2).

To add more complexity to this scenario, the cytokine microenvironment in peripheral organs such as the liver and spleen seems to influence GC response in NK cells by selectively promoting programmed cell death protein 1 (PD-1) expression after an endogenous rise in GCs. This prevents immune overreaction by reducing NK cell activity while preserving antiviral and antimicrobial function in 'resistant' tissue-resident NK cells [60]. PD-1 antibodies are a new addition to cancer therapy, and the discovery of a GC-PD-1 axis could provide further insights into immune checkpoint inhibitor therapy and resistance.

Given this intricate crosstalk between the hypothalamic pituitary axis and NK cells, the profound alterations found in diseases involving GC secretion are easy to understand. In CS, NK cells showed a significantly decreased NKCA with no alterations in $CD56^{+}$ cell count in peripheral blood [61,62] (Table 1). This effect also seemed to depend on changes in the circadian rhythm, as it was more prominent in adrenal and ectopic than in ACTH-dependent CS. In fact, in ACTH-dependent CS and in healthy controls there was a significant difference in spontaneous NK cell activity and IFNγ response between samples taken at 8:00 a.m. and 8:00 p.m., even

though both functions were impaired in patients with hypercortisolism compared with healthy controls [62].

It has also been suggested that circadian system impairment may significantly affect NK cell function in patients with adrenal insufficiency taking multiple daily treatment doses [63,64]. In these patients, NK cells showed a significant decrease in the $CD3^{-}CD56^{+}CD16^{high}$ effector subset, reaching levels close to healthy controls after switching to once-daily modified release therapy, which is more respectful of the endogenous circadian rhythm [63]. This was further confirmed by evidence of circadian gene impairment in patients taking conventional therapy, which was restored after switching the therapy [64]. The mutual relationship between 'low grade inflammation', circadian rhythm disruption, and immune dysfunction has not been yet completely clarified: which comes first? Adrenal disorders alter the circadian rhythm, as GCs are important synchronizers of endogenous clocks, including that of immune cells. A disruption of clock genes in the immune cells has been associated with development of metabolic alterations and a low-grade inflammation. This has not yet been demonstrated in CS, while in adrenal insufficiency, receiving excessive or inadequate GC replacement, this has been shown [63]. An extensive description of the interplay between circadian rhythm and adrenal disorders can be found elsewhere [65].

Eosinophils and Mast Cells

While neutrophils, NK cells, and monocytes account for the majority of innate immune defense strategies, other mediators such as eosinophils and mast cells are also strongly influenced by GCs.

Eosinophils are involved in allergic and inflammatory response. Patients with CS show a decreased total eosinophil count, that reverses after remission [66]. This feature parallels the prompt drop in eosinophil count experienced after exogenous GC administration [67]. The increase in proapoptotic factors and chemokine receptors in eosinophils suggests that GC treatment favors eosinophil extravasation and subsequent apoptosis [67,68]. More recently, a role of eosinophils as adjuvant cells in adipose tissue macrophage polarization has been proposed [69]. Interleukin 4 (IL-4) secretion by adipose tissue resident eosinophils has been demonstrated to polarize macrophages towards an alternatively activated phenotype, ultimately improving glucose homeostasis [69]. Interestingly, a correlation between glucose metabolism, hypercortisolism, and eosinophil count has been described in CS [70]. Thus, an impairing eosinophil function could represent an additional mechanism through which GCs affect the interplay between adipose tissue and infiltrating macrophages, contributing to an altered glucose homeostasis.

Mast cells are responsible for tissue IgE-mediated response, releasing histamine and other powerful inflammatory mediators. Given the preformed nature of their mediators, mast cells can induce immediate inflammatory reactions and are at the basis of allergic diseases.

