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Article

Characterization of Gut-Thyroid Axis in Alcohol Use Disorder: Interplay of Gut-Dysfunction, Pro-Inflammatory Responses, and Thyroid Function

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Abstract: (1) Background: Heavy and chronic alcohol intake causes altered gut-permeability and dysfunction; and exhibits a unique pro-inflammatory state. Thyroid-associated hormones and proteins may be dysregulated by alcohol administration; however, the impact of altered gut-derived changes on thyroid function is unclear. This study investigated the role of alcohol-induced gut dysfunction and pro-inflammatory cytokine profile on thyroid function in patients with alcohol use disorder (AUD). (2) Methods: Male and female AUD patients (n=44) were divided into Gr.1 with normal thyroid stimulating hormone (TSH) levels (n=28, 0.8≤TSH≤3 mIU/L); and Gr.2 with clinically elevated TSH levels (n=16, TSH> 3 mIU/l). Demographics, drinking measures, comprehensive metabolic panel, and candidate thyroid markers (TSH, circulating triiodothyronine [T3] and free thyroxine [fT4]) were tested. Plasma-derived gut-dysfunction associated markers (lipopolysaccharide [LPS], LPS-binding protein [LBP], and soluble LPS-induced pathogen-associated protein [sCD14]), and pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, IL-8, MCP-1, PAI-1) were analyzed and compared with the thyroid, demographics, and drinking markers. (3) Results: Both groups presented with a borderline overweight category of BMI. Gr.2 presented with numerically higher chronic and heavy drinking patterns vs Gr.1. fT4 levels were elevated while T3 was within normal limits in both the groups. Gut-dysfunction markers LBP and sCD14 were numerically elevated in Gr.2 vs Gr.1 suggesting subtle ongoing changes; however, the difference was not statistically significant. All pro-inflammatory cytokines were significantly elevated in Gr.2, including IL-1β, MCP-1, and PAI-1. Gr.2 showed a strong and statistically significant effect of gut-immune-thyroid response (r=0.896, p=0.002) on TSH levels in a multivariate regression model with LBP, sCD14, and PAI-1 levels as upstream variables; this assessment was not significant in Gr.1. In addition, AUROC analysis demonstrated that many of the cytokines strongly predicted TSH in Gr.2, including IL-6 (area=0.774, p<0.001) and TNF-α (area=0.708, p=0.017) among others. This was not observed in Gr.1. Gr.2 demonstrated elevated fT4 as well as TSH, which suggests that there was subclinical thyroiditis with underlying CNS dysfunction and lack of a negative feedback loop. (4) Conclusions: These findings reveal the toxic effects of heavy and chronic drinking that play a pathological role in thyroid gland dysregulation employing the gut-brain axis. These results also strongly emphasize potential directions to strongly consider thyroid dysregulation in the overall medical management of AUD.

Keywords: alcohol use disorder; gut-dysfunction; gut-thyroid axis; pro-inflammatory cytokines; thyroid-associated hormones

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1. Introduction

Alcohol use disorder (AUD) is characterized by compulsive alcohol consumption over an extended period of time combined with a loss of control and negatively associated emotions [1, 2]. According to the 2019 National Survey on Drug Use and Health, 14.1 million adults in the United States meet the criteria for an AUD [3]. Thyroid dysfunction and its association with chronic alcohol use has been described, though its precise pathophysiology has not yet been fully ascertained. One important consideration is that there is a significant contribution from alcohol-induced gut dysfunction and concomitant pro-inflammatory cytokine release to elicit deleterious effects.

Several studies describe the effects of heavy alcohol consumption on thyroid homeostasis. This is initially described in 1960 when Goldberg suggests that a high blood alcohol content exerts direct toxic effects on the vascular thyroid. He also provides an alternative explanation of the possible toxic effects of alcohol on the thyrotropin areas of the hypothalamus or pituitary rather than the thyroid gland itself [4]. This effect of alcohol abuse on the hypothalamic-pituitary-thyroid (HPT) axis is further examined by Zoeller et al, who demonstrate that rats with chronic ethanol treatment develop significantly elevated thyrotropin-releasing hormone (TRH) and TSH levels with a blunted thyrotropic response to cold exposure [5]. Further studies demonstrate similar findings in patients with chronic alcohol use and a dysregulation of the HPT axis by elevation of TRH or TSH with a blunted response [6-9].

The pathogenesis of this response is unknown but has been hypothesized to be either a direct effect of alcohol on the HPT axis or, more interestingly, an indirect mechanism through gut dysfunction and a pro-inflammatory response. Chronic and excessive consumption of alcohol causes gut dysfunction through microbiome dysbiosis and through an increase in intestinal permeability [10, 11]. Alcohol can increase intestinal bacteria through poor digestive and intestinal function secondary to alcohol consumption [10]. There is also dysbiosis with alcohol use, with a change in the ratio between beneficial and pathogenic bacteria [10, 12]; these increases and alterations of the microbiome result in an increase in endotoxin production, including lipopolysaccharide (LPS), lipopolysaccharide binding protein (LBP), and LPS-induced pathogen-associated protein sCD14. Alcohol additionally increases intestinal permeability by alcohol-induced direct cellular damage to gut epithelial cells as well as disruption of tight junctions, cytoskeleton, and other proteins leading to increased leakiness of the gut [13-15].

This increase in gut dysfunction, as well as alcohol metabolism in the liver, further contributes to a pro-inflammatory state in chronic alcohol use. Endotoxins such as LPS bind to toll-like receptor 4 (TLR-4) initiating a cascade of events; this leads to the production of reactive oxygen species (ROS) along with pro-inflammatory cytokines, including tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 1β (IL1-β), plasminogen activator inhibitor 1 (PAI-1), monocyte chemoattractant protein 1 (MCP-1) among others [16-18]. These contribute to systemic inflammation and, the organ dysfunction.

The complexity of alcohol-associated dysregulation in the HPT axis, as well as its relationship to gut-derived alterations, is still being unraveled in the scientific community. The aims of this study were to identify the role of gut dysfunction and a pro-inflammatory state in thyroid dysregulation in patients with AUD; to estimate the levels of dysregulation in thyroid function; and lastly to characterize the gut-thyroid axis for the pathological role of chronic and heavy alcohol drinking.

2. Materials and Methods

Patients Recruitment

This clinical cross-sectional one timepoint study was a secondary project of a larger clinical protocol (NCT#00106106) conducted in the National Institute on Alcohol Abuse and Alcoholism (NIAAA) at the National Institutes of Health (NIH), Bethesda MD. The overarching protocol had a primary aim of determining if acamprosate reduced alcohol

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craving and withdrawal symptoms in patients receiving standard inpatient care for alcohol detoxification. There were several secondary aims of this project at various time points, one which included evaluating alcohol induced gut dysfunction and its effect on the thyroid. This study protocol was Institutional Review Board (IRB) approved through the central neuroscience committee of the NIAAA. The participants were screened, and eligible patients were consented to participate in this study prior to the collection of bodily samples, clinical and research data.

