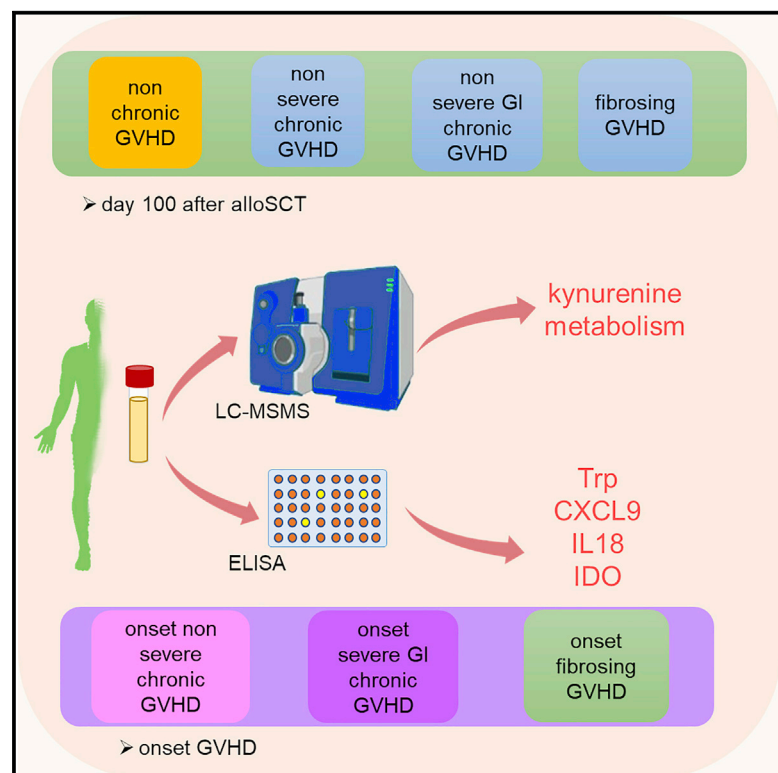


Kynurenine pathway activation and deviation to anthranilic and kynurenic acid in fibrosing chronic graft-versus-host disease

Graphical abstract



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In brief

Orsatti et al. show that severe cGVHD is a debilitating complication after alloSCT. The authors demonstrate that cytokines/chemokines that associate with cGVHD also correlate with metabolic alterations in the kynurenine pathway and the composition of metabolites known to influence fibrosis. Vitamin B2/B6 deficiencies are possibly involved and may be adjusted.

Highlights

- High IDO activity and an activated Kyn pathway are common in all cGVHD subtypes
- Specific Kyn metabolism patterns were identified for gastrointestinal and fibrosing cGVHD
- A pathway shift toward anthranilic and kynurenic acid was found in fibrosing cGVHD
- A rationale for vitamin B2/B6 adjustment for cGVHD prevention is presented



Article

Kynurenine pathway activation and deviation to anthranilic and kynurenic acid in fibrosing chronic graft-versus-host disease

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SUMMARY

Fibrosing chronic graft-versus-host disease (cGVHD) is a debilitating complication of allogeneic stem cell transplantation (alloSCT). A driver of fibrosis is the kynurenine (Kyn) pathway, and Kyn metabolism patterns and cytokines may influence cGVHD severity and manifestation (fibrosing versus gastrointestinal [GI] cGVHD). Using a liquid chromatography-tandem mass spectrometry approach on sera obtained from 425 patients with allografts, we identified high CXCL9, high indoleamine-2,3-dioxygenase (IDO) activity, and an activated Kyn pathway as common characteristics in all cGVHD subtypes. Specific Kyn metabolism patterns could be identified for non-severe cGVHD, severe GI cGVHD, and fibrosing cGVHD, respectively. Specifically, fibrosing cGVHD was associated with a distinct pathway shift toward anthranilic and kynurenic acid, correlating with reduced activity of the vitamin-B2-dependent kynurenine monoxygenase, low vitamin B6, and increased interleukin-18. The Kyn metabolite signature is a candidate biomarker for severe fibrosing cGVHD and provides a rationale for translational trials on prophylactic vitamin B2/B6 supplementation for cGVHD prevention.

INTRODUCTION

Sclerodermatous/fibrosing chronic GVHD (cGVHD) is a debilitating immunological complication after allogeneic stem cell transplantation (alloSCT). It is associated with increased mortality and life-long morbidity in patients who are cured from their malignant disease. The pathophysiology of cGVHD, including development of fibrosis, is poorly understood, similar to fibrosing rheumatological diseases. Current therapeutic standards, such as systemic immunosuppression and extracorporeal photopheresis (ECP) can provide satisfactory symptom relief and control of progressive sclerodermatous transformation only in a few patients.¹ The kynurenine (Kyn) pathway (KP) (Figure 1) is a major modulator of inflammation and fibrosis with numerous active metabolites and may be involved in cGVHD pathophysiology. Kyn is itself a metabolite of tryptophan (Trp), an essential amino acid. The KP of Trp degradation contributes substantially to the control of general inflammation,² and increased indoleamine-2,3-dioxygenase (IDO) activity seems to skew helper T cell polarization toward a T helper 2 (Th2) phenotype.^{3,4} These immune suppressive mechanisms appear to promote chronic infection⁵ and cancer cell escape.⁶

Trp metabolites have been reported to regulate fibrosis. Kyn and kynurenic acid (KA) are endogenous ligands of the aryl hydrocarbon receptor (AhR), which is probably involved in sup-

pressing fibroproliferation and fibrotic phenotypes *in vivo*.⁷ Application of Kyn and KA *in vitro* and in wound models antagonizes fibrosis.⁸ Anthranilic acid (AA) has been associated with suppression of the expression and/or action of the transforming growth factor β (TGF- β) pathway and anti-fibrotic effects; however, that information is derived from experiments with the synthetic analog tranilast (*N*-[3',4'-dimethoxycinnamoyl]-anthranilic acid).⁹ Importantly, KA enhances the kynureninase A (KYNU) reaction (Kyn \rightarrow AA), thereby lowering the 3-hydroxyanthranilic acid (3-HAA)/AA ratio.^{10,11} That ratio is decreased in a variety of disorders and likely represents a protective response to limit primary and secondary damage.^{10,11}

The Kyn catabolism is linked to interferon- γ (IFN- γ)-mediated immune reactions. IDO activity is induced by IFN- γ ,¹² and monokines induced by IFN- γ (MIG, CXCL9, and CXCL9) plasma levels were shown to be significantly increased in patients with cGVHD in two independent, large cohorts.^{13,14} CXCL9 is expressed in response to IFN- γ in macrophages, neutrophils, and even endothelial cells.¹⁵ We have recently reported¹⁶ that CXCL9, at onset of mild cGVHD symptoms, predicts development of later severe forms of the disease. IFN- γ is induced by interleukin (IL)-18, and IL-18 was shown to be increased in cGVHD. Interestingly, a beneficial effect of IL-18 in prevention and treatment of cGVHD in mice has been reported.¹⁷



