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Adverse Effects of Asparaginase in Pediatric Patients with Acute Lymphoblastic Leukemia

Chengcheng Liu

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Adverse Effects of Asparaginase in Pediatric Patients with Acute Lymphoblastic Leukemia

Abstract

Acute lymphoblastic leukemia (ALL) is the most common type of childhood cancer. Asparaginase is a critical treatment component for ALL. However, its use is complicated by adverse effects, such as hypersensitivity, osteonecrosis and pancreatitis.

Hypersensitivity to asparaginase typically requires discontinuation of current formulation and substitution with other formulations, but the differential diagnosis can be challenging, and the diagnostic utility of antibody tests is unclear. We comprehensively analyzed anti-Elspar (native *E.coli* asparaginase) IgG antibodies in 410 pediatric patients treated on an asparaginase-intensive front-line clinical trial. Of 169 patients (41.2%) who exhibited clinical allergy, 147 (87.0%) were positive for anti-Elspar antibody. Of 241 patients without clinical allergy, 89 (36.9%) had detectable antibody. Among those positive for antibody, the antibody titers were higher in those who developed allergy than in those who did not ($P < 1.0 \times 10^{-15}$). Antibody measures at week 7 of continuation therapy had a sensitivity of 87%-88% and a specificity of 68%-69% for predicting or confirming clinical reactions. Antibodies were inversely associated with serum asparaginase activity ($P = 7.0 \times 10^{-6}$). Interestingly, high antibodies were associated with a lower risk of osteonecrosis (odds ratio = 0.83; 95% confidence interval, 0.78-0.89; $P = 0.007$), which is a dose-limiting adverse effect of glucocorticoids but has also been linked to asparaginase treatment. We conclude that antibodies were related to clinical allergy and to low systemic exposure to asparaginase, leading to lower risk of other adverse effects of therapy. Measures of serum antibodies to asparaginase can be useful in patients with ALL.

Osteonecrosis is a common dose-limiting toxicity of glucocorticoids. Data from clinical trials suggest that other medications can increase the risk of glucocorticoid-induced osteonecrosis. Here we utilized a mouse model to study the effect of asparaginase treatment on dexamethasone-induced osteonecrosis. After 6 weeks of treatment, mice receiving asparaginase along with dexamethasone had a higher rate of osteonecrosis than those receiving only dexamethasone (44% vs. 10%, $P = 0.006$). Primary epiphyseal arteriopathy, an initiating event for osteonecrosis, was observed in 58% of mice receiving asparaginase and dexamethasone compared to 17% of mice receiving dexamethasone only ($P = 0.007$). As in the clinic, greater exposure to asparaginase was associated with greater plasma exposure to dexamethasone ($P = 0.0001$). This model also recapitulated other clinical risk factors for osteonecrosis, including age at start of treatment, and association with the systemic exposure to dexamethasone ($P = 0.027$) and asparaginase ($P = 0.036$). We conclude that asparaginase can potentiate the osteonecrotic effect of glucocorticoids.

Acute pancreatitis is a serious complication of asparaginase with no definitive treatment. Risk factors for asparaginase-induced pancreatitis, especially the genetic predisposition, have not been clearly identified. We studied 5398 pediatric patients with ALL and showed that older age, higher exposure to asparaginase, higher Native American ancestry or African ancestry were independent clinical risk factors for pancreatitis. To determine genetic risk factors, we performed a genome-wide association study. A rare nonsense variant rs199695765 in CPA2, a pancreatic enzyme, was highly associated with pancreatitis (odds ratio = 588, 95% confidence interval 66.8- 5166, $P = 9.0 \times 10^{-9}$). A gene-level analysis showed an excess of additional CPA2 variants in those who did versus did not develop pancreatitis ($P = 0.018$). Furthermore, common variants in genes critical to purine metabolism and cytoskeleton function were also associated with development of pancreatitis. Our findings are consistent with a mixed genetic architecture underlying serious adverse drug effects, wherein a combination of rare but highly penetrant and common but weakly penetrant genetic risk factors contribute to genetic risk. For the patients carrying the highly penetrant variants, consideration should be given to treatment with a nonasparaginase

containing ALL chemotherapy regimens.

Overall, we studied the major adverse effects of asparaginase in patients treated for ALL. These findings will provide important guidance for precision medicine.

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**ADVERSE EFFECTS OF ASPARAGINASE IN PEDIATRIC PATIENTS WITH
ACUTE LYMPHOBLASTIC LEUKEMIA**

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Chengcheng Liu
August 2015

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DEDICATION

This work is dedicated to my parents

Haiying Liu

Hua Cheng

and my husband

Hao Wu

Thank you for all of your love and support along the way.

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ABSTRACT

Acute lymphoblastic leukemia (ALL) is the most common type of childhood cancer. Asparaginase is a critical treatment component for ALL. However, its use is complicated by adverse effects, such as hypersensitivity, osteonecrosis and pancreatitis.

Hypersensitivity to asparaginase typically requires discontinuation of current formulation and substitution with other formulations, but the differential diagnosis can be challenging, and the diagnostic utility of antibody tests is unclear. We comprehensively analyzed anti-Elspar (native *E.coli* asparaginase) IgG antibodies in 410 pediatric patients treated on an asparaginase-intensive front-line clinical trial. Of 169 patients (41.2%) who exhibited clinical allergy, 147 (87.0%) were positive for anti-Elspar antibody. Of 241 patients without clinical allergy, 89 (36.9%) had detectable antibody. Among those positive for antibody, the antibody titers were higher in those who developed allergy than in those who did not ($P < 1.0 \times 10^{-15}$). Antibody measures at week 7 of continuation therapy had a sensitivity of 87%-88% and a specificity of 68%-69% for predicting or confirming clinical reactions. Antibodies were inversely associated with serum asparaginase activity ($P = 7.0 \times 10^{-6}$). Interestingly, high antibodies were associated with a lower risk of osteonecrosis (odds ratio = 0.83; 95% confidence interval, 0.78-0.89; $P = 0.007$), which is a dose-limiting adverse effect of glucocorticoids but has also been linked to asparaginase treatment. We conclude that antibodies were related to clinical allergy and to low systemic exposure to asparaginase, leading to lower risk of other adverse effects of therapy. Measures of serum antibodies to asparaginase can be useful in patients with ALL.

Osteonecrosis is a common dose-limiting toxicity of glucocorticoids. Data from clinical trials suggest that other medications can increase the risk of glucocorticoid-induced osteonecrosis. Here we utilized a mouse model to study the effect of asparaginase treatment on dexamethasone-induced osteonecrosis. After 6 weeks of treatment, mice receiving asparaginase along with dexamethasone had a higher rate of osteonecrosis than those receiving only dexamethasone (44% vs. 10%, $P = 0.006$). Primary epiphyseal arteriopathy, an initiating event for osteonecrosis, was observed in 58% of mice receiving asparaginase and dexamethasone compared to 17% of mice receiving dexamethasone only ($P = 0.007$). As in the clinic, greater exposure to asparaginase was associated with greater plasma exposure to dexamethasone ($P = 0.0001$). This model also recapitulated other clinical risk factors for osteonecrosis, including age at start of treatment, and association with the systemic exposure to dexamethasone ($P = 0.027$) and asparaginase ($P = 0.036$). We conclude that asparaginase can potentiate the osteonecrotic effect of glucocorticoids.

Acute pancreatitis is a serious complication of asparaginase with no definitive treatment. Risk factors for asparaginase-induced pancreatitis, especially the genetic predisposition, have not been clearly identified. We studied 5398 pediatric patients with ALL and showed that older age, higher exposure to asparaginase, higher Native American ancestry or African ancestry were independent clinical risk factors for

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Overall, we studied the major adverse effects of asparaginase in patients treated for ALL. These findings will provide important guidance for precision medicine.

TABLE OF CONTENTS

CHAPTER 1. BACKGROUND.....	1
Acute Lymphoblastic Leukemia (ALL)	1
Asparaginase	2
Introduction	2
Formulations	2
Native <i>E.coli</i> asparaginase	2
PEG-asparaginase	3
<i>Erwinia</i> asparaginase	3
Adverse effects.....	3
Hypersensitivity	4
Acute pancreatitis.....	4
Drug interaction with glucocorticoids	6
Effect on glucocorticoid-induced osteonecrosis	6
Pharmacogenomics: A Brief Introduction	8
Cohort study	8
Case-control study	8
Candidate-gene approach	9
GWAS	9
CHAPTER 2. ASPARAGINASE HYPERSENSITIVITY.....	10
Introduction.....	10
Methods	10
Patients	10
Asparaginase regimen and sample collection	10
Phenotyping of clinical allergy to asparaginase.....	12
Anti-asparaginase antibodies	12
Antibody assay.....	12
Data analysis	14
Estimation of antibody area-under-the-curve (AUC)	14
Asparaginase activity	15
<i>Ex vivo</i> asparaginase neutralization assay.....	16
Prognostic value of antibody tests	16
Sensitivity, specificity and predictive values.....	16
Receiver-operator-characteristic (ROC) curves.....	16
Asparaginase adverse effects in Total XV	17
Statistical analysis	17
Results.....	17
Asparaginase allergy	17
Antibodies and allergy	18
Antibodies and serum asparaginase activity	30
Antibodies and adverse effects	30
Discussion	34
Conclusion	36