Eligibility Criteria and Randomization

All study patients were diagnosed with AUD based on DSM-IV TR edition. Altogether 44 male and female AUD patients between the ages of 23-63 years of age participated in this investigation. Primary exclusion criteria included : clinical diagnoses of liver disease which included alcohol associated hepatitis, cirrhosis, viral hepatitis, autoimmune hepatitis; clinical diagnosis of pancreatic disease which included acute pancreatitis, chronic pancreatitis, pancreatic cancer; diagnosis of a severe physical or psychiatric disease, such as unstable cardiovascular disease (with decompensation demonstrated through chest X-ray, or a pathological echocardiogram), renal failure with creatinine clearance <30 ml/min, and/or advanced lung disease. Other exclusion criteria were the following - a diagnosis of HIV, pregnancy, or ongoing breastfeeding, use of biotin supplements, and/or positive urine drug screen for illicit substances. Further inclusion and exclusion criteria are detailed in a previous publication [19]. Thyroid stimulating hormone (TSH) levels were used as reference factor to stratify patients into two groups – normal TSH levels between and including 0.8 and 3 mIU/L (Group 1 [Gr.1]); and TSH levels greater than 3 mIU/L (Hypothyroid, Group 2 [Gr.2]) [20, 21]. It should be noted that the upper limit for normal TSH of 3 mIU/L is lower than the more commonly used 5 mIU/L. This was deliberately chosen due to several proposals that the upper limit for normal should be lowered [20, 21]. One argument discusses that there is a higher level of antithyroid antibodies detected in patients who have a serum TSH between 3 and 5 mIU/L [20]. Another proposal argues that after excluding patients with antithyroid antibodies, goiter, and family history of thyroid disease, the mean TSH is 1.5 mIU/L; when this is extrapolated to be Gaussian curve, the upper limit for the 97.5th percentile is actually 2.5 mIU/L [20].

Demographics, Drinking Profile, Laboratory Evaluations

Data was collected on demographics indices (age, sex, body mass index [BMI]), alcohol drinking inventory (Table 1). Timeline Follow-back Instrument [22] and Lifetime Drinking History assessment [23] were employed to collect information on recent drinking history and chronic alcohol misuse respectively. Drinking markers derived from these assessments included - total drinks in the past 90 days (TD90), heavy drinking days in the past 90 days (HDD90), number of drinking days in the past 90 days (NDD90), average drinks per day in the past 90 days (AvgD90). Further information on the collection of drinking history information is detailed in our previous publication (19). All laboratory assays were analyzed at the Department of Laboratory Medicine of the National Institutes of Health with a guideline set-up at the Medline Plus, set until 2014 when samples were assayed.

Laboratory Analyses

Serum samples were collected at baseline, and the following panels and values were collected: complete metabolic panel (CMP), complete blood count (CBC), thyroid panel (triiodothyronine (T3), free thyroxine (fT4), TSH, inflammatory markers (c reactive protein (CRP), immunoglobulin A (IgA), IgG, IgM). Multiplex kits (Millipore, Billerica, MA USA) on the Luminex (Luminex, Austin, TX USA) platform were utilized for cytokine assays (TNF-α, IL-6, IL-8, IL1-β, PAI-1, MCP-1). LPS, LBP, and sCD14 were tested using

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Kinetic Chromogenic Limulus Aoebocyte Lysate Assay (Lonza, Walkersville, MD USA), per manufacturer recommendations.

Statistical Analyses

SPSS v 28.01 (IBM, Chicago, IL) and Microsoft 365 Excel (MS Corp, Redmond, WA) were utilized for statistical analysis. Independent samples ANOVA was utilized to compare Gr.1 and Gr.2 overall by sex for demographics, drinking history, liver injury markers, thyroid function markers, blood cell measures, inflammatory markers, pro-inflammatory cytokines, and gut dysfunction markers. Univariate and multivariate regression analyses, further stratified by sex, were conducted to correlate drinking history with gut dysfunction markers and pro-inflammatory cytokines, cytokines with thyroid function markers, and cytokines with gut dysfunction. Receiver operating characteristic (ROC) curves were developed to determine the sensitivity and specificity of the cytokines and gut dysfunction markers in predicting thyroid function in the elevated TSH group (Gr.2) when compared to Gr.1. A p-value < 0.05 was used as a reference point to establish statistical significance. Data presented as mean with standard deviation (Mean±SD) unless otherwise noted.

3. Results

3.1. Demographics and Drinking Profile

Gr.2 (elevated TSH) patients were older by approximately 5 years than patients in Gr.1 (normal TSH), though this was not significant statistically (Table 1). Both Gr.1 and Gr.2 had a borderline overweight BMI; females in Gr.2 were significantly more overweight compared to Gr.1 females, while Gr.1 females had a significantly higher BMI than the males (Table 1). All measures of chronic and recent drinking history were elevated in Gr.2 compared to Gr.1 with statistically significant unique sex-differences within and between the subgroups (Table 1).

Table 1. At baseline - demographic, drinking history, liver injury measures, nutritional status, candidate blood panel measures, cytokines, gut-dysfunction markers of alcohol use disorder patients tabulated by normal versus elevated TSH levels.