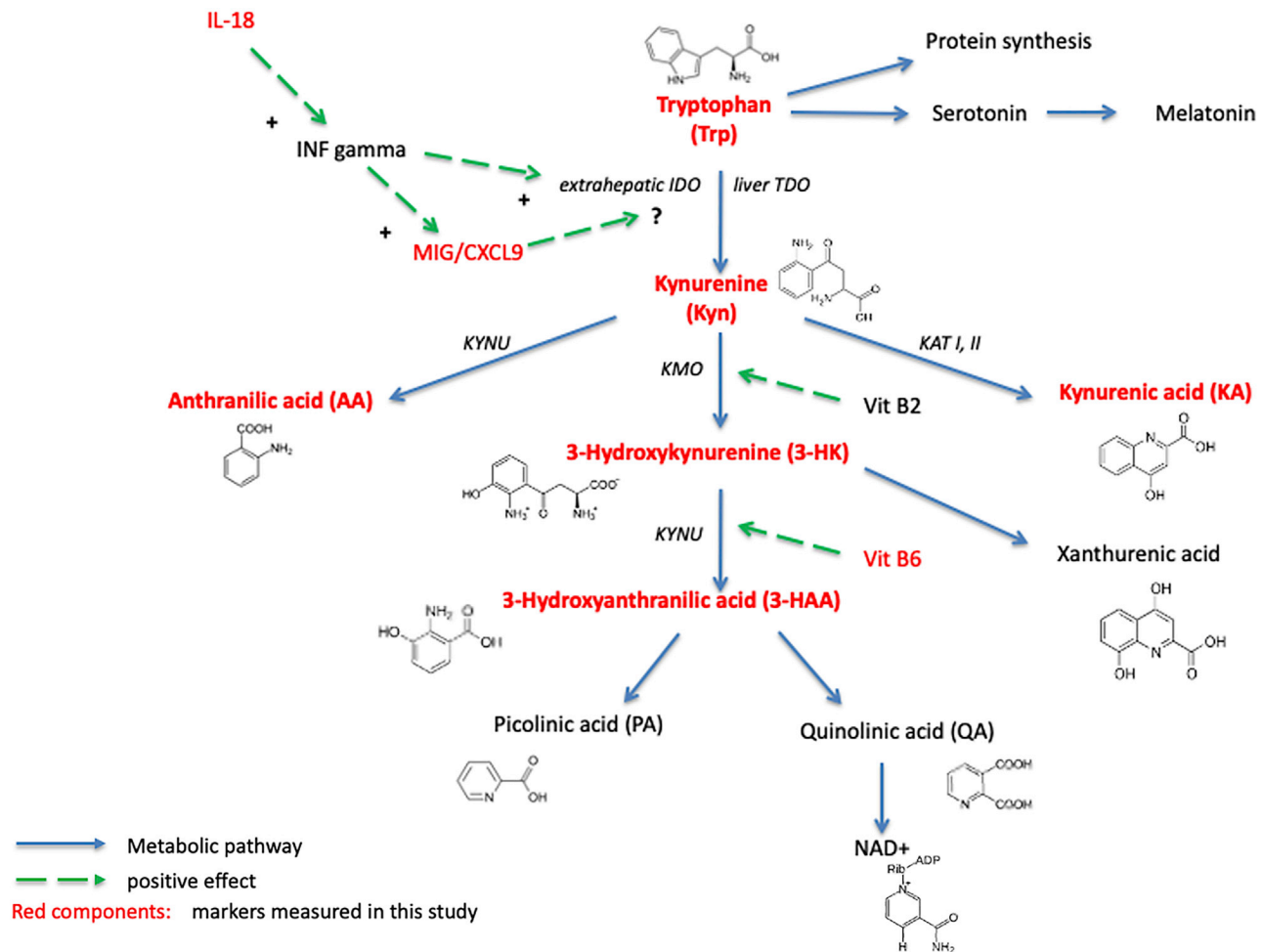


Figure 1. Kynurenine pathway

The kynurenine pathway (KP) constitutes the main route of tryptophan (Trp) degradation. More than 95% of dietary Trp is metabolized via the KP, and only 1% is converted to serotonin. The rate-limiting first step of the KP is the opening of the indole ring by either Trp-2,3-dioxygenase (TDO; liver enzyme) or indoleamine-2,3-dioxygenase (IDO; many cell types, induced by IFN- γ). IL-18 is an inducer of IFN- γ , and IFN- γ induces CXCL9. L-kynurenine (Kyn) is the key compound metabolized by three different enzymes in mammalian tissues: (1) kynurenine-3-hydroxylase (or -monooxygenase [KMO]), which forms 3-hydroxykynurenine (3-HK), quinolinic and picolinic acid, and finally nicotinamide adenine dinucleotide⁺ (NAD⁺); (2) kynureninase (KYNU), which forms anthranilic acid (AA) and 3-hydroxyanthranilic acid (3-HAA); and (3) kynurenine aminotransferases (KAT I and KAT II), which form kynurenic acid (KA). KMO metabolism of Kyn to 3-HK is enhanced by vitamin B2, whereas the conversion of 3-HK to 3-HAA is enhanced by vitamin B6.

In summary, the KP participates in most pathophysiological mechanisms involved in acute and chronic GVHD, such as chronic inflammation and fibrosis.

The aim of the present work was to study the contribution of the IFN- γ pathway and Kyn-derived metabolites to human cGVHD, in particular, the severity and organ manifestations (fibrosing cGVHD versus severe gastrointestinal [GI] cGVHD). Given the species-specific differences in the KP under physiological and pathological conditions,¹⁸ this study focuses on human biology. We used a novel liquid chromatography-tandem mass spectrometry (LC-MS/MS) approach in a large cohort of patients with allografts at two different time points. We identified specific Kyn metabolism patterns for non-severe cGVHD, severe GI cGVHD, and fibrosing cGVHD, providing a molecular distinction between fibrosing and non-fibrosing cGVHD.

RESULTS

Patient characteristics and cGVHD subtypes

Patient characteristics according to type of cGVHD are shown in Table 1. The median observation period was 62.8 months (0.3–172). Patient numbers in the different subgroups are represented in Figure S1. cGVHD was more frequent in patients not receiving anti-thymocyte globulin (ATG) prophylaxis and, correspondingly, in matched, related donors. Multiple myeloma was overrepresented in severe GI cGVHD, and female donors were more frequent in severe fibrosing cGVHD. Finally, high-grade, acute GVHD was associated with more-severe cGVHD.

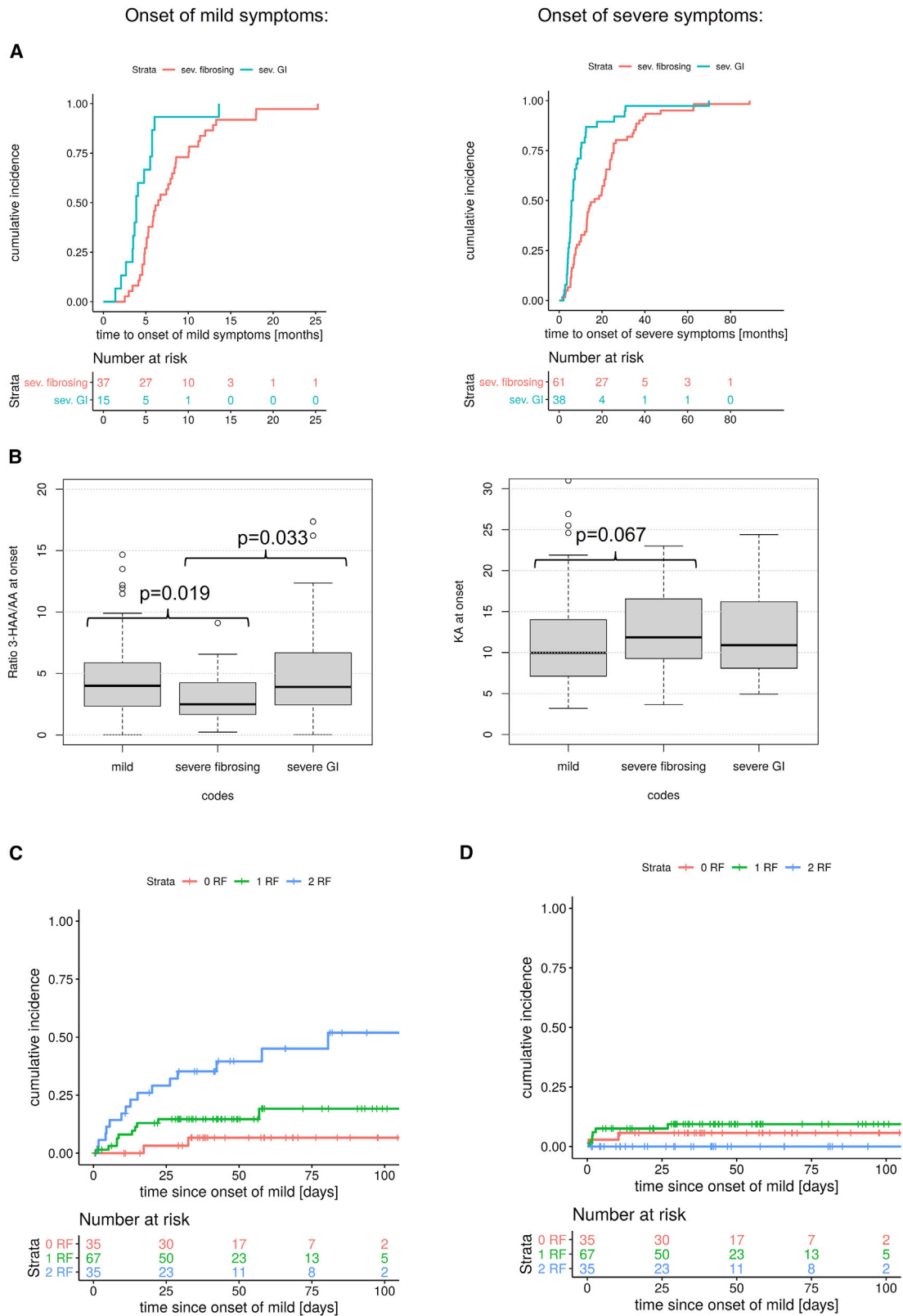
For a first attempt to categorize distinct entities of cGVHD, we distinguished severe fibrosing cGVHD (scleroderma and lung) from severe GI cGVHD (acute-on-chronic GI cGVHD, hepatic