CHAPTER 3. POTENTIATING EFFECT OF ASPARAGINASE ON GLUCOCORTICOID-INDUCED OSTEONECROSIS	38
Introduction.....	38
Methods	38
Chemicals.....	38
Animals	39
Asparaginase and dexamethasone pharmacokinetics	39
Mouse model of dexamethasone-induced osteonecrosis	39
Effect of asparaginase on osteonecrosis.....	41
Plasma dexamethasone concentration and asparaginase activity	41
Histological evaluation of osteonecrosis and arteriopathy	41
Statistical analysis	41
Results.....	42
Plasma dexamethasone concentration was increased by asparaginase treatment.....	42
Asparaginase treatment potentiated osteonecrosis and arteriopathy in dexamethasone-treated mice	42
Arteriopathy was likely the initiating event of osteonecrosis	46
Discussion.....	46
Conclusion	50
CHAPTER 4. ASPARAGINASE-INDUCED ACUTE PANCREATITIS	51
Introduction.....	51
Methods	51
Patients and treatment	51
Diagnosis of acute pancreatitis	55
Genotyping.....	55
SNP-based analysis for pancreatitis.....	58
Gene-level analysis of nonsense variants using SKAT	58
Deep sequencing of selected genes	58
Statistical analysis	63
Results.....	63
Pancreatitis associated with asparaginase	63
Clinical risk factors	63
Gene-level analysis of nonsense variants identified <i>CPA2</i>	68
GWAS of common SNPs.....	68
Deep sequencing detected novel variants in <i>CPA2</i> and other candidate genes	77
Discussion.....	78
Conclusion	81
CHAPTER 5. SUMMARY.....	82
LIST OF REFERENCES	83
APPENDIX A. SUPPLEMENTARY INFORMATION FOR CHAPTER 2	97
Anti-asparaginase Antibody ELISA	97
Positive and negative controls	97

Asparaginase antigens	97
Measurement of anti-asparaginase IgG antibodies by ELISA	97
Normalization of Antibody Readings	98
APPENDIX B. SUPPLEMENTARY INFORMATION FOR CHAPTER 3	111
Mouse Model of Dexamethasone-induced Osteonecrosis	111
Methods	111
Chemicals	111
Animals	111
Treatments	112
Enteric and blood cultures	112
Measurement of plasma concentration of dexamethasone and corticosterone ...	112
Results	113
Body weight differed by substrains	113
Effect of substrain, source, age and treatment regimens on survival and development of osteonecrosis	113
Dexamethasone pharmacokinetics and pharmacodynamics	122
Gender-dependent difference in susceptibility to osteonecrosis	122
Discussion	122
Conclusion	127
Effect of Asparaginase Treatment on Dexamethasone Pharmacokinetics	128
Effect of asparaginase and dexamethasone on plasma protein and lipid levels	129
APPENDIX C. SUPPLEMENTARY INFORMATION FOR CHAPTER 4	139
Deep Sequencing of Selected Genes	139
SNP-based and Gene-based Analysis of Variants Identified by Deep Sequencing	139
VITA.....	171

LIST OF TABLES

Table 2-1.	Clinical features of patients with (n = 410) and without (n = 88) samples evaluable for anti-asparaginase antibodies.....	11
Table 2-2.	Number of patients with evaluable blood samples at each time point.....	13
Table 2-3.	Timing of allergic reaction to Elspar	19
Table 2-4.	Clinical features and allergic reactions to Elspar.....	20
Table 2-5.	Clinical features and anti-Elspar antibody status.....	21
Table 2-6.	Sensitivity, specificity and predictive values of three anti-Elspar antibody tests for predicting or confirming clinical reactions to Elspar	25
Table 2-7.	Multivariate analysis showed that low anti-Elspar antibody area-under-curve (AUC) was a risk factor of osteonecrosis (n = 360).....	32
Table 4-1.	Comparison of asparaginase regimens.....	52
Table 4-2.	Number of patients included in this study	56
Table 4-3.	Putative genes associated with pancreatitis (n = 42) selected for deep sequencing based on GWAS result or literature review.....	59
Table 4-4.	Multivariate analysis for clinical risk of pancreatitis in the cohort study (n = 5185).....	69
Table 4-5.	Six genes associated with pancreatitis from the gene-level analysis of nonsense SNPs	72
Table 4-6.	Top 20 common SNPs (cohort risk allele frequency > 1%) significantly associated with pancreatitis in the cohort.....	75
Table A-1.	Comparison between antibody OD readings obtained at SJCRH (n = 1539) and UTHSC (n = 665).....	99
Table A-2.	Sensitivity, specificity and predictive values of anti-Elspar antibody test at week 7 for predicting or confirming clinical reactions to Elspar using current positivity cutoff and optimized cutoff by recursive partitioning ...	107
Table B-1.	Incidence of ON in male BALB/c mice from different experiments.....	118
Table B-2.	Enteric culture isolates from BALB/cJ mice from vendor and in-house breeding colony	121

Table B-3.	Inhibition of plasma protein level in human and mouse by asparaginase treatment.....	136
Table C-1.	Top 10 SNPs from genome-wide analysis of nonsense SNPs; n = 2,357 SNPs.....	142
Table C-2.	Top 10 SNPs from genome-wide analysis of missense SNPs (excluding nonsense SNPs); n = 144,349 SNPs.....	143
Table C-3.	Top 10 SNPs from genome-wide analysis of synonymous and non-coding SNPs; n = 774,238 SNPs.....	144
Table C-4.	All 380 <i>CPA2</i> variants identified in the cohort (n = 4217) and the case-control group (n = 162)	145
Table C-5.	Top genes ($P < 0.05$) in the gene-level analysis of nonsense variants identified in putative pancreatitis (n = 42) and non-pancreatitis genes (n = 241) based on variants identified by deep sequencing.....	162
Table C-6.	SNPs (n = 81) identified by deep sequencing in previously reported pancreatitis-associated candidate genes (n = 8) at the $P < 0.05$ level (highlighted) in either the cohort or the case-control group.....	165

LIST OF FIGURES

Figure 1-1. Proposed mechanisms of glucocorticoid-induced osteonecrosis	7
Figure 2-1. Total XV asparaginase regimen and anti-asparaginase antibody measurements	13
Figure 2-2. Enzyme reactions in asparaginase activity assay	15
Figure 2-3. Frequency of hypersensitivity to Elspar	19
Figure 2-4. Antibody levels in patients with and without clinical reactions	22
Figure 2-5. Anti-Elspar antibody level relative to the time of clinical reaction to Elspar	24
Figure 2-6. ROC curves of the antibody tests.....	26
Figure 2-7. Association between week 7 anti-Elspar antibody level and the proportion of patients reacting to Elspar around week 7	28
Figure 2-8. The frequency of hypersensitivity to Oncaspar and Erwinase.....	29
Figure 2-9. Correlation between serum asparaginase activity and antibody level	31
Figure 2-10. <i>Ex vivo</i> neutralization of patient sera with antibodies	32
Figure 2-11. Cumulative incidence of osteonecrosis based on anti-asparaginase antibody AUC	33
Figure 3-1. Dexamethasone and asparaginase treatment regimens	40
Figure 3-2. Plasma dexamethasone concentration was positively associated with asparaginase activity in PK experiment	43
Figure 3-3. Asparaginase treatment potentiated osteonecrosis and arteriopathy in dexamethasone-treated mice	44
Figure 3-4. Osteonecrosis was associated with higher plasma dexamethasone and asparaginase levels in mice receiving both dexamethasone and asparaginase treatment	45
Figure 3-5. Histology of osteonecrosis and arteriopathy	47
Figure 4-1. Study design.....	54
Figure 4-2. Race group assignments based on the ancestral composition of patients in the cohort (n = 5185) by treatment protocol	57

Figure 4-3. Incidence of pancreatitis differed by protocols.....	64
Figure 4-4. Time of pancreatitis in the cohort (n = 5185) during the first year of therapy	65
Figure 4-5. The incidence of pancreatitis based on clinical characteristics	67
Figure 4-6. Association between Native American ancestry and pancreatitis	70
Figure 4-7. CART analysis of clinical risk factors for pancreatitis in the cohort (n = 5185).....	71
Figure A-1. Anti-Elspar OD readings obtained at SJCRH (n = 1539) and UTHSC (n = 665) relative to the time of clinical reaction	100
Figure A-2. Fitted curves for (A) anti-Elspar, (B) anti-Erwinase and (C) anti-Oncaspar OD readings of the samples (n = 194) measured at both SJCRH and UTHSC	101
Figure A-3. UTHSC OD readings before and after normalization.....	105
Figure A-4. Determination of AUC threshold.....	106
Figure A-5. Week-7 optimal threshold of OD value to predict or to confirm clinical reactions	108
Figure A-6. Elspar has stronger cross-reactivity with Oncaspar than with Erwinase	109
Figure A-7. Sensitivity of antibody tests within 30 days of reactions.....	110
Figure B-1. Body weight of mice before and after treatment	114
Figure B-2. Incidence of osteonecrosis increased with longer treatment duration	115
Figure B-3. Similar histologic appearance of osteonecrosis in mice of different substrain, age and treatment groups	117
Figure B-4. Survival differed by substrains, sources, regimens and age of mice	119
Figure B-5. Plasma concentrations of dexamethasone.....	123
Figure B-6. Inverse association between plasma corticosterone and dexamethasone levels.....	124
Figure B-7. Gender-dependent differences in susceptibility to osteonecrosis	125
Figure B-8. Plasma dexamethasone concentration was positively associated with asparaginase activity in osteonecrosis experiment.....	128

Figure B-9. Mouse plasma amylase level after treatment with dexamethasone and asparaginase	129
Figure B-10. Mouse plasma lipase level after treatment with dexamethasone and asparaginase	130
Figure B-11. Mouse plasma triglyceride level after treatment with dexamethasone and asparaginase	132
Figure B-12. Mouse plasma albumin level after treatment with dexamethasone and asparaginase	133
Figure B-13. Mouse plasma cortisol level after treatment with dexamethasone and asparaginase	135
Figure B-14. Correlation between asparagine content in the protein and the extent of inhibition by asparaginase treatment.....	138
Figure C-1. CADD score in all SNPs on exome array (n = 162586) and pancreatitis-associated SNPs ($P < 0.05$; n = 8018).....	141
Figure C-2. Top pathways and functions of pancreatitis-associated genes (n = 42).....	163
Figure C-3. Top pathways and functions of pancreatitis-associated genes identified by GWAS (n = 34)	164