	Group 1 (Normal TSH, Gr. 1)			Group 2 (Elevated TSH, Gr. 2)			*Between group*
	Males (n=21; 75%)	Females (n=7; 25%)	Total (n=28; 63.6%)	Males (n=10; 62.5%)	Females (n=6; 37.5%)	Total (n=16; 36.4%)	*p* – value
Demographics							
Age[c] (years)	41.6 ± 8.5	41.3 ± 13.8	41.5 ± 9.8	47.3 ± 12.1	44.7 ± 11.6	46.3 ± 11.6	ns
BMI[bc] (kg/m²)	26.3 ± 4.4	29.5 ± 10.5	27.1 ± 6.5	27.8 ± 4.6	26.3 ± 2.7	27.3 ± 4.0	ns
Drinking History							
TD90	1091 ± 450	915 ± 664	1052 ± 496	1159 ± 686	1084 ± 604	1131 ± 637	ns
HDD90	71.43 ± 22.02	61.33 ± 21.26	69.19 ± 21.87	73.60 ± 21.58	75.83 ± 18.44	74.44 ± 19.85	ns
AvgDPD90[ac]	14.32 ± 3.89	14.91 ± 9.26	14.45 ± 5.31	15.68 ± 7.65	13.57 ± 6.10	14.89 ± 6.97	ns
NDD90	75.14 ± 18.26	62.50 ± 20.93	72.33 ± 19.22	75.50 ± 22.48	77.67 ± 16.07	76.31 ± 19.76	ns
LTDH[c]	15.80 ± 9.60	9.86 ± 5.21	14.26 ± 8.98	21.90 ± 9.69	11.83 ± 9.00	18.13 ± 10.42	ns
Liver Panel							
ALT (IU/L)	81.86 ± 40.93	58.57 ± 77.88	76.04 ± 51.90	80.00 ± 41.00	94.50 ± 108.70	85.44 ± 70.71	ns
AST (IU/L)	92.57 ± 72.12	88.14 ± 134.12	91.46 ± 88.62	114.40 ± 103.13	158.33 ± 122.50	130.88 ± 108.93	ns
AST:ALT	1.091 ± 0.553	1.408 ± 0.459	1.170 ± 0.541	1.300 ± 0.626	1.970 ± 0.937	1.551 ± 0.800	ns
TBili (mg/dL)	0.786 ± 0.869	0.743 ± 0.365	0.775 ± 0.767	0.690 ± 0.247	0.733 ± 0.398	0.706 ± 0.300	ns
Albumin (g/dL)	4.181 ± 0.423	4.029 ± 0.206	4.143 ± 0.382	4.080 ± 0.358	4.217 ± 0.458	4.131 ± 0.389	ns
Thyroid Function							
TSH (mIU/L)	1.781 ± 0.609	1.793 ± 0.711	1.784 ± 0.622	3.931 ± 0.951	4.656 ± 1.001	4.173 ± 0.996	0.046
T3 (ng/dL)	106.45 ± 19.22	109.33 ± 30.12	107.47 ± 22.72	131.88 ± 20.32	146.25 ± 17.75	136.67 ± 19.97	ns

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fT4 (ng/dL)	5.782 ± 1.154	5.700 ± 1.101	5.753 ± 1.101	5.575 ± 1.456	7.950 ± 0.507	6.367 ± 1.669	0.037
Nutritional Status							
Zinc (ug/dL)	79.53 ± 12.19	64.43 ± 12.30	75.46 ± 13.78	71.90 ± 13.37	92.67 ± 46.26	79.69 ± 30.47	ns
Blood Cell Measures							
AMC[c] (K/uL)	0.492 ± 0.233	0.489 ± 0.126	0.491 ± 0.209	0.535 ± 0.268	0.518 ± 0.076	0.528 ± 0.212	ns
ANC (K/uL)	3.684 ± 2.063	5.216 ± 1.872	4.067 ± 2.095	3.266 ± 1.799	3.370 ± 1.280	3.305 ± 1.578	ns
Inflammatory markers							
CRP[c] (mg/L)	2.07 ± 1.61	0.57 ± 0.04	1.42 ± 1.39	4.47 ± 7.16	0.88 **	3.75 ± 6.40	0.039
IgA (pg/ml)	295.9 ± 113.7	268.3 ± 128.4	289.0 ± 115.7	281.1 ± 173.7	249.7 ± 86.0	269.3 ± 144.3	ns
IgG (pg/ml)	1102 ± 439	1152 ± 213	1114 ± 391	1169 ± 314	1010 ± 254	1109 ± 295	ns
IgM[d] (pg/ml)	123.4 ± 63.5	106.9 ± 48.7	119.3 ± 59.7	113.8 ± 41.6	156.8 ± 90.2	129.9 ± 64.9	ns
Candidate Cytokine Response							
IL-6 (pg/ml)	2.588 ± 2.537	3.771 ± 3.101	2.861 ± 2.659	4.268 ± 2.250	6.102 ± 5.357	5.002 ± 3.743	ns
IL-8[cd] (pg/ml)	4.013 ± 3.247	15.886 ± 27.849	6.753 ± 13.753	5.581 ± 2.410	15.463 + 22.137	9.534 ± 14.263	ns
TNF-α[d] (pg/ml)	1.743 ± 0.693	1.458 ± 0.779	1.677 ± 0.708	2.215 ± 0.562	2.659 ± 1.624	2.393 ± 1.083	ns
IL-1β[b] (pg/ml)	0.451 ± 0.227	0.268 ± 0.196	0.409 ± 0.230	0.688 ± 0.378	0.772 ± 0.689	0.722 ± 0.503	0.005
MCP-1[a] (pg/ml)	96.62 ± 32.57	91.47 ± 39.87	95.43 ± 33.60	123.92 ± 63.64	149.90 ± 107.31	134.31 ± 81.24	0.013
PAI-1[a] (ng/ml)	35.91 ± 13.80	40.36 ± 11.98	36.94 ± 13.31	56.86 ± 24.65	38.34 ± 21.69	49.46 ± 24.56	0.031
Candidate Gut Dysfunction Markers							
LPS (EU/ml)	0.106 ± 0.060	0.091 ± 0.058	0.102 ± 0.059	0.097 ± 0.064	0.109 ± 0.069	0.102 ± 0.064	ns
LBP[abcd] (ng/ml)	1119 ± 1430	3989 ± 4989	1781 ± 2838	2822 ± 3480	751 ± 624	2132 ± 2986	ns
sCD14[d] (ng/ml)	9063 ± 1970	9375 ± 1323	9141 ± 1812	8554 ± 1840	11321 ± 733	9592 ± 2031	ns

Between group p-values compare totals between Gr.1 and Gr.2. Significant between group analyses were also conducted for : [a]males only between Gr. 1 and Gr. 2, [b]females only between Gr.1 and Gr.2, [c]between sex in Gr. 1 only, and [d]between sex in Gr. 2 only. BMI: Body mass index; TD90: Total drinks past 90 days; HDD90: heavy drinking days past 90 days; AvgDPD90: Average drinks per drinking day past 90 days; NDD90: number of drinking days past 90 days; NNDD90: number of non-drinking days past 90 days, LTDH: lifetime drinking history (in years), ALT: serum alanine aminotransferase, AST: serum aspartate aminotransferase, AST:ALT: ratio of AST by ALT, Tbili: Total bilirubin, TSH: thyroid stimulating hormone, T3: triiodothyronine, fT4: free thyroxine, WBC: white blood cells count, AMC: absolute monocyte count, ANC: absolute neutrophil count, CRP: c reactive protein, IgA: immunoglobulin A, IgG: immunoglobulin G, IgM: immunoglobulin M, Ω6/Ω3: omega-6 to omega-3 ratio, IL-6: interleukin 6, IL-8: interleukin 8, TNF-α: tumor-like necrotic factor alpha, Il-1β: interleukin 1 beta, MCP-1: monocyte chemoattractant protein-1, PAI-1: plasminogen activator inhibitor-1, LPS: lipopolysaccharide, LBP: LPS binding protein, sCD14: LPS-induced pathogen-associated protein. ***There was only one data point for this particular category.*

3.2. Gut Dysfunction Markers and Pro-Inflammatory Response

All pro-inflammatory cytokine levels were higher in Gr.2 compared to Gr.1 (Table 1). In Gr.2, IL-6 was almost 2-fold increased while both IL-8 and TNF-α were close to 1.5-fold increased versus Gr.1 (Figure 1a). IL-1β was significantly higher in Gr.2, as well as uniquely among the females of Gr.2 (Table 1). MCP-1 and PAI-1 were both significantly higher in Gr.2 compared to Gr.1 (Figure 1b), both in overall patients as well as in males (Table 1). LPS was unchanged between the two groups. LBP and sCD14 were numerically higher in Gr.2 and had significant sex-differences within and between subgroups (Table 1).