Table 1. Patient, disease, and transplant characteristics

Parameter	No cGVHD n = 190	Non-severe cGVHD n = 176	Severe fibrosing cGVHD n = 51	Severe GI cGVHD n = 36	p value
Age, years, at alloSCT, median (range)	54 (17–75)	53 (20–70)	53 (20–68)	46 (20–65)	0.045
Patient gender, n (%) male	115 (60)	109 (62)	35 (69)	19 (53)	0.506
Diagnosis, n (%)					
AML	63 (33)	48 (27)	15 (29)	6 (17)	0.001
ALL/lymphoma	75 (39)	69 (39)	16 (31)	10 (28)	
MDS/MPN	32 (17)	23 (13)	13 (26)	5 (14)	
MM	20 (11)	36 (21)	7 (14)	15 (42)	
Disease score, n (%)					
0	62 (32)	60 (39)	19 (37)	11 (31)	0.661
1	51 (27)	47 (27)	9 (18)	5 (14)	
2	68 (26)	63 (36)	20 (39)	17 (47)	
N/A	9 (5)	6 (3)	3 (6)	3 (8)	
Donor type, n (%)					
MRD	36 (19)	80 (46)	23 (45)	17 (47)	<0.001
MUD	102 (53)	63 (36)	16 (31)	13 (36)	
MMUD	48 (25)	27 (15)	11 (22)	5 (14)	
MMRD	4 (3)	6 (3)	1 (2)	1 (3)	
Donor gender, n (%)					
Male	147 (77)	109 (62)	27 (53)	23 (64)	0.001
Stem cell source, n (%)					
PB	176 (93)	163 (93)	49 (96)	34 (94)	0.816
BM	14 (7)	13 (7)	2 (4)	2 (6)	
Conditioning regimen,^a n (%)					
RIC	159 (83)	145 (82)	41 (80)	33 (92)	0.521
MAC	31 (17)	31 (18)	10 (20)	3 (8)	
First-line immunosuppression					
ATG, n (%)	160 (84)	83 (47)	20 (39)	15 (42)	<0.001
MTX, n (%)	63 (34)	59 (34)	19 (37)	13 (36)	
MMF, n (%)	127 (67)	117 (67)	32 (63)	23 (64)	0.942
CsA, n (%)	155 (82)	151 (86)	41 (80)	32 (89)	
Tacrolimus, n (%)	35 (18)	25 (14)	10 (20)	4 (11)	0.511
Statin+UDCA prophylaxis, n (%)	102 (53)	82 (47)	17 (33)	10 (28)	0.006
Acute GVHD, n (%)					
No	129 (68)	97 (55)	23 (45)	19 (53)	0.017
Grade 1–2	33 (17)	56 (32)	13 (26)	10 (29)	
Grade 3–4	15 (8)	14 (8)	9 (18)	4 (11)	
Steroid-refractory	13 (7)	9 (5)	6 (12)	3 (8)	
DLI	41 (21)	35 (20)	2 (4)	2 (6)	0.005

Abbreviations: ALL, acute lymphoblastic leukemia; alloSCT, allogeneic stem cell transplantation; AML, acute myeloid leukemia; ATG, antithymocyte globulin; aGVHD, acute graft-versus-host-disease; BM, bone marrow; cGVHD, chronic graft-versus-host-disease; CsA, cyclosporine A; DLI, donor leukocyte infusion; MDS, myelodysplastic syndrome; MAC: myeloablative conditioning; MM, multiple myeloma; MMRD, mismatched related donor; MMUD, mismatched unrelated donor; MPN, myeloproliferative neoplasm; MRD, matched related donor; MTX, methotrexate; MMF, mycophenolate mofetil; MUD, matched unrelated donor; N/A, not applicable; PB, peripheral blood; RIC, reduced intensity conditioning; UDCA, ursodeoxycholic acid.

^aAccording to Bacigalupo et al.¹⁹ and Bornhäuser et al.²⁰



(legend on next page)

cGVHD, and severe weight loss > 15%). These groups differed strongly in their median time to onset of mild symptoms (fibrosing: 6.5 months; GI: 3.9 months) and onset of severe symptoms (fibrosing: 16.5 months; GI: 6.1 months) (Figure 2A).

Increased day+100 CXCL9 and KP activity in patients with subsequent cGVHD

Day+100 peripheral-blood Kyn-metabolite levels and cytokine levels (CXCL9 and IL-18) were compared between patients who did not develop cGVHD and those who developed cGVHD within the observation period. Patients with any grade cGVHD harbored increased serum levels of CXCL9, but not IL-18, on day+100 (Table 2). In addition, Trp and AA serum levels were significantly higher (Table 2). Patients with later severe fibrosing cGVHD had higher serum levels of AA and IL-18 than later severe GI cGVHD and a lower 3HAA/AA ratio than later non-severe cGVHD at this day+100 time point (Table S1).

The Kyn pathway at onset of cGVHD

We analyzed metabolite serum level changes between day+100 and onset of cGVHD individually for each subgroup. We found that, in patients with later cGVHD, Trp and AA were already elevated on day+100. Both metabolites remained high but did not increase any further until the onset of (any grade) cGVHD (Tables S2–S4). In contrast, CXCL9 was already elevated on day+100 and rose further until onset of non-severe cGVHD, severe fibrosing cGVHD, and severe GI cGVHD, similar to IL-18 (Tables S2–S4). Although median serum levels were highest at onset of GI cGVHD, there were no significant differences in CXCL9 between the cGVHD subgroups (Table 3). However, as shown previously,¹⁶ patients with severe cGVHD (fibrosing and GI combined) had higher CXCL9 serum levels on day+100 and at onset of cGVHD as compared with patients with non-severe cGVHD. Accordingly, IDO levels increased between day+100 and onset in all cGVHD entities, suggesting an IFN- γ -mediated induction (Tables S2–S4).

In addition, the flux from Kyn to 3-HK and further toward 3-HAA was significantly increased in all three entities (27.3% in non-severe cGVHD, 33.3% in severe fibrosing cGVHD, and 42.4% in severe GI cGVHD) between day+100 and onset (Table 3).

This is contrasted by a shift in fluxes (trend) specifically in fibrosing cGVHD toward AA (33.3%) and KA (46.2%) (Table 3),

whereas, in non-severe cGVHD, the shifts toward AA and KA fluxes were 7.9% and 5.7%, and -5.1% and 9.8% , respectively, in severe GI cGVHD (Table 3). This specific difference is further underscored by direct comparison of cGVHD entities in Table 3. We observed significantly increased AA serum levels and a lower ratio of 3-HAA/AA at the onset of severe fibrosing cGVHD as compared with non-severe or severe GI cGVHD. There was a trend toward higher KA levels at the onset of fibrosing cGVHD compared with non-severe cGVHD. The differences in the relevant Kyn metabolites in severe fibrosing cGVHD as compared with non-severe or severe GI cGVHD are shown in Figure 2B.

3-HAA/AA and KA levels at onset of mild symptoms and risk of progression to severe fibrosing cGVHD

Reduced 3-HAA/AA and a pronounced increase in the fluxes toward KA are specifically associated with severe fibrosing cGVHD. We, therefore, hypothesized that these two parameters predict risk of ensuing severe fibrosing cGVHD at first onset of mild symptoms. We identified $n = 137$ patients with serum available at onset of mild cGVHD symptoms. Twenty-seven of those patients progressed to severe fibrosing cGVHD, whereas eight progressed to severe GI cGVHD. The remaining 102 patients with mild cGVHD symptoms did not progress to severe cGVHD within the observation period.

Because of the limited number of events, we dichotomized the two variables according to their medians at onset of mild cGVHD symptoms ($n = 137$) (ratio of 3-HAA/AA: median = 3.64 [range, 0.01–101.8], KA: median = 10.4 nM [range, 3.2–69.5 nM]). Multivariable Cox regression analysis, including both binary variables, revealed that high KA and low 3-HAA/AA were both associated with severe fibrosing cGVHD (Table S5A). This allowed us to establish a score with two independent risk factors (RFs): high (> median) KA and low (< median) 3-HAA/AA, yielding three groups: 0 versus 1 versus 2 RFs. Figure 2C shows that this score predicted risk of severe fibrosing cGVHD but not the risk of severe GI cGVHD. Multivariable Cox regression analysis, with human leukocyte antigen (HLA) mismatch, ATG, donor gender, diagnosis (multiple myeloma), and previous acute GVHD as co-variables, revealed a strong and significant association of the risk score with severe fibrosing cGVHD (Table 4).

Of note, the risk score based on KA and 3-HAA/AA predicted severe fibrosing cGVHD much better in patients without ATG

Figure 2. Differences in cGVHD subtypes

(A) Comparison of the onset of mild symptoms (left panel) and the onset of severe symptoms (right panel) in patients with severe fibrosing cGVHD and severe gastro-intestinal (GI) cGVHD. Left: all patients who develop first mild, and then severe, cGVHD during the observation period are included. Start of follow-up: day 0 of alloSCT; event, onset of mild symptoms, no censoring. Median time until onset of mild symptoms, fibrosing: 6.5 months ($n = 37$), GI: 3.9 months ($n = 15$). Right: included are all patients who develop severe cGVHD during observation. Start of follow-up: day 0 of alloSCT; event, onset of severe symptoms, no censoring. Median time until onset of severe symptoms, fibrosing: 16.5 months ($n = 61$), GI: 6.1 months ($n = 38$).