LIST OF ABBREVIATIONS

ABL	Abelson tyrosine protein kinase
ACTN2	Actinin-2
ALL	Acute lymphoblastic leukemia
ANTI-ASP	Anti-asparaginase antibodies
AP	Acute pancreatitis
ASNS	Asparagine synthetase
ASP	Asparaginase
AUC	Area-under-the-curve
BCR	Breakpoint cluster region
CADD	Combined Annotation Dependent Depletion
CART	Classification and regression tree
CASR	Calcium sensing receptor
CFTR	Cystic fibrosis trans-membrane conductance regulator
CI	Confidence interval
CLDN2	Claudin-2
CNS	Central nervous system
COG	Children's Oncology Group
CPA1	Carboxypeptidase A1
CPA2	Carboxypeptidase A2
CPB	Carboxypeptidase B
CSF	Cerebrospinal fluid
CTRC	Chymotrypsinogen C
CYP	Cytochrome P450
DEX	Dexamethasone
DI	Delayed intensification
DOCK5	Dedicator of cytokinesis 5
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ExAC	Exome Aggregation Consortium
FDA	U.S. Food and Drug Administration
FHIT	Fragile histidine triad protein
FN	False negative
FP	False positive
GEF	Guanine nucleotide exchange factor
GWAS	Genome-wide association study
HLA	Human leukocyte antigen
HOGA1	4-hydroxy-2-oxoglutarate aldolase
HPLC	High-performance liquid chromatography
HR	Hazard ratio
IACUC	Institutional Animal Care and Use Committee
Ig	Immunoglobulin
IM	Interim maintenance
I.m.	Intramuscularly

I.p.	Intraperitoneally
IU	International unit(s)
I.v.	Intravenously
LOQ	Limit of quantitation
LR	Low-risk
MAF	Minor allele frequency
MICAL2	Molecule interacting with CasL protein 2
MRD	Minimal residual disease
MRI	Magnetic resonance imaging
MS	Mass spectrometric
MSC	Mesenchymal stem cells
NADH	Nicotinamide adenine dinucleotide (reduced)
NCI CTCAE	National Cancer Institute Common Toxicity Criteria for Adverse Events
NHP	Normal human plasma
NPV	Negative predictive value
OD	Optical density
ON	Osteonecrosis
OR	Odds ratio
P24 (or P28)	Postnatal day 24 (or 28)
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
PK	Pharmacokinetics
PPV	Positive predictive value
PRSS1	Protease serine 1 (cationic trypsinogen)
PRSS2	Protease serine 2 (anionic trypsinogen)
PSTI	Pancreatic secretory trypsin inhibitor
RAF	Risk allele frequency
ROC	Receiver-operator-characteristic
RSID	Reference SNP identification number
SD	Standard deviation
SE	Standard error
SHR	Standard/high-risk
SJCRH	St. Jude Children's Research Hospital
SKAT	Sequence kernel association test
SMZ	Sulfamethoxazole
SNP	Single nucleotide polymorphism
SPINK1	Serine protease inhibitor kazal type 1
TCN	Tetracycline
TMP	Trimethoprim
TN	True negative
TP	True positive
TPMT	Thiopurine S-methyltransferase
UTHSC	University of Tennessee Health Science Center
UTR	Untranslated region
UV	Ultraviolet

CHAPTER 1. BACKGROUND

Acute Lymphoblastic Leukemia (ALL)

ALL is a malignant disorder of lymphoid progenitor cells, characterized by proliferation of immature white blood cells – known as lymphoblasts. The lymphoblasts invade peripheral blood, bone marrow and other organs, causing tissue damage and inhibition of normal cells. The symptoms of ALL include weakness, fever, pain in bone and joints, bruising and bleeding, petechiae and others. Diagnostic methods include blood tests, bone marrow aspiration and biopsy, lumbar puncture and chest X-ray.¹ The immunophenotype of lymphoblasts (B- or T-lineage) can be identified by their surface antigens using flow cytometry.²

ALL is the most common childhood cancer and accounts for 25% of cancer diagnoses in children younger than 15 years, with peak prevalence between 2 and 5 years.³ The incidence of new diagnosis every year is approximately 4 cases per million for children and adolescents under 20 years, with an upward trend over the past 40 years.⁴ The increasing incidence may be associated with changes in environmental factors, such as industrialization. In addition, the improved diagnosis and access to medical care over time may also play a role.⁴

Although the precise pathogenesis of ALL remains unknown, this disease has been associated with genetic alterations in tumor-suppressors, proto-oncogenes and other key regulatory genes of hematopoietic stem cells and progenitors.^{1,5} In about 75% of childhood ALL cases, recurring chromosomal alterations have been detected.⁶ For example, the t(9;22) chromosomal translocation results in fusion of the breakpoint cluster region (*BCR*) on chromosome 22 with the Abelson tyrosine protein kinase 1 (*ABL1*) on chromosome 9, which is a tyrosine kinase whose activity is well regulated under normal conditions. The fused BCR-ABL protein, however, has high constitutive activity that leads to enhanced proliferation and self-renewal of hematopoietic stem cells.⁷

There has been dramatic increase in the survival rate of children and adolescents with ALL. In the past few decades, the five-year event-free survival rate of pediatric patients has been improved from less than 5% to over 80%, and the overall survival rate has reached 90%.^{5,8} Success was achieved by the intense use of chemotherapeutic drugs, such as asparaginase, glucocorticoids and thiopurines. Meanwhile, with such a high cure rate, investigators and clinicians have also turned their attention to improving the quality of life of ALL survivors. In recent years, many efforts have been made to modify ALL therapies in attempts to achieve optimal efficacy and avoid serious toxicities, which requires a clear understanding of the mechanisms of drug response and the risk factors of adverse drug reactions.

Asparaginase

Introduction

In 1953, Kidd^{9,10} first observed that injections of normal guinea pig serum induced regression of transplanted lymphoma in mice and rats. At that time it was believed to be an immunological phenomenon. In 1963, Broome¹¹ found out that an enzyme, asparaginase, was responsible for the anti-tumor activity. Mashburn and Wriston¹² subsequently showed that L-asparaginase derived from *Escherichia coli* had similar tumor inhibitory effect. The first clinical use of asparaginase, which was purified from guinea pig serum, showed a short therapeutic effect in a pediatric patient with relapsed ALL.¹³ Later the development of isolation and purification procedures of L-asparaginase from *E.coli* has enabled large-scale production of this effective chemotherapeutic agent.¹⁴

Asparaginase is an enzyme consisting of four identical subunits and has a molecular weight of 140000 Da. It catalyzes the hydrolysis of a non-essential amino acid, asparagine, to aspartic acid and ammonia. Unlike other tissues, leukemic lymphoblasts are deficient of asparagine synthetase (ASNS) and therefore are sensitive to depletion of circulating asparagine. It has been reported that asparaginase sensitivity was correlated with ASNS protein content in ALL cell lines.¹⁵ Moreover, bone marrow-derived mesenchymal stem cells (MSCs), which form the microenvironment for leukemic cells to grow, have been shown to have high expression of ASNS.¹⁶

Asparaginase also shows glutaminase activity, which catalyzes the hydrolysis of glutamine to glutamate. For *E.coli*- and *Erwinia chrysanthemi*-derived asparaginase, glutaminase activity is about 5% of asparaginase activity.¹⁷

Formulations

There are currently three formulations of asparaginase available for the treatment of patients with ALL: the native *E.coli* asparaginase, a pegylated (polyethylene glycol-conjugated) form of native *E.coli* asparaginase (PEG-asparaginase), and *Erwinia chrysanthemi*-derived asparaginase.

Native *E.coli* asparaginase

In 1978, Native *E.coli* asparaginase was approved by the U.S. Food and Drug Administration (FDA) as a component of a multi-agent chemotherapeutic regimen for the treatment of patients with ALL. It has been the most widely used asparaginase formulation, and common brand names include Elspar (USA), Kidrolase (UK), Leunase (France), Crasnitin (Germany) and Asparaginase Medac (Japan). Some of the brand

names have been discontinued, and Native *E.coli* asparaginase is no longer available as a pharmaceutical preparation in the U.S.

The half-life of native *E.coli* asparaginase is approximately 1.2 days.¹⁸ Elspar is stored as lyophilized powder, and is typically given intramuscularly (i.m.) at 6000-10000 U/m² three times a week. Although asparaginase cannot cross the blood-brain barrier, it can deplete asparagine in the cerebrospinal fluid (CSF).¹⁹ Serum level of asparaginase at 100 international units (IU)/L was considered adequate to fully deplete asparagine in blood and CSF.²⁰ It has been reported that the presence of neutralizing antibodies against native *E.coli* asparaginase was associated with higher risk of relapse in the central nervous system (CNS).²¹

PEG-asparaginase

PEG-conjugation has substantially prolonged the circulating half-life and decreased the immunogenicity of native *E.coli* asparaginase.²² PEG-asparaginase (or pegaspargase, brand name: Oncaspar) was first used as a second-line option for patients who developed hypersensitivity to native *E.coli* asparaginase. It was approved for the first-line use by FDA in 2006.²³ PEG-asparaginase has replaced native *E.coli* asparaginase in some recent ALL trials²⁴ because it has a longer half-life (about 5.7 days),¹⁸ thus requires a reduced dose and frequency (2500-3500 U/m² every two weeks) comparing to native *E.coli* asparaginase. The disadvantages of PEG-asparaginase include its high cost and short shelf-life (about 6-8 months).