GCs are often used in the management of such diseases. Their effect on mast cells can be divided into genomic and rapid modulation. The first is mediated by conventional binding to the GC receptor and subsequent over- or underexpression of specific compounds, while the second seems to involve surface or cytoplasm GC receptors, as the response of mast cells is almost immediate and is insensitive to transcriptional and translational inhibitors [71]. The genomic effects of GCs on mast cells (Table 2) include the inhibition of transcription factors such as NF-κB and AP1, downregulation of IgE receptor expression, and upregulation of specific inhibitory phosphatases such as Src-like adaptor protein (SLAP) and Doking protein 1 (Dok1), thus indirectly inhibiting the mitogen-activated phosphatase (MAP) kinase signaling cascade [71].

GCs also play a significant role in the differential modulation of members of the dual specificity phosphatase (DUSP) family, which can also interfere with the MAP kinase cascade [71]. However, the nongenomic effects of GCs on mast cells significantly reduce degranulation and calcium release after IgE-mediated activation. These effects seem to depend on GC-mediated alterations of plasma cell membrane capacity [71]. However, mast cells have seldom been investigated in CS and there is little information on this topic in endogenous hypercortisolism.

Dendritic Cells (DCs)

DCs originate from the monocyte/macrophage lineage and are the most efficient antigen-presenting cells. They are divided into four different subsets: conventional DC 1 (cDC1), which stimulates Th1 response; conventional DC 2 (cDC2), which promotes Th2 and Th17 response; plasmacytoid DCs (pDC), specialized in antiviral immunity; and inflammatory DCs (iDC), which may rise during chronic and acute inflammation [72]. GCs are strongly involved in DC modulation, mostly through the action of GC-induced leucine zipper protein (GILZ). GILZ expression has been demonstrated in many other immune cells. It directly inhibits inflammation by modulating the expression of several inflammatory mediators, such as NF-κB and AP1, and can promote an anti-inflammatory phenotypic shift in numerous immune cells. By inducing GILZ expression, GCs can hinder the ability of DCs to activate Th1 response (thus contributing to the Th2 shift in hypercortisolism, discussed below) and promote a tolerogenic profile [72] (Table 2). The Th1-Th2 shift promoted by chronic GC exposure could play a role in delaying the immune response to viruses and bacteria and, possibly, immune surveillance in early stage malignancies.

Cytokines and Other Circulating Mediators

Cytokines, like many other components of the immune system, show a low-grade inflammation profile during the active phase of chronic hypercortisolism, despite the ability of GCs to suppress classical inflammation. These alterations seem to persist after remission and have been associated with increased truncal fat deposition and altered adipokine profile [73].

In several studies comparing CS patients with healthy controls, patients with chronic hypercortisolism showed lower levels of adiponectin and increased proinflammatory adipokines, soluble tumor necrosis factor α receptors (sTNF-R1 and sTNF-R2), interleukin 6 (IL-6), and C reactive protein [73,74]. TNFα has been associated with increased cardiovascular risk, mostly due to its fundamental role in regulating lipid metabolism, adipocyte function, and insulin resistance, while also contributing to perivascular fat deposition and increased adhesion molecule expression [74]. While there is conflicting evidence of its role in CS, TNFα regulation seems to be altered in chronic hypercortisolism, leading to inappropriately high circulating levels [74] (Table 1).

From a clinical perspective, these alterations have been associated with the increased cardiovascular risk in CS and its persistence after remission [73]. While the exact mechanism of low-grade inflammation in CS is not yet completely understood, it has been speculated that it involves a vicious circle of visceral fat deposition, adipocyte dysfunction, and increased inflammatory cytokine secretion, ultimately escaping the anti-inflammatory effects of high corticosteroid exposure. Interestingly, the hypothesis of an altered interplay between inflammatory cytokines and the hypothalamic–pituitary axis in metabolic diseases has been also supported by evidence that interleukin 1 (IL-1) agonists reduce ACTH secretion, blood pressure, and heart rate in obese individuals [75]. A hallmark of CS is the visceral adipose tissue expansion. Several mechanisms are involved: GR expression is more abundant in truncal adipocytes, but in this site GCs induce less lipolysis; conversely, GCs increase lipoprotein lipase activity, acting on depot-specific fat storage [76].