(B) Boxplots of the ratio of 3HAA/AA and the concentration of KA at the onset of cGVHD, $n = 161$. The median is indicated by a horizontal line. The upper and lower border of the box correspond to the 3rd and 1st quartiles. The whiskers correspond to the most extreme data points, which are no more than 1.5 times the interquartile range away from the upper and lower border of the box.

(C and D) Cumulative incidence of severe cGVHD after onset of mild symptoms, stratified by the Kyn metabolite score (0, 1, or 2 risk factors [RFs]). Included are all patients who developed mild cGVHD symptoms. Follow-up starts at the time of onset of mild cGVHD symptoms. (C) Event: onset of severe fibrosing cGVHD (27 events), censored at last follow-up without severe fibrosing cGVHD or at death without severe fibrosing cGVHD. Cox regression two RFs versus < 2 RFs: HR, 3.92; 95% CI, 1.83–8.37; $p < 0.001$. (D) Event: onset of severe GI cGVHD (eight events), censored at last follow-up without severe GI cGVHD or at death without severe GI cGVHD. Cox regression two RFs versus < 2 RF: n.s.

RF: ratio of 3HAA/AA < median, and KA > median.

Table 2. Concentrations on day+100 after alloSCT in cGVHD-naïve patients with or without subsequent cGVHD development

	Concentration (nM), median (IQR)		Kruskal-Wallis p value	No. of patients	
	Day+100 without subsequent cGVHD	Day+100 with subsequent cGVHD		No subsequent cGVHD	Subsequent cGVHD
Tryptophan	46.5 (31.8–64.6)	56.3 (43.1–70)	<0.001	163	212
Kynurenine	551 (419–694.5)	578 (436–747)	0.238	159	185
Kyn/Trp ^a	12.3 (8–18.2)	10.2 (7.4–17.3)	0.078	133	162
IDO	0.18 (0–0.34)	0.18 (0–0.47)	0.807	145	206
AA	0.75 (0.54–1.1)	0.95 (0.63–1.3)	0.001	160	186
3-HAA	2.6 (1.9–3.9)	3 (2–4.1)	0.286	160	186
3-HAA/AA ^a	3.9 (2.4–5.4)	3.3 (1.8–5.3)	0.211	160	186
KA	9.5 (7.2–12.8)	9.8 (7.2–13.1)	0.714	159	185
3-HK	11.7 (8.4–15.6)	10.8 (8.1–16.9)	0.883	160	186
MIG (CXCL9) ^b	243.8 (135.7–424.4)	318.8 (179.1–633.7)	0.002	170	213
IL-18 ^b	396 (294.2–555.1)	410.3 (269.7–614.9)	0.640	162	201

cGVHD, chronic graft-versus-host disease; IQR, inter quartile range; IDO, indoleamine-2,3-dioxygenase; MIG, monokine induced by interferon- γ ; AA, anthranilic acid; 3-HAA, 3-hydroxy-anthranilic acid; KA, kynurenic acid; 3-HK, 3-hydroxy-kynurenine.

^aWithout concentration units.

^bpg/mL.

prophylaxis (2 RFs versus 0 + 1 RF: hazard ratio [HR], 4.57; 95% confidence interval [95% CI], 1.92–10.86; $p < 0.001$) than in patients receiving ATG (2 risk factors versus 0 + 1 RF: HR, 2.86; 95% CI, 0.58–14.17; $p = 0.198$) (Figure S2).

Association of survival with 3-HAA/AA and KA

We next tested whether the metabolic changes that are associated with severe fibrosing cGVHD also mediate a survival advantage (suggesting a beneficial counter-regulation). In multivariable Cox regression analysis (endpoint overall survival [OS]) with the two markers (ratio of 3-HAA/AA and KA) and the covariables, as in Table 4, a significantly increased mortality was observed for patients with high KA on day+100 and at onset of cGVHD, whereas low 3-HAA/AA did not significantly associate with increased mortality (Tables S5B and S5C).

Possible mechanism for KP deviation in severe fibrosing cGVHD: Low Kyn 3-monooxygenase (KMO) activity (vitamin B2 dependent) and vitamin B6 deficiency

We hypothesized that the deviation toward AA and KA in fibrosing cGVHD was caused by a spillover due to a reduced drain of Kyn toward 3-HK and 3-HAA. The Kyn degradation toward 3-HK and 3-HAA is catalyzed by KMO and KYNU, and these enzymes depend on the co-enzymes vitamin B2 and vitamin B6. To underpin our hypothesis, we asked whether an increase in KA and AA was associated with reduced KMO activity and vitamin B6 deficiency. For that purpose, the ratio of 3-HK/Kyn was used as a readout for KMO activity.²¹ Vitamin B6 was measured in sera taken on day+100 from 324 patients; of whom, 198 (61%) were identified to have a vitamin B6 deficiency (<15 nM, retrospective analysis). Because a “reference range” or a validated cutoff defining high and low KMO activities does not exist, we considered each KMO activity value detected in our dataset as a potential cutoff to separate patients with high KMO activity from patients with low KMO activity. For each possible cutoff value, we

compared the concentrations of the metabolites (AA, KA, and the ratio of 3-HAA/AA) in the patients with KMO activity greater than, and the patients with KMO activity less than, the cutoff. Following that approach, low KMO activity on day+100 was significantly correlated with increased AA serum levels within a wide range of KMO cutoffs tested (0.004–0.028) (Figure S3A). To assess the combined influence of low KMO activity and vitamin B6 deficiency, the group of patients with low KMO activity was further subdivided according to the vitamin B6 concentration in their serum (concentrations < 15 nM were considered a deficiency) (Figure S3). This demonstrated that the AA serum levels were further increased if low KMO activity was combined with low vitamin B6 serum levels (<15 nM) (Figure S3B). Similarly, low KMO activity and low vitamin B6 correlated with low 3-HAA and a reduced ratio of 3-HAA/AA (Figures S3C–S3F). In contrast, neither low KMO activity nor vitamin B6 deficiency was associated with increased KA (Figure S3G). To assess the full Kyn \rightarrow 3HK \rightarrow 3-HAA axis (representing both KMO activity and vitamin B6 deficiency), the 3-HAA/Kyn ratio was calculated and correlated across all 344 (d 100) or 164 (onset) possible cutoffs with AA and KA. We observed the expected significant correlation of a low 3-HAA/Kyn ratio with increased AA, but not with KA levels on day+100 (Figure S4A). Importantly, this finding was similar, and even more pronounced, at the onset of chronic GVHD (Figure S4B). A summary of those results is visualized in Figure 3A.

Possible mechanism for KP deviation in severe fibrosing cGVHD: CXCL9 and IL-18

The two cytokines involved in the IFN- γ pathway were measured on day+100 and at onset of cGVHD. Although there was a significant correlation with both markers (Spearman ρ coefficient day+100: 0.397, $p < 0.001$; onset mild cGVHD symptoms: 0.456, $p < 0.001$), distinct associations with the Kyn metabolism were observed. On day+100, CXCL9 was increased in all patients with subsequent cGVHD and did not distinguish cGVHD

Table 3. Concentrations and fluxes of metabolites at the onset of non-severe, severe fibrosing, and severe GI cGVHD compared with each other

	Concentration, nM at onset, median (IQR)			p value (Kruskal-Wallis)			No. of patients		
	Non-severe cGVHD	Severe fibrosing cGVHD	Severe GI cGVHD	Non-severe versus severe fibrosing cGVHD	Non-severe versus severe GI cGVHD	Severe fibrosing versus severe GI cGVHD	Non-severe cGVHD	Severe fibrosing cGVHD	Severe GI cGVHD
Trp	57 (37.4–79.3)	59.8 (45.2–79.1)	59.6 (47–74.6)	0.56	0.487	0.908	119	37	34
Kyn	720 (532.8–1,022.5)	868.5 (618.5–1,285)	681 (493–946)	0.034	0.819	0.086	102	32	27
Kyn/Trp ^a	14.1 (8.1–30)	15.9 (11.8–19.5)	10.3 (7.4–17.8)	0.731	0.156	0.075	100	29	27
IDO	0.56 (0.13–1.7)	0.75 (0.2–1.2)	0.49 (0.02–1.8)	0.788	0.648	0.635	116	37	34
AA	1.2 (0.71–1.8)	1.5 (1.2–3.2)	1.0 (0.49–1.6)	0.016	0.256	0.013	102	32	27
3-HAA	4.2 (2.7–5.2)	4.8 (2.5–6)	4.2 (2.4–5.5)	0.514	0.824	0.513	102	32	27
3-HAA/AA ^a	4.0 (2.4–5.8)	2.5 (1.8–4.1)	3.9 (2.5–6.7)	0.019	0.658	0.033	102	32	27
KA	10 (7.1–14)	11.9 (9.5–16.3)	10.9 (8.1–16.2)	0.067	0.287	0.626	102	32	27
3-HK	12.5 (9–21.2)	15.7 (10.8–28.1)	14.5 (8–21.1)	0.102	0.596	0.429	102	32	27
MIG (CXCL9) ^b	678.1 (308.9–1450.2)	765.8 (346.7–2,115.8)	1,438.6 (268.5–2,799.5)	0.27	0.288	0.945	118	37	34
IL-18 ^b	580.8 (423–735.2)	633.3 (517.9–750)	620.1 (503.6–750)	0.211	0.366	1.000	116	27	14
Kyn → AA	7.9% (–34.5% to 107.8%)	33.3% (–41.6% to 214.1%)	–5.1% (–48.3% to 35%)	0.009 ^c	0.766 ^d	0.157 ^e	83	21	18
Kyn → KA	5.7% (–20.6% to 54.5%)	46.2% (–22.3% to 74.2%)	9.8% (–8.8% to 40.6%)	0.016 ^c	0.099 ^d	0.009 ^e	83	21	18
Kyn → 3-HK	27.3% (–2.8% to 146.7%)	33.3% (5.3% to 181.6%)	42.4% (0.43% to 190.2%)	<0.001 ^c	0.004 ^d	<0.001 ^e	83	21	18
3-HK → 3-HAA	27.3% (–2.8% to 146.7%)	33.3% (5.3% to 181.6%)	42.4% (0.43% to 190.2%)	<0.001 ^c	0.004 ^d	<0.001 ^e	83	21	18