***Erwinia* asparaginase**

Erwinia asparaginase (brand name: Erwinaze in USA or Erwinase in UK) was approved by FDA as second-line therapy when patients develop allergy to native *E.coli* asparaginase or PEG-asparaginase for ALL in 2011.²⁵ *Erwinia* asparaginase has a short half-life of 0.6 day.¹⁸ It has been shown to have lower serum activity and therapeutic efficacy than native *E.coli* asparaginase.²⁶ In EORTC-CLG 58881 trial, patients receiving *Erwinia* asparaginase had inferior outcome comparing to those receiving native *E.coli* asparaginase at the same dose (10000 U/m² twice weekly).²⁷ Therefore, higher dose (20000-30000 U/m²) and more frequent administration (three times a week) is recommended when using *Erwinia* asparaginase. Recently, a pegylated recombinant *Erwinia* asparaginase (PEGcrisantaspase) has been reported to have similar efficacy and lower immunogenicity than native *Erwinia* asparaginase in a preclinical study.²⁸

Adverse effects

The use of asparaginase is complicated by its adverse effects. Common adverse effects include hypersensitivity, thrombosis, coagulopathy, pancreatitis, altered lipid metabolism, glucose intolerance, hepatotoxicity and neurotoxicity.²⁹ Drug interactions

between asparaginase and other antileukemic agents, such as glucocorticoids, have also been observed.³⁰

Hypersensitivity

Because asparaginase is a bacteria-derived protein, hypersensitivity reaction is the most frequent adverse effect. Up to 45% patients can develop clinical allergy to native *E. coli* asparaginase, mostly upon re-exposure.³¹ Some patients may also develop anti-asparaginase antibodies without any clinical signs of hypersensitivity, which is referred to as “silent hypersensitivity”.³² Anti-asparaginase antibodies can be detected by enzyme-linked immunosorbent assay (ELISA) in patients with hypersensitivity to asparaginase. The majority of anti-asparaginase antibodies are of the immunoglobulin G (IgG) class, and the presence of IgM and IgE has been reported in several cases.³³⁻³⁶

Allergic reactions typically require discontinuation of the offending formulation (e.g., native *E. coli* asparaginase) and substitution with other formulations (e.g., *Erwinia* asparaginase or PEG-asparaginase). Only 20-25% of patients will develop allergic reactions to the substituting formulation.¹⁸ However, allergic reactions caused by asparaginase should be differentiated from acute reactions caused by other drugs. So far only few studies have focused on using a diagnostic test, such as anti-asparaginase antibodies, to confirm allergic reactions.³⁷

Attempts have been made to predict asparaginase hypersensitivity, especially the life-threatening anaphylactic reactions, before asparaginase is given. Some studies used a small test dose to determine the reactivity of patients; however, this method seemed to lack sensitivity and the test dose itself may pre-sensitize the patients.^{38,39}

Anti-asparaginase IgG antibodies were associated with lower serum asparaginase activity and a decreased half-life of asparaginase.^{32,40,41} Moreover, because of the cross-reactivity between native and pegylated *E. coli* asparaginase, the activity of PEG-asparaginase as a second-line treatment was also reported to be associated with anti-*E. coli* asparaginase antibody levels.⁴² The presence of IgG antibodies has been shown to affect the therapeutic outcome of ALL,^{21,43} although there are conflicting data.^{31,36,44-46} There was no correlation between serum asparaginase activity and IgM or IgE antibody levels.³³

Thus, we studied the effect of anti-asparaginase (Elspar) IgG antibodies on serum asparaginase activity, and investigated the clinical utility of anti-Elspar antibodies (see Chapter 2).

Acute pancreatitis

Acute pancreatitis is one of the most serious adverse events of asparaginase treatment. It occurs in up to 18% of patients,^{40,47-54} and can be life-threatening for some

patients who develop pseudocysts or necrosis.^{55,56} Common symptoms associated with pancreatitis include nausea, vomiting, and upper abdominal pain especially after eating.⁵⁷ The diagnostic criteria include (a) upper abdominal pain, (b) serum level of amylase or lipase ≥ 3 times the upper limit of normal, and (c) radiographical findings consistent with pancreatitis. The grading system is based on the National Cancer Institute Common Toxicity Criteria for Adverse Events (NCI CTCAE)⁵⁸ or the Atlanta Classification system.⁵⁹

Development of pancreatitis during asparaginase treatment can result in temporarily or permanently withholding of asparaginase based on the severity of the initial episode. Some of the patients may be allowed to receive asparaginase after recovery if their initial episode is considered mild. However, up to 63% of patients may experience a second episode of pancreatitis after being re-challenged.⁶⁰ There is no definitive treatment for pancreatitis; only supportive care is given.^{57,61}

The pathophysiology behind asparaginase-induced pancreatitis remains unknown. It is hypothesized that asparaginase treatment may be associated with amino acid imbalance and altered pancreatic secretion. After pediatric patients with ALL received the first dose of asparaginase, an imbalance of plasma amino acid levels was observed along with elevations of trypsin and pancreatic secretory trypsin inhibitor (PSTI), a sign of pancreatic injury.⁶² In a rat model of asparaginase-induced “pre-pancreatitis”, which was characterized by slight morphologic change without enzyme elevation, it was observed that zymogen granules accumulated within pancreatic acinar cells, indicating that asparaginase may alter exocrine function of pancreas.⁶³ Asparaginase has also been shown to affect insulin response, which can be attributed to an impairment of exocrine secretion.⁶⁴ However, the underlying mechanism has not been elucidated.

Older age and intensive asparaginase therapy have been indicated to increase the risk of pancreatitis in pediatric patients with ALL,^{47-50,56,65} although most studies of asparaginase-induced pancreatitis lack sufficient power to robustly assess risk factors.⁴⁶⁻⁴⁹ Genetic predispositions may also play a role, because some patients develop pancreatitis after only few doses of asparaginase. Several genetic factors have been identified for other forms of pancreatitis, mostly associated with premature trypsin activation or sustained trypsin activity in the pancreas. All of these studies focused on pancreatitis of other etiologies, such as alcoholic, idiopathic and chronic pancreatitis. There has been no genetic study on asparaginase-induced pancreatitis.⁸² Gain-of-function mutations in cationic and anionic trypsinogen genes (*PRSS1* and *PRSS2*) have been associated with pancreatitis.⁶⁶⁻⁶⁹ Polymorphisms in serine protease inhibitor kazal type 1 (*SPINK1*), a gene encoding an inhibitor that prevents premature trypsin activation, have been shown to be associated with pancreatitis.⁷⁰⁻⁷² Cystic fibrosis trans-membrane conductance regulator (*CFTR*), which regulates elimination of trypsin through the pancreatic duct, has also been linked to pancreatitis.⁷³⁻⁷⁵ Other risk genes include chymotrypsinogen C (*CTRC*), calcium sensing receptor (*CASR*), claudin-2 (*CLDN2*), carboxypeptidase 1 (*CPA1*), and *HLA-DRB1*07:01*.^{72,75-81}

In our current study, we aimed to identify the clinical and genetic risk factors for asparaginase-induced acute pancreatitis in pediatric patients with ALL (see Chapter 4).

Drug interaction with glucocorticoids

Because asparaginase inhibits the synthesis of protein, including enzymes responsible for drug metabolism and DNA synthesis, it may have interactions with accompanying antileukemic medication, such as glucocorticoids.

Glucocorticoids (e.g. dexamethasone and prednisone) are a class of steroid hormones with anti-inflammatory activities, thus are used extensively in adult and pediatric oncology.⁸³⁻⁸⁶ Asparaginase exposure has been shown to be associated with decreased clearance and increased exposure of dexamethasone.³⁰ Moreover, a clinical trial reported that the development of an antibody response against asparaginase was associated with a decreased incidence of glucocorticoid-induced osteonecrosis,⁸⁷ a common adverse effect of glucocorticoids.⁸⁸⁻⁹²

Effect on glucocorticoid-induced osteonecrosis

Osteonecrosis is one of the primary dose-limiting toxicities of ALL therapy.⁸⁸⁻⁹² The development of osteonecrosis can result in debilitation and surgical intervention, and seriously affect the quality of life. Proposed mechanisms (**Figure 1-1**) of glucocorticoid-induced osteonecrosis include inhibition of angiogenesis, bone marrow adipogenesis, hypercoagulation, and apoptosis of endothelial cells and osteocytes.⁹³ Our study recently suggested that damage to blood vessels supplying bone (referred to as arteriopathy) may play a primary role in the pathogenesis of osteonecrosis.⁹⁴

Evidence is accumulating that asparaginase can increase the risk of glucocorticoid-induced osteonecrosis. A recent ALL trial showed that additional doses of asparaginase during interim maintenance therapy caused more osteonecrosis in patients receiving prednisone.⁹⁵ The hypoalbuminemia caused by asparaginase was also associated with osteonecrosis risk.⁹⁰ However, these clinical data are not definitive, in that they do not come from a trial in which patients receive identical glucocorticoid regimens with different exposures to asparaginase.

Because any modification of current chemotherapy to decrease the risk of adverse events must be weighed against the possible compromised antileukemic effect, preclinical models are needed to characterize the contribution of concurrent medications, different dosing schedules and genetic variations to glucocorticoid-induced osteonecrosis, and will be coupled with studies to evaluate the influence of these risk factors for antileukemic effectiveness. Our lab has developed the first murine model for glucocorticoid-induced osteonecrosis.⁹⁶ Male BALB/cJ mice were treated with oral dexamethasone continuously for 12 weeks, and the incidence of osteonecrosis was approximately 45%. In past few years we have further optimized the murine model,

Figure 1-1. Proposed mechanisms of glucocorticoid-induced osteonecrosis
 Solid arrows represent the relationships that have been experimentally confirmed, and dashed arrows represent hypothesized relationships. Red arrows show the mechanism that involves damage to blood vessels supplying bone (arteriopathy).⁹⁴

and here we utilized this model to study the effect of asparaginase on glucocorticoid-induced osteonecrosis (see Chapter 3).

Pharmacogenomics: A Brief Introduction

Pharmacogenomics is the study of the role of genetics in drug response. There is substantial interpatient variability in response to many medications, including drug absorption, distribution, metabolism and excretion (“pharmacokinetics”), and the pharmacologic effects of drugs on the body (“pharmacodynamics”). These individual differences can be caused by genetic polymorphisms (e.g. single-nucleotide polymorphism, SNP) in genes encoding drug-metabolizing enzymes, drug transporters or drug targets or modifiers. Given the narrow therapeutic index of anticancer drugs, especially for pediatric patients, it is particularly important to optimize the treatment for both safety and efficacy.⁹⁷ Moreover, pharmacogenomic studies also yielded new insights into the pathogenesis of disease and underlying mechanisms of drug response.^{66,69,80,90,98} The ultimate goal of pharmacogenomics is to provide personalized treatment with maximal efficacy and minimal toxicity for individual patients – also known as “personalized medicine”.