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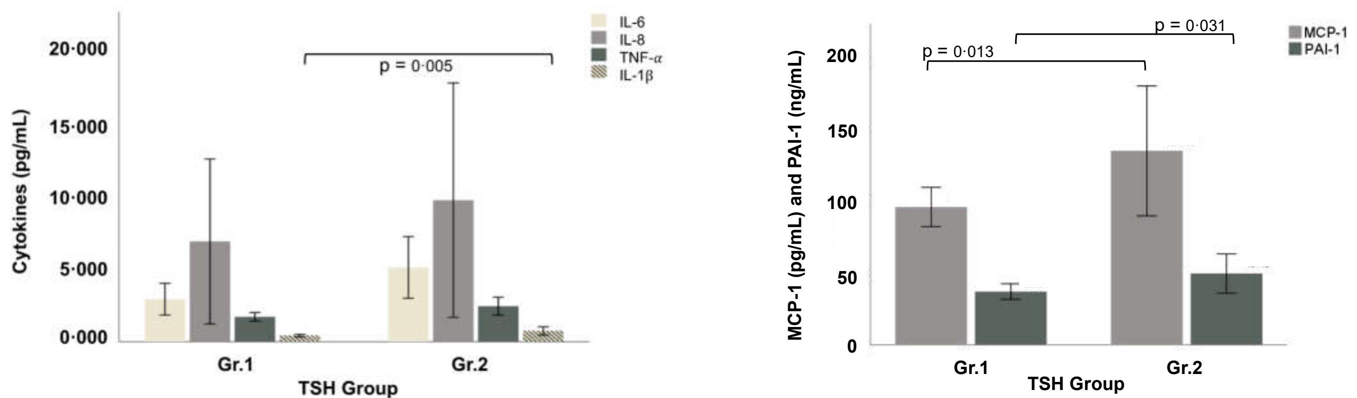

Figure 1. Proinflammatory cytokine levels in Gr. 1 vs Gr. 2 patients. (a) IL-6, IL-8, TNF-α, IL-1β, and (b) MCP-1 and PAI-1. Significant differences between groups are denoted by p values, and seen with IL-1β, MCP-1, PAI-1.

3.3. Thyroid Function and Non-Specific Inflammatory Markers

TSH was significantly higher by more than two-fold in Gr.2 compared to Gr.1, p = 0.046 (Table 1). fT4 was above normal levels in both groups, and significantly higher in Gr.2 compared to Gr.1. T3 was within normal limits in both groups and higher by approximately 30 ng/dL in Gr.2, though this was not statistically significant.

In Gr.2, patients had an elevated AMC while they had a lower ANC, both not statistically significant (Table 1). CRP was significantly higher by approximately 2.5-fold in Gr.2, p=0.039. IgA, IgG, and IgM levels were minimally varied between the two groups with insignificant differences.

3.4. Association of Drinking Markers and Measures of Gut-Dysfunction and Proinflammatory Status

In all the AUD patients (Gr.1 and Gr.2), HDD90 and NDD90 were significantly and independently associated with both LPS and LBP. TD90 was significantly associated with LBP. Correspondingly, unique significant relationships were found within Gr.2 patients. HDD90 and NDD90 were significantly associated with LBP, with p = 0.012 and p = 0.025 respectively. TD90 also significantly correlated with sCD14 within Gr.2, p = 0.038. In multivariate analyses, the same regression model yielded higher effects and significance in Gr.2; HDD90 demonstrated significant association with LBP and sCD14 combined (r=0.658, p =0.033).

Alcohol drinking markers also presented significant positive relationships with proinflammatory cytokines. TNF-α, one of the earliest cytokines in the pro-inflammatory cascade associated with AUD-related gut dysfunction[21], was significantly associated with NDD90 (p = 0.018) in Gr.2 patients and close to significance with HDD90 (p = 0.050). Among all groups, TNF-α was similarly significantly correlated with elevated NDD90 and HDD90. Further downstream in the inflammatory cascade, IL-6, IL-8, and IL-1β also significant increased with alcohol use. In both groups combined, IL-6 was significantly correlated with NDD90, while IL-8 correlated significantly with TD90. Within both groups and in Gr.2 specifically, IL-1β correlated significantly with TD90 and AvgDPD90.

Multivariate regression demonstrated several significant relationships between alcohol drinking markers, gut dysfunction, and cytokine production (Table 2). In Gr.2, TD90 correlated significantly with IL-1β in the setting of gut dysfunction markers LBP and sCD14. NDD90 correlated significantly with several cytokines in the setting of elevated LBP and sCD14; with IL-6 (r=0.736, p =0.043), IL-1β (r=0.741, p=0.040), and PAI-1 (r=0.786, p=0.018) all within Gr.2 (Table 2). HDD demonstrated similarly significant associations in

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Gr.2 with cytokines IL-6, IL-8, MCP-1, TNF-α, IL-1β, and PAI-1 when combined with LBP and sCD14 (Table 2).