The greater release of proinflammatory cytokines by visceral fat contributes to the increased cardiovascular risk but also to other CS-related comorbidities, such as osteoporosis. In fact, the increased IL-1 and TNFα levels enhance RANKL expression, increasing osteoclast activity and bone resorption [77]. Thus, the immune dysregulation appears pivotal to explain the increased risk fractures observed in CS or prolonged GC therapy [78], but could also represent an additional mechanism for metabolic impairment. In fact, osteocalcin, which carries independent beneficial effects on glucose homeostasis, is markedly reduced in CS [78]. Finally, TNFα and CXCL-10 overexpression may play a significant role in inflammatory myopathy and muscle atrophy, which are additional complications of CS [79,80].

Adaptive Immune System

Adaptive, specific, and acquired immunity are synonyms for the most recent evolutionary part of the immune system. Adaptive immunity is found in vertebrates only and comprises both cellular and humoral components. It acts mainly through lymphocytes, with antibody production by B lymphocytes accounting for the humoral component.

As previously mentioned, there are many connections and interactions between innate and adaptive immunity. CS has been associated with several alterations in acquired immunity, leading to dangerous unresponsiveness to harmful pathogens.

T Lymphocytes

T lymphocytes are a heterogeneous immune cell population with many different functions and phenotypes. They are classified, on the basis of their surface receptors, as $CD4^+$ or $CD8^+$ lymphocytes, along with other smaller subsets. $CD4^+$ lymphocytes, also known as T-helper lymphocytes, exert their immunomodulatory function after antigen recognition by MHC-II binding, while the cytotoxic $CD8^+$ subset are responsible for MHC-I-mediated binding and cytolysis of virally infected or tumor endogen cells. After antigen recognition, T-helper lymphocytes can differentiate into Th1 or Th2 phenotypes, with different regulatory effects and cytokine secretion profiles.

GCs play a significant role in lymphocyte suppression and have been extensively used in lymphoid proliferative diseases such as lymphoid leukemia or lymphoma. GC treatment can influence lymphocytes from the early stages of their development in the thymus. Double positive intermediate thymocytes are the major target of GCs, with increased apoptosis and significantly hindered proliferation rates [81]. These effects, however, seem to be dose-dependent, as GCs can also enhance lymphocyte proliferation by altering T cell receptor (TCR)-mediated apoptosis, thus impairing physiological screening for self-responsiveness [81] (Table 2).

GCs can also significantly influence the Th1/Th2 balance and induce apoptosis in mature T lymphocytes. Th1/Th2 differentiation is regulated by many different cytokines and cellular interactions and GCs inhibit one of the main Th1 shift inductors, the macrophage IL-1 system, producing a significant shift towards Th2 differentiation (Table 1). The consequent production of IL-4 amplifies Th2 polarization by inhibiting Th1 differentiation [82].

Interestingly, T cells seem to exhibit a sensitivity gradient to GC exposure, which further enforces Th2 polarization. Th1 cells are sensitive to GC-induced apoptosis and cytokine secretion, while Th2 cells are only sensitive to the latter. Another subset of T cells, the Th17 population, seems to be resistant to both. This could account for the GC resistance observed in Th2/Th17 dominant diseases such as asthma [83].

Patients with CS predictably show significant lymphopenia [25,37], mostly due to a reduction in the $CD4^+$ subset, with a concomitant reduction in the CD4/CD8 ratio [61] (Table 1). They are also more prone to developing opportunistic and viral infections, which are usually kept at bay by the Th1 subset of helper lymphocytes [8,25]. Despite the alterations in lymphocyte count, the function of $CD4^+$ and $CD8^+$ lymphocytes seems to be preserved [61]. The increase in autoimmune diseases during and after CS remission could also be determined by Th2 polarization and alterations in thymocyte development in these patients.