Over the past half century, advances of genomic technology have enabled a more concise, comprehensive and systematic view of the genome. Currently, the major challenge is to use appropriate study designs and analysis strategies to elucidate the complex genotype-phenotype relationships. Two primary types of designs are cohort and case-control studies, and two common strategies are genome-wide association study (GWAS) and the candidate-gene approach.⁹⁸

Cohort study

A cohort means a group of people who share a common characteristic (e.g. pediatric patients treated for ALL). Cohort studies assess the outcome status of each individual in the cohort, thus limit the potential of biases and provide strong generalizability power. Cohort studies can be prospective (from the present time to the future) or retrospective (from the present time to the past). Prospective studies have well-designed data collection methods that allow the investigators to collect complete and specific data, but it may be less efficient when studying diseases with long follow-up period. In contrast, retrospective studies are more cost- and time-effective, but the investigators have very limited control over data collection,⁹⁹ including data on factors needed for correcting bias.

Case-control study

Case-control studies identify subjects by their outcome status, therefore are retrospective studies. Subjects are categorized as “cases” and “controls”, and the

association between outcomes and exposures are determined. Comparing with cohort studies, case-control studies requires fewer subjects; however, their biggest disadvantage is their susceptibility to biases. The selection of matching controls is thus critical – each control subject should be paired with a case with respect to the background variables in order to ensure comparability.¹⁰⁰ However, because important covariates are not always known, controls may not be appropriately matched to cases, which could lead to bias. In addition, as the interpretation largely hinges on the definitions of “cases” and “controls”, great caution must be taken when generalizing to study population.

Candidate-gene approach

The candidate-gene approach has been the traditional strategy of pharmacogenomics studies, especially before the development of large-scale and high-throughput genotyping techniques. The selection of single-gene candidates is based on our knowledge about the medication, and most of the candidates are key metabolizing enzymes. One of the well-known successes was thiopurine S-methyltransferase (*TPMT*), an enzyme responsible for inactivation of thiopurine drugs. Polymorphisms in *TPMT* have been found to be associated with increased levels of active thiopurine metabolites and bone marrow suppression in patients receiving thiopurines.¹⁰¹ It has been recommended that *TPMT* variants should be tested before starting treatment with thiopurines.¹⁰² The disadvantage of candidate gene approach is that it requires *a priori* knowledge, which may limit the ability to include all possible causative genes and polymorphisms.¹⁰³

GWAS

In contrast to candidate-gene approach, GWAS involves a scan of the genome, interrogating all genetic variations for phenotype association. The advantage of GWAS is clear – it can identify totally novel genes and lead to important insights into the overall mechanisms of drug response. With high sample sizes, GWAS has adequate power to allow detection of common genetic variations with modest to high effect sizes,¹⁰⁴ but the ability of testing rare variation by GWAS approach can be limited by genotyping platforms, because most DNA microarrays have been designed to capture common variations.¹⁰⁵ Deep re-sequencing of the regions implicated by GWAS may be effective in revealing the functional rare variants.¹⁰⁶

There are currently fewer genome-wide studies on adverse effects of drugs than there are on drug response, probably because of the limited sample sizes.¹⁰⁷ However, prediction of the potential toxicity, especially the serious and rare adverse events, may provide important guidance for personalized medication. Understanding the genetic factors that predispose patients to adverse effects can help us discover the underlying mechanism, identify patients at risk, and develop clinical tests and treatment regimens to reduce the toxicity.¹⁰⁸ In Chapter 4, we will present a comprehensive study of genetic risk factors associated with asparaginase-induced pancreatitis in pediatric patients with ALL.

CHAPTER 2. ASPARAGINASE HYPERSENSITIVITY*

Introduction

Asparaginase is a critical treatment component for ALL,¹⁰⁹⁻¹¹³ but its use is complicated by the development of clinical hypersensitivity.^{33,37,114-117} Differentiating allergy to asparaginase from other acute reactions can sometimes be challenging. Serum asparaginase antibody has been associated with clinical allergy, although few studies have focused on its utility as a diagnostic test.³⁷

Some previous studies have indicated that serum antibodies, even in the absence of clinical allergy, may inhibit serum asparaginase activity⁴⁰ and attenuate its anticancer effect,⁴³ although there are conflicting data.^{31,36,44-46} However, many studies lack properly timed control samples from patients whose asparaginase therapy is identical to that of patients who do develop antibodies.

Here, we prospectively measured IgG antibodies to asparaginase at predetermined time points among 410 pediatric patients treated on a front-line trial of ALL, St. Jude Total XV protocol, and evaluated the predictive utility of antibody measures for allergy, and their association with asparaginase activity and adverse effects.

Methods

Patients

Between 2000 and 2007, 498 patients with newly diagnosed childhood ALL were enrolled in St. Jude Children's Research Hospital (SJCRH) front-line Total XV protocol: 239 treated on the low-risk (LR) arm and 259 on the standard/high-risk (SHR) arm. All patients received asparaginase treatment, and 410 (197 LR and 213 SHR) had serum samples evaluable for anti-asparaginase antibodies (**Table 2-1**). The risk arm assignment and detailed treatment regimens have been described previously.⁸ Genetic ancestry was estimated using STRUCTURE¹¹⁸ and race was categorized as white (European ancestry > 90%), black (African ancestry > 70%) and other.

Asparaginase regimen and sample collection

During remission induction, Elspar was administered i.m. at a dose of 10000 U/m² thrice weekly, for a total of 6 doses (on days 6, 8, 10, 12, 14, and 16) or 9 doses

*Adapted with permission. Liu C, Kawedia JD, Cheng C, et al. Clinical utility and implications of asparaginase antibodies in acute lymphoblastic leukemia. *Leukemia*. Nov 2012;26(11):2303-2309.

Table 2-1. Clinical features of patients with (n = 410) and without (n = 88) samples evaluable for anti-asparaginase antibodies

Clinical features	Patients with samples n (%)	Patients without samples n (%)	<i>P</i>
Age			
< 10 years	302 (81.2)	70 (18.8)	0.28
≥ 10 years	108 (85.7)	18 (14.3)	
Sex			
Male	228 (81.7)	51 (18.3)	0.72
Female	182 (83.1)	37 (16.9)	
Race ^a			
White	282 (88.4)	37 (11.6)	0.0001
Black	65 (91.5)	6 (8.5)	
Other	63 (58.3)	45 (41.7)	
Immunophenotype ^b			
B-lineage	345 (81.6)	78 (18.4)	0.33
T-lineage	65 (86.7)	10 (13.3)	
Treatment arm			
Low-risk	197 (82.4)	42 (17.6)	1.0
Standard/high-risk	213 (82.2)	46 (17.8)	

^aGenetically determined race as described.¹¹⁸ ^bALL immunophenotype.

(additionally on days 19, 21, 23) in patients with high levels (i.e., 1% or more) of leukemic cells in bone marrow on day 19 of remission induction.

Patients on the LR arm received 9 doses of Elspar during reinduction I (weeks 7-9 from start of continuation treatment), and 9 doses during reinduction II (weeks 17-19; **Figure 2-1**). Patients on the SHR arm received Elspar at 25000 U/m² weekly for 19 doses in continuation treatment (weeks 1-19). For SHR patients with Philadelphia chromosome-positive ALL or induction failure, an additional dose of 25000 U/m² Elspar was given in the reintensification phase (after consolidation or after reinduction I based on minimal residual disease [MRD] status). Patients with clinical allergy to Elspar were subsequently given *Erwinia* asparaginase (Erwinase) or PEG-asparaginase (Oncaspar) by i.m. injection, based on their availability (Erwinase was used preferentially when both were available), which was influenced by manufacturer-related drug shortages. Erwinase was given at 20000 U/m² thrice weekly during remission induction for both LR and SHR patients, 20000 U/m² thrice weekly during reinduction for LR patients, and 25000 U/m² twice weekly in weeks 1-19 of continuation therapy for the SHR patients. Oncaspar was given at 2500 U/m² weekly according to treatment phase. If allergy was confirmed for all three forms, asparaginase was discontinued.

Blood was collected into tubes without anticoagulant for asparaginase measures on days 5, 19, and 34 of remission induction, day 1 of reinduction I, and day 1 of reinduction II. Samples were collected before the asparaginase injection if given on the same day of sampling. Serum was frozen at -80°C until analysis.

Phenotyping of clinical allergy to asparaginase

The allergic reactions to asparaginase were characterized by local (pain, swelling, erythema) and/or systemic manifestations (fever, urticaria, or edema) and graded using the NCI CTCAE version 3.0; only patients who developed grade 2 or higher reactions received drug substitution.¹¹⁹

Anti-asparaginase antibodies

Antibody assay

A total of 2010 serum samples (**Table 2-2**) were measured for antibodies to three forms of asparaginase (Elspar, Erwinase, and Oncaspar). Of these, 471 samples were assayed at the University of Tennessee Health Science Center (UTHSC) and 1345 samples were assayed at SJCRH. Antibodies to three forms of asparaginase (Elspar, Erwinase, and Oncaspar) were measured by ELISA based on a modification of a previously reported assay.¹²⁰ The details are presented in **Appendix A (Table A-1 and Figures A-1 through A-3)**.