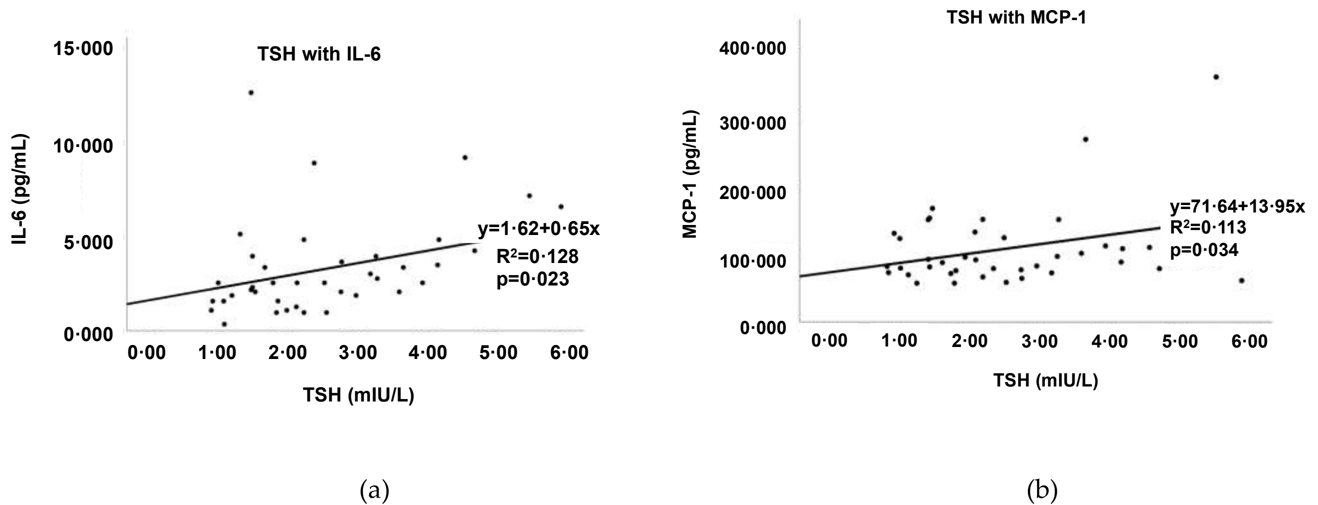
Table 2. Multivariate regression analysis between drinking markers, gut dysfunction markers, and pro-inflammatory cytokine response. R values are reported along with the associated p value if significant.

	LBP + sCD14												LPS + LBP + sCD14	
	TNF-α		**IL-6**		**IL-8**		**IL-1β**		**MCP-1**		**PAI-1**		**IL-1β**	
TD90	..	..	..	..	..	..	0.827	*p=0.007*	..	..	..	..	0.827	*p=0.023*
NDD90	..	..	0.736	*p=0.043*	..	..	0.741	*p=0.040*	..	..	0.786	*p=0.018*	..	..
HDD	0.759	*p=0.030*	0.788	*p=0.018*	0.769	*p=0.025*	0.774	*p=0.023*	0.757	*p=0.031*	0.798	*p=0.014*	..	..

TD90: Total drinks past 90 days; NDD90: number of drinking days past 90 days; HDD90: heavy drinking days past 90 days; TNF-α: tumor necrosis factor-α; IL-6: interleukin 6; IL-8: interleukin 8; IL-1β: interleukin 1β; MCP-1: monocyte chemoattractant protein-1; PAI-1: plasminogen activator inhibitor-1; LPS: lipopolysaccharide; LBP: LPS binding protein; sCD14: LPS-induced pathogen-associated protein.

3.5. Characterization of Thyroid Dysregulation by the Gut-Immune-Brain, and Gut-Thyroid Axis

Several pro-inflammatory cytokines were significantly associated with thyroid function among both groups of patients combined. TSH levels significantly correlated with IL-6 (Figure 2a), MCP-1 (Figure 2b), and TNF-α in both groups. Within Gr.2 particularly, TSH was significantly correlated with IL-6 (r = 0.615, p = 0.019) (Figure 3).



(a) (b)

Figure 2. Correlation of TSH with pro-inflammatory cytokines among both Gr.1 and Gr.2 combined. (a) correlation between TSH and IL-6, (b) correlation between TSH and MCP-1.

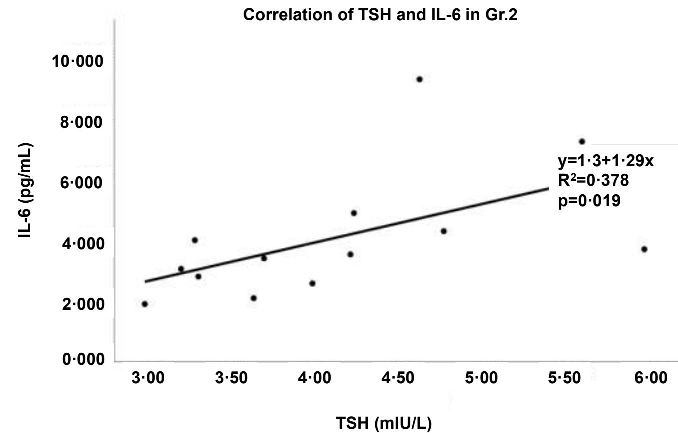


Figure 3. Regression analysis demonstrates the correlation between TSH and IL-6 in Gr.2 (elevated TSH). The r^2 value and p-value are denoted.

When combined with gut-dysfunction markers in multivariate analysis, thyroid function demonstrated greater correlations with the pro-inflammatory cytokines (Table 3). Within Gr.2 patients, in the setting of gut dysfunction (elevated LPS and LBP), TSH was significantly correlated with IL-6 and PAI-1 individually (Table 3). T3 and fT4 also demonstrated increased correlation with PAI-1 (Table 3). Thyroid function also correlated significantly with several cytokines when analyzed with LBP and sCD14 combined; in particular, PAI-1 had strong correlations with TSH (r = 0.896, p = 0.002), T3 (r = 0.914, p = 0.004), and fT4 (r = 0.903, p = 0.006) (Table 3). These significant correlations were not seen in Gr.1 patients. Similarly, with all gut dysfunction markers combined – LBP, sCD14, and LPS – thyroid function correlated significantly with PAI-1 in particular; this significance was seen with TSH (r = 0.905, p = 0.005), T3 (r = 0.919, p = 0.013), and fT4 (r = 0.906, p = 0.020) (Table 3).

Table 3. Multivariate regression analysis between gut dysfunction markers, pro-inflammatory cytokine response, and thyroid function. R values are reported along with the associated p value if significant.

	LPS + LBP				LBP + sCD14				LPS + LBP + sCD14			
	IL-6		PAI-1		IL-6		PAI-1		IL-6		PAI-1	
TSH	0.756	*p=0.046*	0.800	*p=0.022*	0.816	*p=0.016*	0.896	*p=0.002*	0.819	*p=0.043*	0.905	*p=0.005*
T3	..	..	0.878	*p=0.012*	..	..	0.914	*p=0.004*	..	..	0.919	*p=0.013*
fT4	..	..	0.854	*p=0.021*	..	..	0.903	*p=0.006*	..	..	0.906	*p=0.020*

IL-6: interleukin 6; PAI-1: plasminogen activator inhibitor-1; LPS: lipopolysaccharide; LBP: LPS binding protein; sCD14: LPS-induced pathogen-associated protein; TSH: thyroid stimulating hormone; T3: triiodothyronine; fT4: free thyroxine.