cGVHD, chronic graft-versus-host disease; IQR, inter quartile range; IDO, indoleamine-2,3-dioxygenase; MIG, monokine induced by interferon- γ ; AA, anthranilic acid; 3-HAA, 3-hydroxy-anthranilic acid; KA, kynurenic acid; 3-HK, 3-hydroxy-kynurenine.

^aWithout concentration units.

^bpg/mL.

^cp value for non-severe cGVHD.

^dp value for severe GI cGVHD.

^ep value for severe fibrosing cGVHD → flux of metabolites d 100 to onset cGVHD (paired Wilcoxon test).

Table 4. Multivariate Cox regression analysis for developing severe fibrosing cGVHD, including KA and 3-HAA/AA serum levels at the onset of non-severe cGVHD symptoms (n = 137, number of events = 27)

	HR	95% CI low	95% CI high	p value
Low ratio of 3-HAA/AA ^a and high KA ^a at onset				
2 RFs versus 0 = ref	8.46	1.90	37.63	0.005
2 RFs versus 1 = ref	3.12	1.39	6.97	0.006
Donor gender (male versus female = ref)	0.94	0.43	2.05	0.876
ATG (no ATG = ref)	0.25	0.08	0.76	0.014
Multiple myeloma (no = ref)	0.91	0.33	2.52	0.854
HLA-mismatch (8/8 = ref)	2.08	0.63	6.81	0.227
Previous aGVHD (no = ref)	0.90	0.41	2.00	0.804

Observation period: onset of non-severe cGVHD symptoms until end of follow-up. Abbreviations: HR, hazard ratio; 95% CI, confidence interval. Included are all patients who develop mild cGVHD. Start of follow-up at onset of mild symptoms, event: onset of severe fibrosing cGVHD, censored at last follow-up without severe fibrosing cGVHD or at death without severe fibrosing cGVHD.

^aHigh > median value; low < median value.

subtypes. Increased IL-18 serum levels at that time point were measured in patients with subsequent fibrosing cGVHD (median, 460.1 pg/mL) as compared with subsequent GI cGVHD (median, 345.6 pg/mL; $p = 0.030$) (Table S1). Both cytokines increased further between day+100 and the onset of (any subtype) of cGVHD (Tables S2–S4). To assess the effects of high versus low cytokine levels on the activity of the Kyn metabolic pathway, CXCL9 and IL-18 were correlated with IDO, AA, KA, and 3-HAA across all possible cutoffs. In all possible cutoffs, high CXCL9 correlated with a significant induction of IDO and IDO activity (ratio of Kyn/Trp), both on day+100 and at the onset of cGVHD, whereas IL-18 showed a much weaker association (Figures S5A and S5B; visualized in Figure 3B). Moreover, high CXCL9 serum levels were associated with increased AA, but not KA, on day+100 and at the onset of cGVHD (Figures S6A and S6B). In contrast, IL-18 correlated with significantly increased AA and KA on day+100 and was even more pronounced at the onset of cGVHD (Figures S7A and S7B; visualized in Figure 3C).

DISCUSSION

Chronic GVHD is an immune-mediated disease. A substantial proportion of patients do not respond sufficiently to immunosuppressive medications, but rather, progress in flares and breakthroughs after tapering the initial steroid therapy. Nevertheless, patients transplanted for malignant disorders experiencing non-severe cGVHD have a better overall outcome than do patients without any cGVHD because of relapse prevention by graft-versus-tumor activity.^{16,22} Thus, the primary medical goal should be to avoid the morbidity and mortality associated with severe cGVHD rather than preventing cGVHD in general.

Chronic GVHD often occurs in protracted phases. Initially, mild symptoms worsen gradually, and fibrotic changes accumulate

with each flare-up. Progressive fibrosis leads to extended periods of intensive immune suppression and increased infection-related mortality. Current grading systems are in place to guide immunosuppressive therapy and predict outcome of the actual flare-up. The grading systems do not reflect either pathophysiology or long-term prognosis of cGVHD.

In this study, we have analyzed three groups of patients: those with non-severe, severe fibrosing, and severe GI cGVHD. That gradation was based on clinical observations: better survival for patients with non-severe cGVHD compared with patients without cGVHD¹⁶ and different times of symptom onset in fibrosing and GI cGVHD. We observed features shared by all types of cGVHD, such as increased CXCL9 levels, increased IDO levels, and increased KP activity. That is consistent with the presence of a common IFN- γ response and underscores the important role of T cells in the pathophysiology of cGVHD.²³

The KP constitutes the main route of Trp degradation and is associated with the production of neuroactive compounds: quinolinic acid (QA) is an excitotoxic agonist at the *N*-methyl-D-aspartate (NMDA) receptor,²⁴ whereas KA is a broad-spectrum glutamate receptor antagonist and neuroprotective.^{25,26} More than 95% of dietary Trp is metabolized via the KP, and only 1% is converted to serotonin.²⁷ The rate-limiting first step of the KP is the opening of the indole ring by either Trp-2,3-dioxygenase (TDO) or IDO (Figure 1). TDO is expressed mainly in the liver and is induced by a variety of stimuli (e.g., cortisol). IDO is expressed by various cells; its main inducer is the Th1-cytokine interferon, IFN- γ .²⁸ L-Kyn is metabolized by three different enzymes in mammalian tissue: (1) Kyn hydroxylase (or monooxygenase [KMO]), which forms 3-HK, QA, phosphatidic acid (PA), and finally, nicotinamide adenine dinucleotide (NAD⁺); (2) KYNU, which forms AA; and (3) Kyn aminotransferase (KAT), which forms KA. KP metabolites were reported to participate in fibrosis.^{7,9,26,29}

Severe GI cGVHD and non-severe cGVHD showed only gradual differences regarding the pathways investigated in this study, although the clinical courses were different. In contrast, fibrosing courses were associated from the onset of symptoms with a qualitative shift within the Kyn catabolism, with enhanced metabolic flux toward AA (resulting in a low 3-HAA/AA ratio) and toward KA. High KA and low 3-HAA/AA, indeed, predicted the risk of severe fibrosing cGVHD after the onset of mild cGVHD symptoms but not the risk of severe GI cGVHD.

Thus, our study provides a molecular distinction between two entities of severe cGVHD. Although our LC-MS/MS-based methodology for measuring Kyn metabolites is still not readily available for clinical routine, it might be a valuable assessment tool for scientific trials. All processing steps were validated. In addition, sample handling and processing were fully automated to reduce total workup time and possible errors from manual sample manipulation. Our results open perspectives for a better understanding of the pathophysiology of fibrosing cGVHD. Specifically, the observation of a deviation in the Kyn metabolism raises a number of new questions. Importantly, it remains to be settled whether the increased production of AA and KA is causally related to fibrosis or whether it is a counter-regulation of the organism trying to amend the impending tissue fibrosis.^{10,11} The increased mortality in patients with high KA does not support the latter; however, there