Figure 2-1. Total XV asparaginase regimen and anti-asparaginase antibody measurements

All patients received Elspar (ASP) at 10000 U/m² per dose during remission induction; patients on the SHR arm received 25000 U/m² per week at weeks 1-19; those on the LR arm received 10000 U/m² thrice weekly at reinduction weeks 7-9 and 17-19. Serum samples for anti-asparaginase antibodies (ANTI-ASP) were collected on days 5, 19, and 34 of remission induction and weeks 7 and 17 of continuation therapy. The area under the antibody-concentration-versus-time curve (ANTI-ASP AUC) was estimated for induction (between day 5 and day 34) and continuation (between week 7 and week 17) using OD readings tested against Elspar.

Table 2-2. Number of patients with evaluable blood samples at each time point

Time point of antibody test	Number of patients (samples)
Day 5 of induction	401 (420)
Day 19 of induction	392 (396)
Day 34 of induction	384 (399)
Week 7 of continuation	381 (408)
Week 17 of continuation	367 (387)

Data analysis

Anti-asparaginase antibodies were analyzed as a continuous variable (optical density [OD] readings at 1:400 dilution after normalization across instruments) and also classified as positive or negative. Those with positive antibodies in the absence of clinical allergy were considered to have silent hypersensitivity to asparaginase.

The positive control reference pool was prepared from patients known to have received the specific form of asparaginase and who exhibited clinical signs of allergic reaction and had high titers of antibodies, represented by high OD readings. The negative control reference pool was prepared from healthy volunteers who had never received asparaginase treatment. A sample assayed at SJCRH was considered positive if the natural log of its 1:400 OD reading was greater than 2.58 standard deviations above the negative control process mean. A sample assayed at UTHSC was considered positive if the natural log of its 1:3200 OD reading was greater than 2 standard deviations above the negative control process mean. Samples that were not positive were considered negative. Because the magnitudes of the scale of antibody OD readings obtained at the two facilities differed, we normalized the OD readings based on a comparison of 194 serum samples measured for antibody levels at both SJCRH and UTHSC. The normalization of UTHSC OD readings to the same scale was applicable to the SJCRH readings was described by an exponential model using **Equation 2-1** (Elspar), **Equation 2-2** (Erwinase), and **Equation 2-3** (Oncaspar) to maintain consistency of OD readings between UTHSC and SJCRH. All of the samples measured at UTHSC had their OD readings normalized to the scale of the readings obtained at SJCRH. The details of data normalization are shown in **Appendix A**.

$$\log (\text{normalized OD}) = -5.066 + 3.509 \cdot \exp [0.526 \times \log (\text{original OD})] \quad (\text{Eq. 2-1})$$

$$\log (\text{normalized OD}) = -4.360 + 3.518 \cdot \exp [0.806 \times \log (\text{original OD})] \quad (\text{Eq. 2-2})$$

$$\log (\text{normalized OD}) = -5.280 + 3.684 \cdot \exp [0.520 \times \log (\text{original OD})] \quad (\text{Eq. 2-3})$$

Estimation of antibody area-under-the-curve (AUC)

To estimate the long-term exposure to antibodies, the area under the antibody-concentration-versus-time curve (AUC) was estimated using the method of trapezoids in the 360 patients who had at least four out of five scheduled antibody tests over the first 35 weeks of therapy (including week 7 and week 17 tests in the continuation phase and at least two antibody tests in the induction phase). Induction AUC (antibody AUC between day 5 and day 34 of remission induction) and continuation AUC (antibody AUC between week 7 and week 17 of continuation) were estimated separately using OD readings for each patient's serum tested against Elspar, the front-line asparaginase preparation. Analyses were performed using the sum of the induction AUC and continuation AUC to Elspar (**Figure 2-1**).

Patients were considered to have detectable antibody if the AUC was greater than 2.12 (OD value $\times$ days), which corresponds to 2.58 standard deviations above the mean value of negative cases (i.e., the antibody AUCs of the patients negative for anti-Elspar antibody consistently during therapy; **Figure A-4**).

Asparaginase activity

Serum asparaginase activity was determined in a subset of samples from patients in the SHR arm: 77 week-7 samples and 69 week-17 samples which were collected 6-8 days after the last Elspar administration of 25000 U/m²; 9 week-7 samples and 10 week-17 samples which were collected 3-4 days after the last Erwinase administration of 25000 U/m²; and 14 week-7 samples and 16 week-17 samples which were collected 6-8 days after the last Oncaspar administration of 2500 U/m².

We used a kinetic spectrophotometric assay similar to a previously reported assay,³² with a faster and more controlled process as well as improved linearity range without sample pretreatments. The asparaginase activity in serum was measured by an enzymatically coupled oxidation of reduced nicotinamide adenine dinucleotide (NADH). The reactions were shown in **Figure 2-2**. When monitored by spectrophotometry, the oxidation of NADH leads to a corresponding decrease in absorbance at 340 nM. The rate of change of absorbance is directly proportional to asparaginase activity.

The enzyme reaction mixture was prepared by combining 200 mL of 10x Tris-buffered saline, 400 mL of glycerol, 200 mg of α -ketoglutaric acid, 264 mg of asparagine, 200 mg of β -NADH, 400 μ L of glutamic oxaloacetic transaminase, 200 μ L of malic dehydrogenase, and 1400 mL distilled and deionized water, and stored at -80°C. (All the reagents above were from Sigma-Aldrich, St. Louis, MO.) Before plate reading, the enzyme reaction mixture was incubated in a 37°C water bath for 30 min. Then 10 μ L patient sera were added to 96-well UV-transparent plates (Corning. Inc, NY) in quadruplicate.

Figure 2-2. Enzyme reactions in asparaginase activity assay

The reaction was started by adding 190 μ L of enzyme reaction mixture to each well. The enzyme reaction mixture was added one row at a time, with a 20 second interval between each addition. The plate was read using a BioTek ELx808IU Microplate Reader (BioTek, Winooski, VT) at 340 nm with a 20 second interval for 15 min. The activity was estimated by the slope of the change of optical density over time. The linearity range of this assay was 0.011-2.2 U/mL. Samples higher than 2.2 U/mL were diluted with sample dilution buffer (5% bovine serum albumin in deionized water). Calibrators were established with asparaginase standard solutions: 0.025-2.0 U/mL native *E. coli* asparaginase (BioVendor Laboratory Medicine, Inc, Czech Republic) in sample dilution buffer.

***Ex vivo* asparaginase neutralization assay**

Patient serum samples were tested for their neutralizing effect against asparaginase using an *ex vivo* neutralization assay. Elspar and Oncaspar were diluted with sample dilution buffer (5% bovine serum albumin in deionized water) to 1.0 IU/mL, and then mixed with patient sera at 1:1 volume. The mixture was incubated at 37°C for 1 hour. The remaining asparaginase activity was then measured as described above.

Prognostic value of antibody tests

Sensitivity, specificity and predictive values

Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated using the numbers of patients with true positive (TP), false positive (FP), true negative (TN) and false negative (FN) antibody results. For this purpose, patients who had clinical reactions attributed to Elspar over a given time period (e.g., weeks 7-9) were classified as TP or FN, depending on whether they were positive for anti-Elspar at an adjacent time point (e.g., week 7); the patients who never had a clinical reaction were classified as TN or FP, depending on whether they were ever positive for anti-Elspar during therapy. Sensitivity was defined as the percentage of reacting patients who were positive for anti-Elspar (calculated as $TP / [TP + FN]$); specificity was defined as the percentage of non-reacting patients who were negative for anti-Elspar (calculated as $TN / [TN + FP]$); PPV was defined as the likelihood that a patient who was positive for antibody had a clinical reaction (calculated as $TP / [TP + FP]$); and NPV was defined as the likelihood that a patient who was negative for antibody never had a clinical reaction (calculated as $TN / [TN + FN]$).

Receiver-operator-characteristic (ROC) curves

The prognostic capacity of antibody tests at different threshold levels were also evaluated in terms of the ROC curve. The ROC curves of antibody tests were generated

by plotting the true positive rate (sensitivity) and corresponding false positive rate (1-specificity) for every possible threshold OD value (to separate positive from negative samples). The AUC of the ROC curve is a relative measure of the diagnostic accuracy of a test. An AUC of 1.0 would indicate a perfect test (100% TP, 0% FP, 100% TN, 0% FN), whereas 0.5 would indicate a random classification. A value greater 0.9 represents a rather high accuracy, a value less than 0.7 represents a low accuracy, and a value in between would be considered fair to good.¹²¹

Asparaginase adverse effects in Total XV

Other than allergy, the adverse effects possibly related to asparaginase include pancreatitis, thrombosis, and osteonecrosis, which exposure were uniformly assessed in all patients. Patients were prospectively screened by magnetic resonance imaging (MRI) for osteonecrosis of the hips and knees, which was graded as 0 (absent), 1 (asymptomatic), 2 (symptomatic), 3 (severe), and 4 (disabling) based on the NCI CTCAE version 3.0, as previously reported.⁹⁰ All cases of grade 2-4 thrombosis and pancreatitis before week 20 of continuation therapy were considered possible adverse effects of asparaginase.

Statistical analysis

Statistical analyses were conducted using R 2.11.1 (R Development Core Team, <http://www.r-project.org>) and SAS 9.2 (SAS Institute Inc., Cary, NC, USA). Comparisons of continuous variables between groups were conducted with Wilcoxon rank sum test. Fisher's exact test was used to determine the difference in frequencies of categorical variables (e.g., allergy or antibody positivity). Nonlinear and linear regression analyses were used to describe the change of antibody OD readings with time or with the frequency of clinical reaction. Recursive partitioning approach (using with the *rpart* function of R) was performed to determine the optimal threshold to differentiate allergy status based on OD values. Logistic regression was applied for multivariate analysis of risk factors of osteonecrosis; and the cumulative incidence of osteonecrosis was compared between groups by log-rank test. Pancreatitis and thrombosis were compared between groups using time-dependent analysis,¹²² a Cox proportional-hazards regression model where antibody status was treated as a time-dependent covariate.