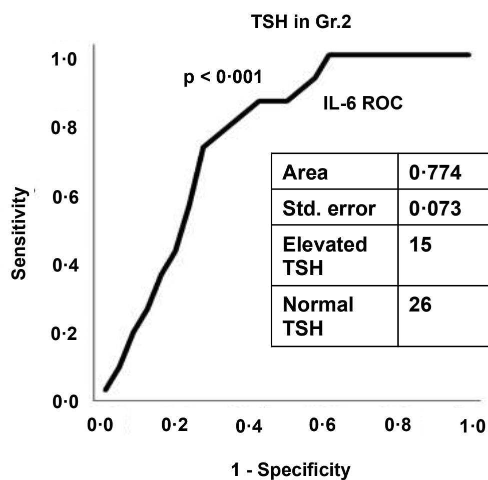
3.6. Assessment of Alcohol and Its Effect on the Gut-Brain-Thyroid Axis

When analyzing the effect of alcohol induced gut-dysfunction and pro-inflammatory state on thyroid function, there were highly significant correlations demonstrated within Gr.2 in particular. Thyroid function demonstrated significant correlations with TD90, gut dysfunction markers sCD14, LBP, and with pro-inflammatory cytokine IL-6 (r = 0.893, p = 0.007). A similar strong relationship was seen with PAI-1 (r = 0.914, p = 0.003). Thyroid function also correlated significantly with NDD90, sCD14 and LBP with multiple pro-inflammatory cytokines; this includes with IL-6 (r = 0.902, p = 0.005), IL-8 (r = 0.887, p = 0.009), MCP-1 (r = 0.926, p = 0.002), TNF-α (r = 0.911, p = 0.004), IL-1β (r = 0.894, p = 0.007), and PAI-1 (r = 0.905, p = 0.005). Thyroid function had similarly significant correlations with HDD and with all of the above pro-inflammatory cytokines in the setting of gut dysfunction; this includes IL-6 (r = 0.891, p = 0.007), IL-8 (r = 0.885, p = 0.009), MCP-1 (r = 0.922,

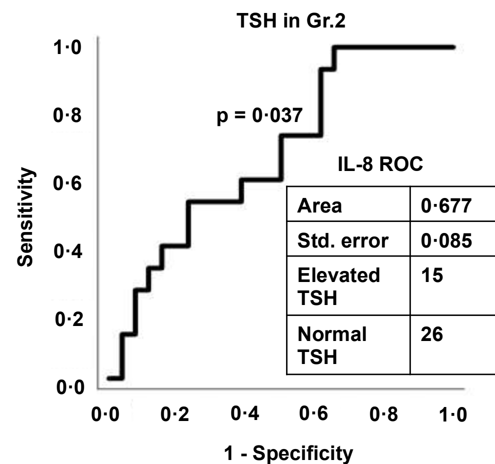
p = 0.002), TNF-α (r = 0.907, p = 0.004), IL-1β (r = 0.888, p = 0.008), and PAI-1 (r = 0.893, p = 0.007).

3.7. Diagnostic Assessment of Inflammation and Gut Dysfunction on Thyroid Function

Within Gr.2, several inflammatory cytokines had high predictive efficacy of hypothyroidism by elevated TSH. Within Gr.2, TNF-α significantly predicted an elevated TSH, area 0.708, p = 0.017 (Figure 4c). Further downstream in the inflammatory pathway, IL-6, IL-8, IL-1β, and MCP-1 significantly predicted an elevated TSH (Figure 4a,b,d,e). PAI-1 also had high predictive efficacy, though this was not statistically significant. These associations were not seen in Gr.1.



(a)



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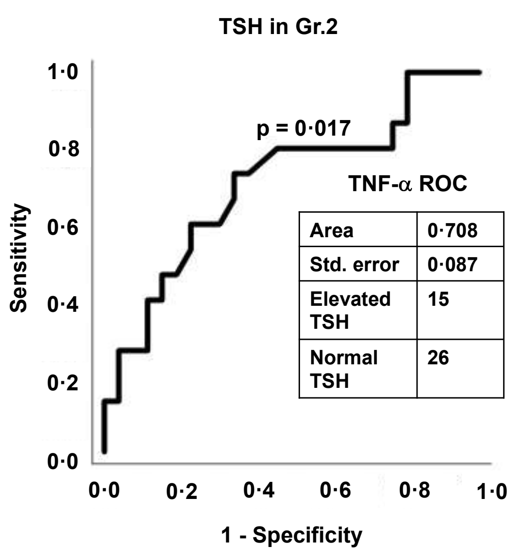
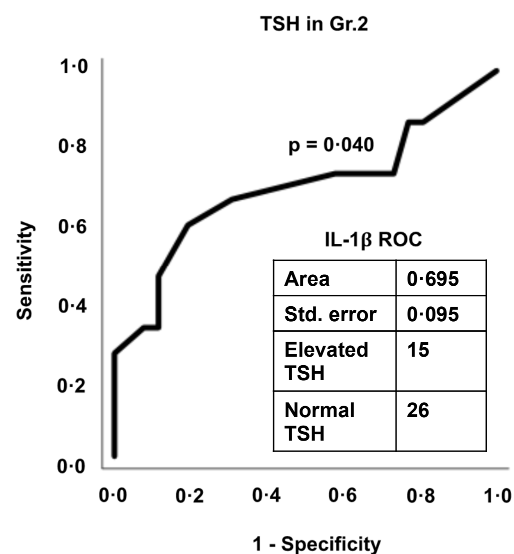


(c)



(d)

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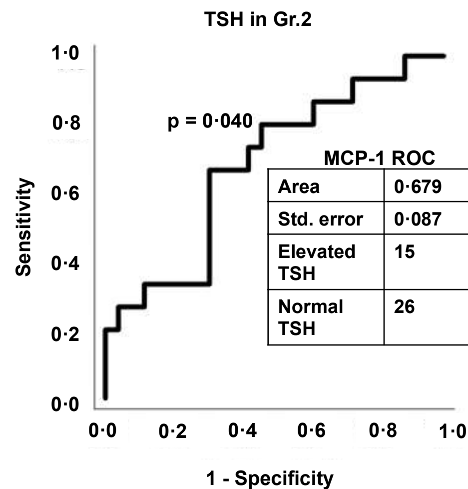

(e)

Figure 4. AUROC analysis demonstrating the diagnostic ability of several of the cytokines to predict TSH levels within Gr.2: (a) IL-6, (b) IL-8, (c) TNF-α, (d) IL-1β, (e) MCP-1. Area under the curve, standard error, number of patients within Gr.2 elevated TSH, and number of patients within Gr. 1 normal TSH are reported for each analysis.