Other T lymphocyte subpopulations, such as $CD4^+CD25^+$ regulatory (Treg) lymphocytes, have been evaluated in several studies on patients taking DEX for underlying conditions. There is conflicting evidence of the effects of GCs on this subpopulation. Some studies showed a resistance of Tregs to DEX-induced cell death [84,85], while others found a decrease in the Treg count after DEX administration [86]. However, these results were arguably comparable. In a large cohort of cancer patients undergoing DEX treatment, Tregs dropped considerably but showed a proliferative advantage compared with the effector subset, possibly due to their greater affinity for IL-2 [87]. Since Tregs can lead to immune tolerance to neoplasms, an increase in their count might be related to shortened life expectancy in cancer patients undergoing GC therapy. However, there is a lack of evidence on Treg lymphocytes in CS.

B Lymphocytes

Although B lymphocyte responsiveness to GCs in lymphoproliferative diseases has been extensively evaluated, there is less evidence on the effects of chronic endogenous hypercortisolism in these cells. Therapeutic B cell depletion is often sought and achieved in autoimmune diseases too and artificial steroids, such as DEX, are efficient in reducing B cell proliferation and cytokine production [88].

GCs seem to exert different effects on B lymphocytes, depending on their grade of differentiation (Table 2). *In vitro* studies demonstrated strong antiproliferative effects of GCs in the early stage of B cell development, while steroids did not seem to influence or even enhance immunoglobulin production by differentiated B cells [89]. The altered T lymphocyte function observed in CS could also account for the possible reduction in B lymphocyte numbers and activity (Table 1).

Concluding Remarks

Since the discovery of endogenous hypercortisolism, Harvey Cushing noted that 'the malady seems to leave the patients with a definite susceptibility to infections' [90]. However, the underlying immune dysfunction of CS has been often overlooked and patients left untreated until infections progress and put their life at risk.

While recent experimental and clinical advances have provided many new insights into immunity in hypercortisolism, the complexity of this topic is far from being completely unveiled (see Outstanding Questions), and targeting hypercortisolism does not completely restore a normal immune response in affected patients.

After remission, patients continue to experience problems either due to rebound autoimmune diseases and/or persistence of low-grade inflammation. Whether this is secondary to metabolic comorbidities, such as 'sick adipose tissue' depots, glucose intolerance, bone dysfunction, or more profound GC-induced bone marrow changes, is yet to be clarified.

Epigenetic modifications such as reduced DNA methylation in genes involved in GC receptor binding have been found in CS patients after remission. The persistence of enhanced intracellular

Outstanding Questions

The alterations of innate immunity observed in CS are associated with the loss of circadian rhythmicity of glucocorticoids, are more prevalent in adrenal and ectopic CS, and, albeit with different features, have also been described in other conditions characterized by glucocorticoid dysregulation, such as adrenal insufficiency. Could immune system alterations be used as biomarkers of excess GC or to monitor response to treatment?

Exogenous GC treatments are given to reduce mast cell degranulation and calcium release after IgE-mediated activation. In patients with endogenous hypercortisolism, is mast cell function affected in the same way as when GCs are given exogenously? Should mast cells be targeted prophylactically to prevent post-treatment rebound?

The response of T regulatory lymphocytes ($CD4^+CD25^+$ Treg) to exogenous glucocorticoids is unclear: some studies showed their resistance to dexamethasone-induced cell death, while others documented a decline. How does CS affect Treg lymphocytes? How does immune regulation differ between endogenous and exogenous GC exposure? What are the implications for the diseases that cause CS?

The mechanistic pathways though which excess GC causes end-organ damage are only partially elucidated. What are the roles of inflammation and altered immune response on the metabolic pathways affected by excess GC? How do they contribute to increased cardiovascular and infectious disease mortality? What are the implications for the treatment of CS patients?