Results

Asparaginase allergy

Among 410 patients, 169 (41%) had clinical allergy to Elspar, of whom 72 were first re-challenged with Erwinase and 94 with Oncaspar, and 3 were switched to other drugs. Thirty-three patients experienced a second episode of allergy (9 to Erwinase and

24 to Oncaspar), and 2 patients developed allergy to all three preparations. Patients in the LR arm were more likely than those in the SHR arm to experience hypersensitivity to Elspar, with cumulative incidences of clinical allergy being 51% versus 32% ($P = 0.0002$), a finding consistent with that of our prior report.¹¹⁹ Antibody against Elspar was also more common in patients in the LR arm than the SHR arm: 69% versus 47%, $P = 6.6 \times 10^{-6}$ (**Figure 2-3**).

The majority of allergic reactions occurred upon first re-exposure to Elspar following a 10- to 16-week hiatus after the induction phase: 78% (54 of 69) of patients in the SHR arm who had clinical allergy reacted during continuation weeks 1-6, and 96% (96 of 100) of patients in the LR arm who had clinical allergy reacted at reinduction I (**Table 2-3**).

We also investigated whether other clinical features such as age, gender, race, or ALL lineage were associated with the risk of developing antibodies or an allergic reaction to Elspar (**Table 2-4** and **Table 2-5**). Age and gender were not associated with allergy or antibody status; age was not related to allergy ($P = 0.68$) or antibody status ($P = 0.86$) even when the analysis was restricted to those with B-lineage ALL (adjusting for treatment arm and race). Allergies differed by race: reactions occurred in 45% of white, 31% of black, and 33% of other (Hispanic and Asian) patients ($P = 0.037$), and the frequencies of positive antibodies during therapy were 62%, 55%, and 40% in those patient groups ($P = 0.005$), respectively. All patients with T-cell ALL were enrolled in the SHR arm, and they had significantly fewer clinical reactions (19% vs. 39%, $P = 0.003$) and lower antibodies (35% vs. 52%, $P = 0.02$) to Elspar than did those with B-lineage ALL in the SHR arm.

Antibodies and allergy

Anti-Elspar antibodies were more frequent among the patients with clinical allergy to Elspar: 87.0% (147 of 169 reacting patients) compared to only 36.9% (89 of 241) among non-reacting patients ($P < 1.0 \times 10^{-15}$). Among the 236 patients who were considered positive for anti-Elspar antibody, those who had reactions had higher levels of antibody (based on OD readings) at the end of induction ($P = 0.0002$) and during the continuation phase (week 7, $P = 8 \times 10^{-9}$; and week 17, $P < 1.0 \times 10^{-15}$), and higher cumulative antibody titer based on anti-Elspar antibody AUC (27.8 ± 17.9 vs. 10.8 ± 13.3 , $P < 1 \times 10^{-15}$) than those who did not have reactions (i.e., the group who would be classified as “silent hypersensitivity”; **Figure 2-4**). However, patients with more severe clinical toxicity (grade 3-4 reactions) against Elspar did not have higher cumulative anti-Elspar antibody AUC (22.8 ± 23.0 vs. 28.6 ± 16.9 , $P = 0.11$) than those with mild toxicity (grade 2 reactions). For Oncaspar, similarly, we observed no difference in anti-Oncaspar antibody AUC in those with grade 3-4 vs. grade 2 reactions (5.7 ± 7.8 vs. 5.4 ± 4.8 , $P = 0.83$). For Erwinase, all allergic reactions were grade 2, and thus no comparisons are possible.

Figure 2-3. Frequency of hypersensitivity to Elspar

The frequency of allergic reactions and anti-Elspar antibody (Ab) positivity in patients enrolled on the low-risk (LR) and standard/high-risk (SHR) treatment arms. Compared with those on the SHR arm, patients on the LR arm had more reactions ($P < 0.00001$) and anti-Elspar antibody ($P = 0.0002$).

Table 2-3. Timing of allergic reaction to Elspar

Phase	Low-risk (n = 100)	Standard/high-risk (n = 69)	Total (n = 169)
Induction	2 (2%)	3 (4%)	5 (3%)
Continuation weeks 1-6	0 (0%)	54 (78%)	54 (32%)
Reinduction I	96 (96%)	1 (2%)	97 (58%)
Continuation weeks 10-16	0 (0%)	7 (10%)	7 (4%)
Reinduction II	2 (2%)	0 (0%)	2 (1%)
Reintensification	0 (0%)	4 (6%)	4 (2%)

Number (and percentage) of patients reacting to Elspar at each phase of therapy are compared to the total number of patients who had a reaction by risk groups.

Table 2-4. Clinical features and allergic reactions to Elspar

Clinical features	All patients (n = 410) ^a				Low-risk arm (n = 197)				Standard/high-risk arm (n = 213)			
	n	Allergy n (%)	No allergy n (%)	<i>P</i>	n	Allergy n (%)	No allergy n (%)	<i>P</i>	n	Allergy n (%)	No allergy n (%)	<i>P</i>
Age												
< 10 years	302	129 (42.7)	173 (57.3)	0.36	183	94 (51.4)	89 (48.6)	0.59	119	35 (29.4)	84 (70.6)	0.31
≥ 10 years	108	40 (37.0)	68 (63.0)		14	6 (42.9)	8 (57.1)		94	34 (36.2)	60 (63.8)	
Sex												
Male	228	96 (42.1)	132 (57.9)	0.69	93	53 (57.0)	40 (43.0)	0.12	135	43 (31.9)	92 (68.1)	0.88
Female	182	73 (40.1)	109 (59.9)		104	47 (45.2)	57 (54.8)		78	26 (33.3)	52 (66.7)	
Race ^b												
White	282	128 (45.4)	154 (54.6)	0.037	146	75 (51.4)	71 (48.6)	0.19	136	53 (39.0)	83 (61.0)	0.004
Black	65	20 (30.8)	45 (69.2)		24	15 (62.5)	9 (37.5)		41	5 (12.2)	36 (87.8)	
Other	63	21 (33.3)	42 (66.7)		27	10 (37.0)	17 (63.0)		36	11 (30.6)	25 (69.4)	
Immunophenotype ^c												
B-lineage	345	157 (45.5)	188 (54.5)	0.0001	197	100 (50.8)	97 (49.2)	NA	148	57 (39.2)	91 (60.8)	0.003
T-lineage	65	12 (18.5)	53 (81.5)		0	0 (0.0)	0 (0.0)		65	12 (18.5)	53 (81.5)	

^aPatients with serum samples evaluable for anti-asparaginase antibodies (n = 410). Analysis for all patients (n = 498) has been previously reported.¹¹⁹ ^bGenetically determined race as described.¹¹⁸ ^cAcute lymphoblastic leukemia immunophenotype. NA, not applicable.

Table 2-5. Clinical features and anti-Elspar antibody status

Clinical features	All patients (n = 410)				Low-risk arm (n = 197)				Standard/high-risk arm (n = 213)			
	n	Positive n (%)	Negative n (%)	<i>P</i>	n	Positive n (%)	Negative n (%)	<i>P</i>	n	Positive n (%)	Negative n (%)	<i>P</i>
Age												
< 10 years	302	182 (60.3)	120 (39.7)	0.07	183	129 (70.5)	54 (29.5)	0.14	119	53 (44.5)	66 (55.5)	0.49
≥ 10 years	108	54 (50.0)	54 (50.0)		14	7 (50.0)	7 (50.0)		94	47 (50.0)	47 (50.0)	
Sex												
Male	228	135 (59.2)	93 (40.8)	0.48	93	70 (75.3)	23 (24.7)	0.09	135	65 (48.2)	70 (51.8)	0.67
Female	182	101 (55.5)	81 (44.5)		104	66 (63.5)	38 (36.5)		78	35 (44.9)	43 (55.1)	
Race ^a												
White	282	175 (62.1)	107 (37.9)	0.005	146	104 (71.2)	42 (28.8)	0.40	136	71 (52.2)	65 (47.8)	0.013
Black	65	36 (55.4)	29(44.6)		24	16 (66.7)	8 (33.3)		41	20 (48.8)	21 (51.2)	
Other	63	25 (39.7)	38 (60.3)		27	16 (59.3)	11 (40.7)		36	9 (25.0)	27 (75.0)	
Immunophenotype ^b												
B-lineage	345	213 (61.7)	132 (38.3)	0.0001	197	136 (69.0)	61 (31.0)	NA	148	77 (52.0)	71 (48.0)	0.026
T-lineage	65	23 (35.4)	42 (64.6)		0	0 (0.0)	0 (0.0)		65	23 (35.4)	42 (64.6)	

^aGenetically determined race as described.¹¹⁸ ^bAcute lymphoblastic leukemia immunophenotype. NA, not applicable.

Figure 2-4. Antibody levels in patients with and without clinical reactions
 Left: Anti-Elspar optical density (OD) measures (natural log) for 410 patients at days 5, 19, and 34 of remission induction and weeks 7 and 17 of continuation therapy. At each time point, patients are grouped based on whether they ever (or never) experienced a clinical allergic reaction (Rxn), and whether they were ever (or never) positive for anti-Elspar antibody (Ab) at anytime during therapy. Among the patients positive for anti-Elspar, those who had reactions (white bars) had significantly higher anti-Elspar levels than did those who had silent hypersensitivity (light grey) at day 34, week 7, and week 17. Among the patients negative for anti-Elspar antibodies (as defined by the threshold in the Supplement), those who had allergic reactions (hatched) exhibited slightly elevated anti-Elspar levels compared with those without any allergic reactions (dark grey) at weeks 7 ($P = 0.05$) and 17 ($P = 0.03$). Right: cumulative anti-Elspar antibody AUC over the first 35 weeks of therapy for antibody-positive patients who had allergic reactions versus those who had silent hypersensitivity. Box plots: median, quartiles, non-outlier range.

This study's sampling strategy was designed to have evaluable antibody samples at comparable times relative to asparaginase dosing for all patients (those who developed an allergy and those who did not). Because allergic reactions may have occurred at times during which no samples were obtained, maximal values may not have been captured if antibody levels are maximal at the time of reaction. However, serial samples that happened to be obtained before, during, and after reactions indicated that antibody levels were estimated to be maximal at about 50 days after an allergic reaction (**Figure 2-5**).