4. Discussion

Our study examined the effects of heavy and chronic alcohol use on thyroid function by way of alcohol-induced gut dysfunction and pro-inflammatory cytokine release. Overall, the study patients did not have large differences in age or BMI. Gr.2 patients did drink more chronically as determined by LTDH, more frequently as determined by TD90 and NDD90, and more heavily as determined by HDD90 and AvgDPD90. Correspondingly, this group demonstrated higher gut dysfunction markers and pro-inflammatory cytokine levels. Alcohol has long been known to play a critical role in the activation of the innate and adaptive immune system. There are several studies that have described the correlation between alcohol consumption and associated immune responses. Chronic alcohol use in particular is associated with an upregulation of NF-kB activation by LPS; this leads to an increase in activation of monocytes and macrophages, instigating a large surge in the release of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-8 [24-26]. Specifically, chronic alcohol use results in microbial proliferation and acetaldehyde-mediated opening of intestinal tight junctions, which results in increased endotoxin release into the circulation [27-29]. Endotoxin LPS, with the cooperation of LBP and co-receptor sCD14, binds to TLR4 in the liver and gut; this activates signaling cascades which upregulate NF-kB and eventually increase the production of pro-inflammatory cytokines [30]. In addition to gut dysfunction-induced cytokine production, alcohol has also been shown to directly activate inflammasomes in multiple tissues after chronic use [31, 32]; this results in the induction of caspase-1 which is required to form the mature forms of IL-1β among other cytokines [33, 34]. This alcohol induced dysfunction was similarly demonstrated in our study.

In this study, all of the patients enrolled had baseline AUD with a history of chronic and acute drinking habits. However, it was particularly the acute drinking history, especially HDD90, TD90, and NDD990, which had the strongest correlation with gut-dysfunction and pro-inflammatory cytokine response. This could suggest that in the setting of chronic alcohol abuse, the increase in acute alcohol intake acts as a second-hit, further worsening the gut-dysfunction and cytokine surges. NDD90 and HDD90 had moderately strong and significant correlations in Gr.2 with gut-dysfunction markers, particularly with LBP and sCD14 combined; TD90, NDD90, and HDD had moderately strong correlations with several cytokines, such as IL-6, IL-8, IL-1β, and TNF-α as well. When adding

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cytokines to the alcohol-gut relationship, there was a large increase in strength of correlation. This coincides well with the understanding that alcohol-induced gut dysfunction contributes to the alcohol-induced pro-inflammatory state by the action of endotoxins on toll-like receptors.

In our study, the thyroid response (TSH, T3, and fT4) to the gut-cytokine relationship indicates a positive response, particularly with cytokines further downstream in the inflammatory cascade such as PAI-1 and IL-6. In addition, several inflammatory cytokines also significantly predicted TSH levels within Gr.2, which was not seen in Gr.1. This further contributes to the hypothesis that alcohol induced gut-dysfunction and pro-inflammatory cytokine response has an effect on thyroid function. There are studies that demonstrate relationships between pro-inflammatory cytokines and thyroid function previously. Several studies demonstrate that IL-6 and IL-8 levels are significantly higher in patients with subacute thyroiditis when compared to normal healthy controls [35-37]. This relationship between thyroid hormones and the immune system is thought to be bidirectional or constitute as part of the feedback loop. Some studies elucidate an association between thyroidectomy, hypothyroidism, and thymic growth depression with decreased circulating lymphocytes [38-41]. Furthermore, Blalock et al. initially demonstrate that TSH induced an increase in immune response by increasing the production of MCP-1 and IL-6 [42]. Conversely, other studies reveal that acute infections indirectly increase thyroid hormone release by the action of IL-1, IL-6, and TNF-α on the hypothalamus decreasing TSH release from the pituitary. There is clearly a mutual relationship between these inflammatory molecules and thyroid hormones, and our study furthers this understanding through the statistically significant correlations observed between gut dysfunction, cytokine production, and thyroid dysfunction.

Interestingly Gr.2 also had higher T3, albeit within normal levels, and higher fT4 levels; this is discordant with the classic hypothyroid picture expected with elevated TSH. In fact, when we examined this further, every patient in Gr.2 had an above normal fT4; this indicates that there are multiple factors modulating thyroid function, likely both at the glandular level as well as in the central nervous system (CNS). The relationship between alcohol use and the HPT axis has previously been investigated though there is no consensus on the exact effects of alcohol use on the thyroid or the underlying pathogenesis. Some studies demonstrate a blunted or absent response of TSH to TRH in patients with a history of alcohol abuse [43, 44]. Other studies further explore this effect, and determine that in these patients there are decreased concentrations of TSH at baseline and after stimulation with TRH [45] which insinuates alcohol has some effect on the CNS, likely on the pituitary gland. Several authors also suggest that pituitary TRH receptors are downregulated due to chronically high TRH concentrations in chronic alcohol use, as demonstrated by human and rat models [46, 47]. Other studies also examine the effect of alcoholism on peripheral thyroid hormones. Hegedus et. al, demonstrates a significant reduction in thyroid gland volume in alcohol dependent patients in multiple studies [48, 49]. Ultrasound images of the gland demonstrate not only a decrease in volume of the gland, but also a higher level of fibrosis with even a short duration of excessive alcohol use; the authors suggest that this may be due to a direct effect of alcohol on the gland itself [50, 51]

Our study postulated the effects of alcohol, gut dysfunction, and pro-inflammatory cytokines on the HPT axis (Figure 5). Interestingly, Gr.2 patients had an elevated TSH as well as elevated fT4, while T3 was within normal limits. One possibility is that alcohol abuse results in a direct toxic effect of the thyroid gland resulting in its inflammation, leading to subacute thyroiditis; this would account for the elevated fT4 in both groups, with even higher levels in Gr.2. The likelihood of elevated fT4 being secondary to a subacute thyroiditis is strengthened by the significantly elevated CRP levels in Gr.2. In fact, several studies demonstrate the strong correlation between thyroid function and elevated CRP levels in patients primarily with subacute thyroiditis, with this correlation not necessarily seen in other inflammatory thyroid conditions [52-54]. T3 was normal in both groups which indicates that even with an elevated fT4, there was decreased iodation. This is consistent with several studies that establish that in the setting of elevated inflammatory

cytokines, such as IL-6, or systemic illness, there is decreased conversion of T4 into T3 peripherally regardless of TSH levels [55-57] With elevated fT4 levels, it is expected that TSH levels will be low or normal due to the negative feedback of peripheral thyroid hormones on the pituitary and the hypothalamus [58]. However, in our study, every patient with elevated TSH had an elevated fT4, suggesting there was a disruption of this negative feedback loop; we hypothesize that this could be either at the hypothalamus and/or the pituitary level (Figure 5). Interestingly, this strengthens prior hypotheses that alcohol may not only have toxic effects on the thyroid gland, but also affect the HPT axis more centrally. This is a novel finding in a small population size and would be an interesting relationship to explore in larger studies with well-structured study design and focused hypothesis..