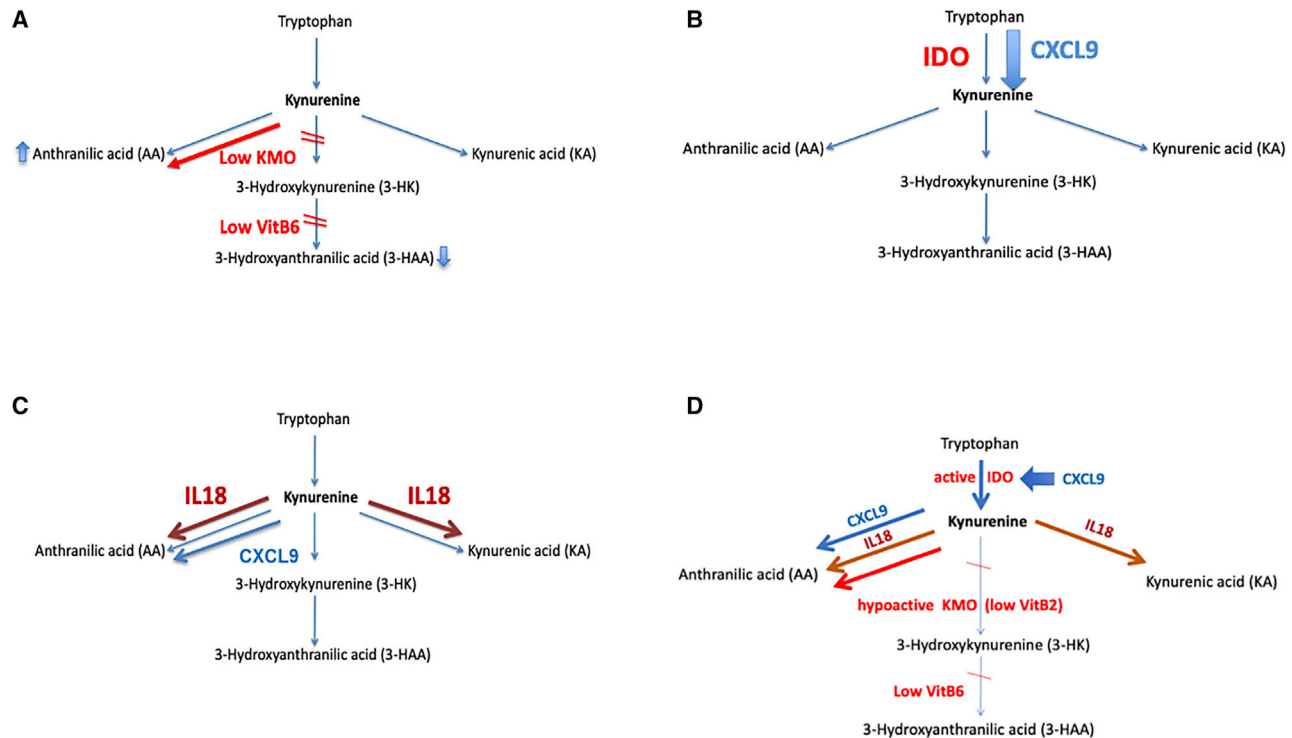


Figure 3. Influence of KMO, vitamin B6, CXCL9, and IL-18 on the kynurenine pathway

(A) Low KMO activity and low vitamin B6 (VitB6) correlate with a low ratio HAA/AA but not with KA (for details, see Figure S3).

(B) High CXCL9 (but not IL-18) correlates with IDO activity (for details, see Figures S5A and S5B).

(C) High IL-18 correlates with high AA and with high KA, and there is no effect of CXCL9 on KA (for details, see Figures S6 and S7).

(D) Triple-hit model of fibrosing cGVHD (hypothesis): severe fibrosing cGVHD is associated with strong CXCL9-induced activation of IDO. The result of this activation is influenced by reduced KMO activity, either because of genetic polymorphisms or because of the lack of vitamin B2, and is further aggravated by a lack of vitamin B6. The resulting metabolic deviation increases AA concentrations and reduces the 3-HAA/AA ratio. Finally, high IL-18 is associated with increased serum KA. These three hits result in a metabolic situation with high KA and a low ratio of 3-HAA/AA, which predicts a risk of fibrosing cGVHD. The number of replicates depend on the available measurements of the respective metabolites (for details, see Figures S3–S7).

was no increased mortality associated with a reduced ratio 3-HAA/AA on day+100 or at onset of cGVHD.

In this regard, it is known that QA and KA act as agonist and antagonist of the NMDA receptor of the excitatory amino acid glutamate.^{24–26} In contrast, a number of Kyn metabolites function as immunomodulators, in particular KA, 3-HK, 3-HAA, QA, and PA.^{10,30} It is, therefore, tempting to hypothesize that QA (metabolic step following 3-HAA) and KA could also have opposite roles in inflammatory conditions, including cGVHD. Darlington et al.¹¹ demonstrated a decrease in the ratio of plasma 3-HAA to AA ([3-HAA]/[AA]) in many inflammatory diseases and proposed that this decrease either reflected inflammatory disease or was an anti-inflammatory response.

Mechanistically, the observed increases in Kyn, AA, and KA in fibrosing cGVHD are reminiscent of reduced KMO activity.³¹ Possible reasons include single-nucleotide polymorphisms in the KMO gene,^{32–34} vitamin B2 (riboflavin) deficiency,^{35–37} or negative regulation by IDO and cytokines.^{38,39} In this context, the involvement of vitamin B2 and vitamin B6 (pyridoxine) in the KP is of particular interest. Vitamin B6 has a strong cofactor effect for KYNU and a weaker effect for KAT.³⁵ Vitamin B deficiencies are frequently observed in patients undergoing alloSCT⁴⁰ and can be

caused by low nutritional intake or inflammation.⁴¹ Vitamin B2 cannot be measured retrospectively in frozen samples, but we were able to perform high-performance liquid chromatography (HPLC)-based analyses of vitamin B6 levels in day +100 sera. Notably, 198 of 324 patients (61%) showed vitamin B6 deficiency (<15 nM) at that time point. Comparing high and low KMO activity in the context of known vitamin B6 serum levels, we observed that both synergized in the drain of Kyn toward 3-HAA, and low activity of both, indeed, was associated with strongly increased AA serum levels. Unexpectedly, this spillover did not reach KA. Thus, although the mechanism remains to be elucidated, our data suggest that vitamin B2/B6 deficiencies may be critically involved in the pathophysiology of severe fibrosing cGVHD.

IL-18, but not CXCL9, turned out to be a marker associated with increased KA, in particular, at onset of cGVHD. The mechanisms behind this observation remain to be clarified, but, in contrast to CXCL9, IDO is not correlated with IL-18. Our data show that both cytokines differentially regulate the Kyn metabolism, although both belong to the inflammatory IFN- γ -associated pathway. A similar functional difference between IL-18 and CXCL9 was observed in mice, in which IL-18 had beneficial effects on the prevention and treatment of cGVHD.¹⁷

Based on the results presented here, the following model for the pathophysiology of fibrosing cGVHD may apply: in the context of a strong CXCL9-based inflammation, IDO with subsequent production of AA and 3-HAA will be induced, which is associated with the development of (any grade) cGVHD. Fibrosing cGVHD may develop if deviations of the KP toward AA and KA occur, specifically because of reduced KMO (low vitamin B2) activity and vitamin B6 deficiency (both, together, reducing the 3-HAA/AA ratio). Third, high IL-18 induces AA and KA elevations, thereby further adding to increased IFN- γ /CXCL9 activity and, thus, cGVHD severity (Figure 3D).

In summary, our study reports specific deviations of the KP in fibrosing cGVHD. The Kyn metabolite signature identified here is a candidate biomarker for distinguishing severe fibrosing cGVHD from other cGVHD forms and may be a valuable tool for characterizing fibrosing cGVHD in clinical trials. In translational terms, this study provides a rationale for prospective assessment of vitamin B2/B6 supplementation in clinical trials

Limitations of study

The limitations of our study include its retrospective nature and the lack of a validation cohort. Furthermore, vitamin B2 levels were not available and neither were vitamin B6 levels at the onset of cGVHD. The mechanisms leading to low KMO activity and a low 3HAA/AA ratio cannot be named, and the mechanism for increased KA serum levels, correlating with high IL-18, remains to be elucidated. Finally, the cytokine network that participates in CXCL9 and IL-18 regulation needs to be studied in a wider scale.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xcrm.2021.100409>.

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AUTHOR CONTRIBUTION

L.O. designed the study, produced and analyzed the data, and wrote the paper; R.S., P.D.P., and E.M. produced and analyzed the data and wrote the paper; K.D., A.R., C.M.-T., and P.D. produced the data and wrote the paper; T.S. designed the study, analyzed the data, developed the mathematical model, and wrote the paper; T.L. designed the study, collected and analyzed the data, and wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We worked to ensure gender balance in the recruitment of human subjects. The author list of this paper includes contributors from the location where the research was conducted who participated in the data collection, design, analysis, and/or interpretation of the work.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Pooled human serum (n = 20)	Sera Laboratories International	N/A
Chemicals, peptides, and recombinant proteins		
L-kynurenine	Evotec	Custom synthesis
3-hydroxy-DL-kynurenine	Evotec	Custom synthesis
Kynurenic acid	Evotec	Custom synthesis
Anthranilic acid	Evotec	Custom synthesis
3-hydroxyanthranilic acid	Toronto Research Chemicals	H801790
L-kynurenine sulfate:H ₂ O (ring-d ₄ , 3,3-d ₂)	Cambridge Isotope Laboratories	DLM-7842-PK
Anthranilic acid-ring- ¹³ C ₆	Sigma-Aldrich	709530
3-Hydroxy-DL-kynurenine ¹⁵ N, ¹³ C ₂	Toronto Research Chemicals	H943697
3,5,6,7,8-d ₅ -KYNA	Qmx Laboratories	D-4391
3-Hydroxyanthranilic acid-d ₃	Toronto Research Chemicals	H801792
Ascorbic acid	Sigma	A92902
Acetonitrile	Fluka	10061044
Water	Li Chrosolv	693520
Formic acid	Sigma	695076
DMSO	Sigma	D2650
Critical commercial assays		
CXCL9 duo set	R&D	DY392
Interleukin-18 duo set	R&D	DY318-05
Tryptophan ELISA	Immundiagnosics, Bensheim, Germany	K7730
IDO ELISA	Immundiagnosics, Bensheim, Germany	KR7727
Deposited data		
Mendeley, https://doi.org/10.17632/c9fmxsctsb.1		https://doi.org/10.17632/c9fmxsctsb.1
Software and algorithms		
Analyst software version 1.6	AB Sciex	N/A
Watson LIMS version 7.6	ThermoFisher Scientific	N/A
R statistical software version 3.44 with package 'survival' version 2.43.	R Foundation for Statistical Computing, Vienna, Austria.	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Thomas Luft (thomas.luft@med.uni-heidelberg.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

All metabolite and cytokine concentrations have been deposited at Mendeley and is publicly available as of the date of publication. DOIs are listed in the [Key resources table](#).