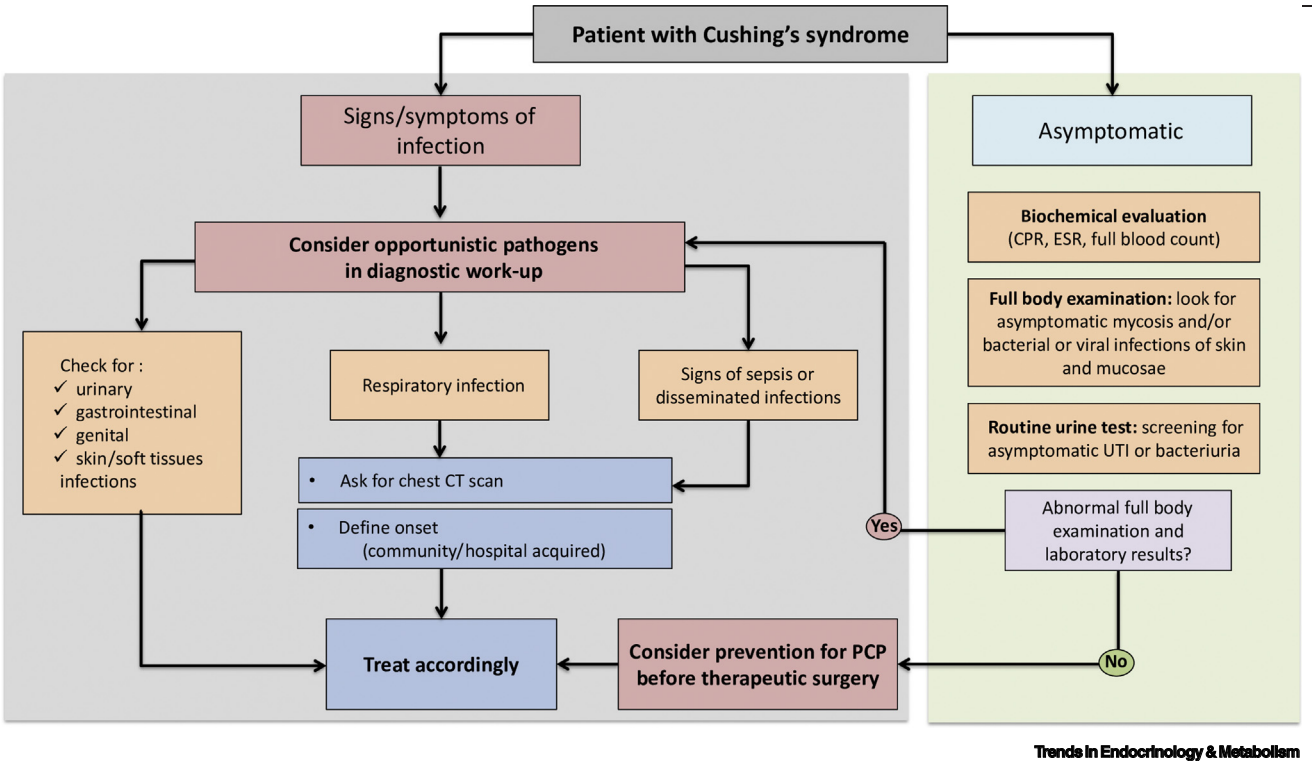

Figure 3. Flow Chart to Reduce the Risk of Infection-Related Morbidity in Cushing's Syndrome. Abbreviations: CSR, C-reactive protein test; CT, computed tomography; ESR, erythrocyte sedimentation rate; PCP, pneumocystis pneumonia; UTI, urinary tract infection.

GC resistance after long-term remission could explain the prolonged effects on the immune system.

In addition, when surgical remission is not achieved or feasible, the aim of medical intervention in CS patients is to restore eucortisolism, but some of the available regimens are unable to control all the features of autonomous cortisol secretion, resulting in the loss of circadian rhythm and increased exposure at anticircadian times of day. Evaluating the impact of different therapies on immune function could therefore significantly contribute to risk stratification in patients with hypercortisolism, providing the basis for more tailored therapies and post-remission follow-up.

In conclusion, taking care of the immune system dysregulation in CS should become a standard of care. A tentative algorithm is proposed to open the discussion around the need of dedicated trials (Figure 3). By acknowledging the immune alterations, including a more thorough evaluation of signs, symptoms, and predisposing factors and developing strategies to prevent the risk of infections, we could improve the prognosis, quality of life, and outcome of patients with hypercortisolism.

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