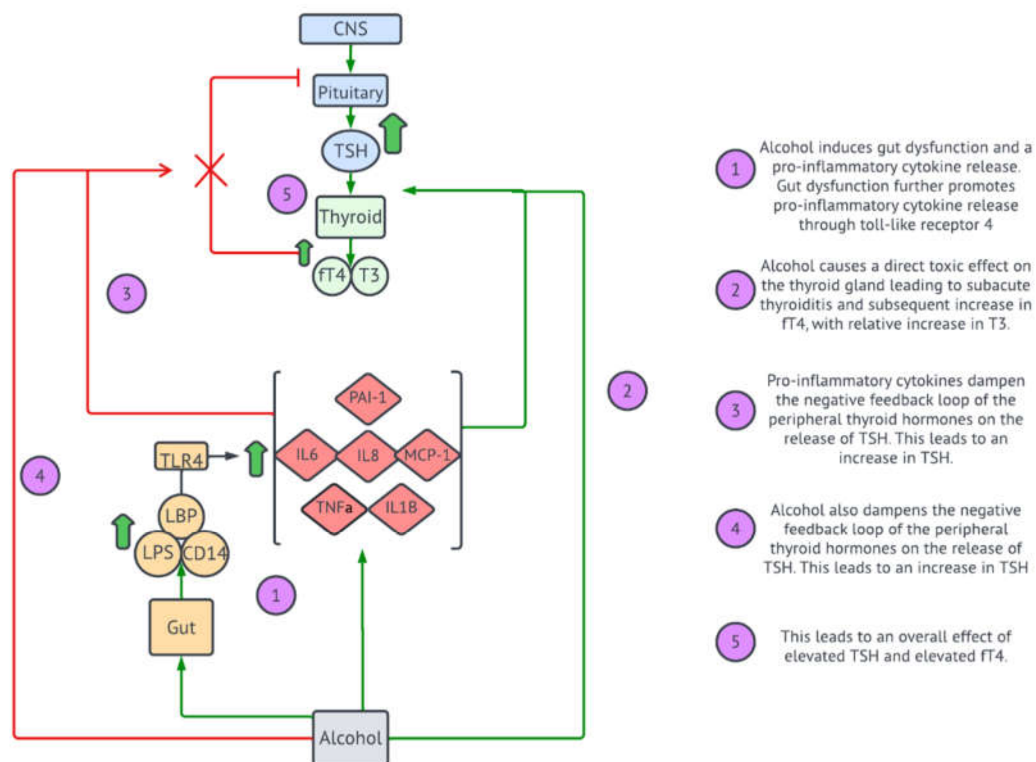

Figure 5. Our study hypothesis on the effects of alcohol on the HPT axis. This demonstrates (1) the pathogenesis of alcohol induced gut dysfunction and pro-inflammatory response, (2) the direct effect of alcohol on the thyroid, (3) the effect of pro-inflammatory cytokines on the HPT axis, (4) the effect of alcohol on the HPT axis, and (5) the resulting presentation of elevated TSH and elevated fT4.

There are several limitations in this study. Part of extent of the limitations lies in the scope of the study. This is a novel finding and was conducted as a clinical-translational paradigm. Thus, the aims were very specific and did not include the offshoots or special sub-categorial group studies. The sample size was small to moderate, and this was not a large-scale study. Thus, many underlying effects were not investigated that were identified during the course of data evaluation. Several findings from this novel study brings interest to address other scientific goals. These may need different study designs independent of this project at a larger scale with well-defined hypothesis. The study was also based on a single time point, so the thyroid function over a period of time was not evaluated. There was no TRH collected on any of the patients which would have provided even more of an understanding of the mechanism of CNS dysfunction. In addition, we did not collect other pituitary induced hormones such as cortisol. However, the likelihood of

AUD causing significant changes in the adrenocorticotropin hormone (ACTH)-cortisol pathway is low considering that would likely require significant disease. These patients were also admitted for treatment of acute alcohol intoxication, but there was no control on the level of intoxication or if the patients had already started the withdrawal process. The different levels of stress associated with these stages may have affected thyroid levels. Future studies can aim to address these limitations when investigating these relationships.

As this is a pilot study examining a largely uninvestigated relationship between alcohol use and thyroid function, there are still several components that will need to be expanded on in future studies. We focused on the functional and pathophysiological pathways involved with alcohol-induced gut inflammation leading to altered thyroid function. However, further examination into the particular microbiota that may be involved in this pathophysiology might contribute to further understanding of these pathways. Gut dysfunction markers in this study were limited to LPS, LBP, and sCD14 which we demonstrate have significant association with gut dysfunction associated cytokines, and we conclude that they are important markers of gut dysfunction. Other markers, such as fecal calprotectin or plasma citrulline, may also complement these primary markers and in determining gut dysfunction. These can and should be evaluated in future studies.

While there are several studies demonstrating the effects of pro-inflammatory cytokines and direct effects of alcohol on the HPT axis on an individual basis, our study was the first of its kind to describe alcohol induced gut-dysfunction and pro-inflammatory cytokine response on thyroid function. There is a high chance that in the AUD patients enrolled in our study, the HPT axis demonstrated dysregulation at several levels (Figure 5). This includes the direct effects on the thyroid gland to produce a sub-acute thyroiditis. These combined with the effects centrally dampen the negative feedback loop of the elevated peripheral thyroid hormones. This is a novel finding of alcohol-induced thyroid dysfunction that has only begun to determine the underlying unique pathogenesis. This study dictates the need for further study of this unique correlation, as well as the long term effects on the thyroid after the discontinuation of alcohol drinking.

Author Contributions: V.V. is the project the principal investigator of this study and designed the study. V.V., M.L.S. and M.K. participated in the collection, processing of the clinical sample and data analyses. M.K., M.S. and V.V. interpreted the results. M.S., A.J.R, and V.V. wrote the manuscript. H.H., A.R., S.R.P, M.K., M.L.S., S.K., D.P., M.C.C., and V.V. critically reviewed the manuscript and contributed scientifically.

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Institutional Review Board Statement: The study was approved by the site/s' Institutional Review Board of the University of Louisville and NIAAA. IRB Statement: NCT00106106 (clinicalTrials.gov Identifier), and approval number 98-AA-0009. The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of NIAAA and University of Louisville (protocol code: 98-AA-0009 and March 21, 2005 as the date of approval).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets analyzed during the current study are available from the corresponding author on reasonable request. Email address for the readers to contact the author to obtain the data: v0vats01@louisville.edu.

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Conflicts of Interest: The authors declare no conflict of interest.

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