All original code has been deposited at Mendeley and is publicly available as of the date of publication. DOIs are listed in the [Key resources table](#).

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All patients provided informed written consent. This study was approved by the local ethics committee of the University of Heidelberg. Patient characteristics are provided in [Tables 1](#) and [S6](#).

METHOD DETAILS

Patient population

Recruited patients who received alloSCT at our institution between 02/2002 and 03/2013 and who survived at least 180 days post alloSCT were included in this analysis. Exclusion criteria were death before day+180, relapse before day+100, or steroid refractory aGVHD ($n = 223$ excluded), resulting in a study population of $n = 453$. Serum at day+100 was available for 430 patients. In addition, serum at onset of cGVHD was available for 196 of 263 patients with a recorded cGVHD episode ([Figure S1](#); [Table S6](#)).

GVHD prophylaxis included anti-thymocyte globulin (ATG) (10 mg/kg/d) d-3 to d-1 for unrelated donor transplantations. Either short-course methotrexate (MTX) (days 1, 3, 6) or Mycophenolate Mofetil (MMF) (days 0-28) were given to all patients according to the actual institutional policy. Cyclosporin A (CsA) was the first calcineurin inhibitor applied to all patients starting day-1. Tacrolimus or Sirolimus were used if CsA was not tolerated. Immune suppression was maintained until d+60 (matched related donors) and d+100 (all others) and then tapered off according to the clinical situation. Acute GVHD and impending relapse individualized this period. Patients receiving donor leukocyte infusions (DLI) were not excluded.

Diagnosis and grading of cGVHD

Clinical characteristics, signs, and symptoms of cGVHD as well as treatment and outcome were assessed by retrospective review of medical charts. cGVHD diagnosis and grading of severity was based on the National Institute of Health's (NIH) 2005 consensus criteria as reported before.^{16,42} In particular, diagnosis of cGVHD was based on presence of a distinctive or diagnostic criterion as delineated by the NIH.⁴² Severe cGVHD was defined according to the NIH consensus criteria, namely involvement of at least one organ system in its severest form (corresponding to 3 points in the NIH grading system with the exception of lung cGVHD in which 2 points are sufficient). Non-severe (moderate or mild) cGVHD was defined as any cGVHD not fulfilling the criteria of severe cGVHD. Isolated hepatitis-like GVHDs were non-severe cGVHDs except for 5 cases. Here only one patient qualified for severity due to high liver enzymes as hepatitis-like cGVHD, the other 4 had both, high bilirubin, and high liver enzymes. In most cases, hepatitis-like cGVHDs coincided with severe GI-cGVHDs, we therefore included this cohort with the GI-cGVHDs.

The onset of cGVHD was defined as the first appearance of a distinctive or diagnostic criterion. For prediction of progression after initially mild symptoms, the onset of severe symptoms was additionally recorded, however, the disease was counted as severe cGVHD starting with the onset of mild symptoms. Details of cGVHD onset and organ involvement for each patient are shown in [Table S6](#).

Serum samples collection

Serum samples were collected in weekly intervals after alloSCT. Sera were transferred into cryotubes and stored at -80°C until further processing. Sera on day+100 (± 7 days) after alloSCT as well as those closest to the day of cGVHD onset were selected for assessment of CXCL9, (IL-18, Trp, and IDO levels using (ELISA CXCL9 and IL-18 duo sets, R&D, UK; Trp and IDO ELISAs, Immunodiagnosics, Bensheim, Germany). ELISA plates were read with the Tecan Sunrise Remote Elisa Microplate Reader (Tecan, Austria).

Assessment of biomarkers

Serum levels of Kyn, 3-HK, KA, AA and 3-HAA were measured by liquid chromatography tandem mass spectrometry (LC-MS/MS). A method for simultaneous quantification of all metabolites in a single run was used ([Methods S1](#)) and was based on a method previously validated for rat plasma.⁴³ The method allows simultaneous determination of the metabolites using small amounts of sera (25 μL). Sample processing was performed by protein precipitation and ascorbic acid was added to the reconstitution solvent to avoid degradation of the most labile 3-HK and 3-HAA. Calibration standards were prepared in solvent and quality controls in human serum. Labeled internal standards were used for all metabolites in order to normalize interindividual variations of recovery and matrix suppression or enhancement. Calibration curves range was chosen based on endogenous level of Kyn, KA, 3-HK, AA and 3-HAA measured in previous work performed by our group.

Serum samples were analyzed in 6 different analytical runs. In each run a set of quality control (endogenous, low, medium, and high) was repeated every 18-20 samples. Solvent, matrix, and control blanks ($n = 2$) were analyzed at the beginning of each run, followed by a set of calibration standards which was repeated at the end of the run. Samples were analyzed in single replicate. Values below the limit of sensitivity were replaced by the minimal value*0.5.

Intra-day accuracy, inter-day accuracy and precision of calibration standards and quality controls of Kyn, KA, 3-HK, AA and 3-HAA are reported along with additional methodological details in [Methods S1](#) or are available upon request.

Flux quantification and mathematical modeling

The purpose of the mathematical model is to quantitatively compare the flux of metabolites through the kynurenine pathway at different time points and in different subtypes of cGVHD. As flux from metabolite X to metabolite Y we define the amount of metabolite X that is transformed into Y per unit of time. The mathematical model is given as a system of ordinary differential equations. Ordinary differential equations are widely used to model biochemical and biological pathways.^{44–46}

We make the following assumptions:

At the time of measurement, the system is in a dynamic equilibrium (equilibrium of flow).

The flux from metabolite X to metabolite Y is given as the product of the concentration of X and the rate parameter k_Y . The rate parameter can depend on other variables of the system.

The clearance rates of anthranilic acid (d_{AA}), 3-OH anthranilic acid (d_{HAA}) and kynurenic acid (d_{KA}) are constant in time and are not affected by the stage of cGVHD and are approximately identical for different individuals.

We consider the reactions as unidirectional, or, respectively, the fluxes as net fluxes.

This leads to the following model:

$$\begin{aligned} \frac{d}{dt}C_{KYN} &= K_{KYN} - k_{AA}(C_{KYN}, C_{AA}, C_{KA}, C_{HKYN}, C_{HAA}) \cdot \\ &C_{KYN} - k_{HKYN}(C_{KYN}, C_{AA}, C_{KA}, C_{HKYN}, C_{HAA}) \cdot C_{KYN} \\ &- k_{KA}(C_{KYN}, C_{AA}, C_{KA}, C_{HKYN}, C_{HAA}) \cdot C_{KYN} \end{aligned}$$

$$\frac{d}{dt}C_{AA} = k_{AA}(C_{KYN}, C_{AA}, C_{KA}, C_{HKYN}, C_{HAA}) \cdot C_{KYN} - d_{AA} \cdot C_{AA}$$

$$\frac{d}{dt}C_{KA} = k_{KA}(C_{KYN}, C_{AA}, C_{KA}, C_{HKYN}, C_{HAA}) \cdot C_{KYN} - d_{KA} \cdot C_{KA}$$

$$\begin{aligned} \frac{d}{dt}C_{HKYN} &= k_{HKYN}(C_{KYN}, C_{AA}, C_{KA}, C_{HKYN}, C_{HAA}) \cdot \\ &C_{KYN} - k_{HAA}(C_{KYN}, C_{AA}, C_{KA}, C_{HKYN}, C_{HAA}) \cdot C_{HKYN} \end{aligned}$$

$$\frac{d}{dt}C_{HAA} = k_{HAA}(C_{KYN}, C_{AA}, C_{KA}, C_{HKYN}, C_{HAA}) \cdot C_{HKYN} - d_{HAA} \cdot C_{HAA}$$

By C_X with $X \in \{KYN, AA, KA, HKYN, HAA\}$ we denote the concentration of metabolite X. By K_{KYN} we denote the kynurenine production per unit of time (flux $\text{Trp} \rightarrow \text{Kyn}$). The rate parameters for the reactions kynurenine \rightarrow anthranilic acid, kynurenine \rightarrow 3OH-kynurenine, kynurenine \rightarrow kynurenic acid and 3OH-kynurenine \rightarrow 3OH-anthranilic acid are denoted by k_{AA} , k_{HKYN} , k_{KA} and k_{HAA} respectively. The clearance rates are denoted as d_Y for $Y \in \{AA, KA, HAA\}$.

Flux quantification

We denote the concentration of metabolite X in the dynamic equilibrium at time t as $C_{X,t}^*$, the corresponding rate parameters in this dynamic equilibrium are denoted as

$$K_{X,t}^* \equiv K_X(C_{KYN,t}^*, C_{AA,t}^*, C_{KA,t}^*, C_{HKYN,t}^*, C_{HAA,t}^*)$$

Here t is either 100 days after transplantation or the time of onset of cGVHD. The flux from metabolite X to metabolite Y in the dynamic equilibrium at time t is given by $C_{X,t}^* \cdot k_{Y,t}^*$. We denote this as $\text{flux}_{X \rightarrow Y,t}^*$. The above equations imply:

$$\begin{aligned} \frac{\text{flux}_{KYN \rightarrow AA,t}^*}{d_{AA}} &= \frac{k_{AA,t}^* \cdot C_{KYN,t}^*}{d_{AA}} = \\ C_{AA,t}^* &: \frac{\text{flux}_{KYN \rightarrow KA,t}^*}{d_{KA}} = \frac{k_{KA,t}^* \cdot C_{KYN,t}^*}{d_{KA}} = \\ C_{KA,t}^* &: \frac{\text{flux}_{HKYN \rightarrow HAA,t}^*}{d_{HAA}} = \frac{k_{HAA,t}^* \cdot C_{HKYN,t}^*}{d_{HAA}} = C_{HAA,t}^* \end{aligned}$$

$$\frac{k_{HKYN,t}^* \cdot c_{KYN,t}^*}{k_{HAA,t}^*} = c_{HKYN,t}^* \Leftrightarrow \frac{\text{flux}_{KYN \rightarrow HKYN,t}^*}{d_{AA}} = c_{HAA,t}^*$$

If we have longitudinal (paired) data from the same patient at times t_1 and t_2 it follows that the respective changes in % from time t_1 to time t_2 are given by

$$\text{flux}_{KYN \rightarrow AA,t_2}^* - \text{flux}_{KYN \rightarrow AA,t_1}^* = \left(\frac{c_{AA,t_2}^*}{c_{AA,t_1}^*} - 1 \right) \cdot 100\% \text{ of } \text{flux}_{KYN \rightarrow AA,t_1}^*$$

$$\text{flux}_{KYN \rightarrow KA,t_2}^* - \text{flux}_{KYN \rightarrow KA,t_1}^* = \left(\frac{c_{KA,t_2}^*}{c_{KA,t_1}^*} - 1 \right) \cdot 100\% \text{ of } \text{flux}_{KYN \rightarrow KA,t_1}^*$$

$$\text{flux}_{HKYN \rightarrow HAA,t_2}^* - \text{flux}_{HKYN \rightarrow HAA,t_1}^* = \left(\frac{c_{HAA,t_2}^*}{c_{HAA,t_1}^*} - 1 \right) \cdot 100\% \text{ of } \text{flux}_{HKYN \rightarrow HAA,t_1}^*$$

$$\text{flux}_{KYN \rightarrow HKYN,t_2}^* - \text{flux}_{KYN \rightarrow HKYN,t_1}^* = \left(\frac{c_{HAA,t_2}^*}{c_{HAA,t_1}^*} - 1 \right) \cdot 100\% \text{ of } \text{flux}_{KYN \rightarrow HKYN,t_1}^*$$

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed using R version 4.02 (R Core Team, R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria). We considered $p < 0.05$ as significant.

Analysis of patient, disease, and transplant characteristics

To assess whether patients with different cGVHD subtypes differ with respect to categorical variables we used Fisher's test. Patient numbers and p values are reported in [Table 1](#). Detailed onset and organ involvement of cGVHD cohort is shown in [Table S6](#). We used the function `fisher.test` from R version 4.02 with default settings and the option `hybrid = TRUE`. This uses the exact test for 2x2 contingency tables and an approximation for larger tables.

Metabolite and cytokine analysis

To test our hypothesis that the activity of the Kyn pathway differs across cGVHD subtypes at day+100 we used the non-parametric multivariate approach.⁴⁷ This is an affine-invariant multivariate analog of the Kruskal-Wallis, the Wilcoxon rank sum, and the signed-rank tests. The multivariate generalization is based on the spatial rank and results in a multivariate non-parametric framework for location testing. This global approach allowed us to reject the null hypothesis that the distribution centers for none of the metabolites differ between the cGVHD cohorts at day+100 at an error rate of $\alpha = 0.05$. We applied the same framework to test for each cGVHD subtype at an error rate of $\alpha = 0.05$ whether the differences of metabolite concentrations between onset and day+100 are all centered at zero. We could reject the null hypothesis for non-severe and severe fibrosing cGVHD and obtained a borderline result ($p = 0.053$) for severe GI cGVHD. The global tests have been implemented in the R-package `SpatialNP` (version 1.1-4). The tests were performed using the function `sr.loc.test` setting `score = rank`. Having obtained evidence for the involvement of the Kyn pathway at day+100 at a global error rate of $\alpha = 0.05$ and for the change of Kyn pathway activity between day+100 and onset (for each cGVHD subtype at a global error rate of $\alpha = 0.05$) we continued with an exploratory analysis that is based on the univariate Kruskal-Wallis and paired Wilcoxon tests. This part of the study has exploratory character; therefore, we did not apply multiple testing corrections in [Tables 2](#) and [3](#).

Changes in metabolite concentrations between two independent patient groups were assessed by the Kruskal-Wallis test. The respective group sizes are reported in [Tables 2, 3](#), and [S1](#). Changes of metabolite concentrations or fluxes between two time points within the same patient group were studied using the paired Wilcoxon test (`wilcox.test` from R version 4.02 with default settings and `alternative = "two-sided"`). This non-parametric test makes no assumptions on the underlying statistical distribution. Patient numbers are reported in [Tables 3](#) and [S2–S4](#).

Based on the exploratory part of the study we hypothesized that the observed changes in Kyn metabolites could be triggered by MIG and IL-18. Driven by this hypothesis we considered these cytokines using univariate tests at error rates $\alpha = 0.05$. The

exploratory analysis has led to patho-physiological hypotheses which we condensed into a risk score (Risk factors¹ 3HAA/AA < median and² KA > median). The ability of this risk score to predict cGVHD has been confirmed using multivariate Cox regression considering the confounders identified in [Table 1](#).

Cox-Regression

Survival analysis was performed using the R package survival (version 3.2-7). Cox-regressions are based on the function `coxph` with default settings, categorical variables were implemented using the `factor` function. The variables included in the Cox-regression were selected based on the p values in [Table 1](#). We only included complete cases in the multivariable analyses. Continuous variables were log₂-transformed. The respective endpoints, patient numbers and event numbers are given in [Tables 4](#) and [S5](#). The proportional hazards assumption was assessed using the `zph` test implemented in the `cox.zph` function from the R package survival. For the Cox analyses presented in [Table S5](#) and [Figures 2C](#) and [2D](#) no violation was detected. For the Cox analysis in [Table 4](#) the global p value of the `zph` test was non-significant, however the p value for the variable ATG was significant, indicating a violation of the proportional hazards assumption. We therefore introduced a stratification with respect to ATG. The variables of the stratified Cox-model did not violate the proportional hazard assumption. The same applies to the Cox-regression presented in [Table S5](#). In the Cox-regression in [Table S5](#), the variables ATG and disease (Multiple Myeloma) violated the proportional hazards assumption. Introducing a stratification with respect to ATG we obtained a Cox model with unchanged significances that does not violate the proportional hazard assumption. We decided to maintain the unstratified analyses to improve readability, since they lead to the same conclusions as the stratified analyses. To assess the linearity assumption of continuous variables we use penalized splines implemented by the `spline` function of the survival package. In all considered cases the nonlinear component has no significant impact when assessed by the `cox.zph` function. Therefore, we conclude that the linearity assumption is fulfilled. All R functions were used with their standard parameters if not indicated differently.

In [Tables 2](#), [3](#), and [S1–S4](#) metabolite concentrations and flux changes for different patient subgroups are reported as median values and inter-quartile range (IQR).

Kaplan-Meier analysis

Cumulative incidence plots were generated using the function `survfit` from the R package survival Version 3.2-7. The incidence plots and tables with the participants at risk were generated using `ggsurvplot` from the package R `survminer` Version 0.4.8 Incidence curves were compared using Cox-regression. Proportional hazard and linearity assumptions were tested as described above.