We investigated the utility of serum anti-Elspar antibody positivity to predict future (if sampling just before) or to confirm (if sampling just after) clinical allergic reactions. The sensitivity, specificity, and predictive values are shown in **Table 2-6**. The greatest utility was evident for the week 7 serum samples, which happened to be obtained closest to the time period during which most reacting patients had their allergic reaction (continuation weeks 7-9 for those in the LR arm and weeks 1-6 for those in the SHR arm). The week 7 anti-Elspar antibody test had a sensitivity of 87-88% and a specificity of 68-69% for predicting clinical reactions to Elspar at weeks 7-9 for LR patients and for confirming clinical reactions to Elspar at weeks 1-6 for SHR patients. Using the optimal OD thresholds determined by recursive partitioning (**Table A-2** and **Figure A-5**), the specificity was slightly improved to 71-74% but the sensitivity of prediction was lowered to 83%. The prognostic utility of the antibody tests at day 34, week 7 and week 17, as evaluated by the AUC of the ROC curves, ranged from 0.70 to 0.91 (**Figure 2-6**). Antibody test obtained at day 34 of induction for future clinical reactions showed a lower prognostic capacity compared with those at week 7 and week 17 (**Figure 2-6**).

Interestingly, we observed a strong positive linear relationship between the anti-Elspar level (natural log of OD reading) at week 7 and the probability of patients reacting to Elspar at weeks 7-9 on the LR arm ($R\text{-squared} = 0.96$, $P = 6 \times 10^{-8}$) or weeks 1-6 on the SHR arm ($R\text{-squared} = 0.83$, $P = 4 \times 10^{-5}$; **Figure 2-7**), consistent with our finding that anti-Elspar level was higher in patients with allergy than those with silent hypersensitivity (**Figure 2-4**).

The frequency of secondary allergy to Oncaspar was higher than to Erwinase (24/94 or 26% vs. 9/72 or 13%, $P = 0.05$), probably due to the cross reactivity between Oncaspar and Elspar (**Figure A-6**). For secondary treatment with Erwinase and Oncaspar, there was less association between clinical reactions to these agents and corresponding antibodies. We measured anti-Erwinase and anti-Oncaspar antibodies in 63 of the 72 patients who went on to be treated with Erwinase and 85 of the 94 patients who went on to receive Oncaspar. Among the 63 patients receiving Erwinase, 9 had an allergy to it, of whom only 4 had detectable anti-Erwinase antibody. Among the 85 patients receiving Oncaspar, 23 had a clinical reaction, of whom 20 had detectable anti-Oncaspar antibody. In addition, silent hypersensitivity occurred among 8 of 63 (13%) patients treated with Erwinase and among 47 of 85 (55%) treated with Oncaspar (**Figure 2-8**).

Figure 2-5. Anti-Elspar antibody level relative to the time of clinical reaction to Elspar

Anti-Elspar OD level in patient samples (dots) relative to the number of days elapsed before (negative) or after (positive) between sample collection and clinical reaction to Elspar. The trend lines of median (solid) and quartiles (dashed) are shown.

Table 2-6. Sensitivity, specificity and predictive values of three anti-Elspar antibody tests for predicting or confirming clinical reactions to Elspar

Risk arm	N of patients	Time of Ab test	Time of Rxn	Time between test and rxn (weeks)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Prediction of future reactions								
LR	185	Day 34	Weeks 7-9	17-20	42.8	92.5	84.8	62.6
SHR	190	Day 34	Weeks 1-6	10-17	26.1	93.1	54.5	79.8
LR	192	Week 7	Weeks 7-9	0-3	87.4	68.0	72.8	84.6
Confirmation of past reactions								
SHR	184	Week 7	Weeks 1-6	0-6	87.8	68.9	50.6	93.9
LR	189	Week 17	Weeks 7-9	7-10	89.4	65.3	71.8	86.1
SHR	174	Week 17	Weeks 1-16	0-16	72.7	88.2	74.1	87.5

SHR, standard/high-risk; LR, low-risk; PPV, positive predictive value; NPV, negative predictive value; Ab, anti-Elspar antibody; Rxn, clinical allergic reaction; Day 34, day 34 of remission induction therapy (34 days from diagnosis of ALL); Week 7, week 7 of continuation therapy (23 weeks from diagnosis of ALL; and Week 17, week 17 of continuation therapy (33 weeks from diagnosis of ALL).

Figure 2-6. ROC curves of the antibody tests

Receiver operating characteristic (ROC) curves of the antibody tests at day 34, week 7 and week 17, showing the true positive rates and respective false positive rates for predicting or confirming clinical allergic reactions. Each point on the curve corresponds to a specific threshold of anti-Elspar OD that putatively divides antibody-positive from antibody-negative samples. Right y-axis shows the color key that visualizes the mapping from the natural log of anti-Elspar OD to colors. The positive threshold dividing positive from negative samples for logOD is -3.2 (circles). The AUC of the ROC curves, which represents overall test performance on predictions, is shown in each graph. LR, low-risk; SHR, standard/high-risk; Ab, anti-Elspar antibody; Rxn, clinical reaction; AUC, area under the curve. Time of antibody tests: D34, Day 34 of induction therapy; W7, Week 7 of continuation therapy; and W17, Week 17 of continuation therapy.

Figure 2-7. Association between week 7 anti-Elspar antibody level and the proportion of patients reacting to Elspar around week 7

Left panels: the distribution of patients with (solid bars) and without (open bars) clinical reactions by their anti-Elspar OD at week 7. (A) Patients on the LR arm (n = 195) with and without reactions during weeks 7-9. (C) Patients on the SHR arm (n = 175) with and without reactions during weeks 1-6. Right panels: the positive correlation between week 7 anti-Elspar OD and the proportion of patients having clinical reaction at (B) weeks 7-9 (LR) and (D) weeks 1-6 (SHR). Trend lines are shown. LR, low-risk; SHR, standard/high-risk.

Figure 2-8. The frequency of hypersensitivity to Oncaspar and Erwinase

The proportion of patients exhibiting secondary allergic reactions and antibody positivity among the patients who had samples tested for anti-Oncaspar (n = 85) or anti-Erwinase (n = 63) after they received Oncaspar or Erwinase. Ab, antibody against Oncaspar (left) or Erwinase (right).

Antibodies and serum asparaginase activity

In addition to testing the clinical utility of asparaginase antibodies as a tool to predict or confirm clinical allergy, antibodies have been hypothesized to be important as indicators of lower systemic exposure to asparaginase. Indeed, we found that *in vivo* serum asparaginase activity after Elspar was lower in the samples positive for antibodies than in those negative for antibodies ($P = 7 \times 10^{-6}$), and was inversely related to the level of antibody (based on OD readings, $P = 7 \times 10^{-11}$; **Figure 2–9**).

To determine whether antibody was also inversely related to asparaginase activity after secondary treatment with Erwinase and Oncaspar, we measured *in vivo* serum asparaginase activity in 19 patients on Erwinase and 30 patients on Oncaspar at the time of the anti-Elspar antibody measurement. Asparaginase activity did not differ by anti-Elspar antibody positivity in patients who received second line treatment with Erwinase ($P = 0.29$), but it was lower (median 0.03 IU/mL, range 0-2.73 IU/mL) in those who were positive for anti-Elspar antibody and received second line treatment with Oncaspar compared to those who were antibody negative (median 2.04 IU/mL, range 1.07-2.77 IU/mL; $P = 0.005$).

The neutralizing effect of asparaginase antibodies was also confirmed by the *ex vivo* experiment (**Figure 2–10**). Patient sera that were positive for anti-Elspar antibodies neutralized up to about 60% of asparaginase activity of Elspar. There was a linear correlation between the level of antibody (based on OD readings) and the neutralizing capacity of patient samples (R-squared = 0.82, $P = 6.9 \times 10^{-21}$; **Figure 2–10A**). Similar neutralizing effect on Oncaspar activity was observed for patient sera with anti-Oncaspar antibodies, although not as strong as Elspar (R-squared = 0.28, $P = 3.4 \times 10^{-5}$; **Figure 2–10B**).

Antibodies and adverse effects

As we have shown that asparaginase can potentiate exposure to dexamethasone,³⁰ we investigated whether cumulative asparaginase antibody titer affected the risk of symptomatic (grade 2 to 4) osteonecrosis. After adjustment for age and treatment arm, risk factors for the osteonecrosis, higher cumulative antibody titer (based on antibody AUC) was associated with lower risk of symptomatic osteonecrosis (odds ratio for log₂ antibody AUC = 0.83; 95% CI, 0.78 – 0.89; $P = 0.007$; **Table 2–7**), consistent with antibodies causing less asparaginase exposure.

We also compared the cumulative risk of symptomatic osteonecrosis between patients negative for anti-Elspar antibody (i.e., anti-Elspar antibody AUC $\leq$ 2.12) and those with detectable antibody (i.e., anti-Elspar antibody AUC $>$ 2.12; **Figure 2–11**). There was a lower cumulative incidence of symptomatic osteonecrosis in those with a higher anti-Elspar antibody AUC who were older than 10 years of age (in the LR arm, $P = 0.048$; and the SHR arm, $P = 0.037$), and in younger patients (in the LR arm, $P = 0.047$; but not on the SHR arm, $P = 1.0$; **Figure 2–11**).

Figure 2-9. Correlation between serum asparaginase activity and antibody level
 Serum asparaginase activity (IU/mL) measured 6-8 days after the last Elspar dose of 25000 U/m². (A) Asparaginase activity was significantly lower in the samples positive than in those negative for anti-Elspar antibodies. Box plots: median, quartiles, non-outlier range. (B) Asparaginase activity inversely correlated with anti-Elspar OD reading of serum samples. Individual data points and the regression line are shown for all cases (n = 146).

Figure 2-10. *Ex vivo* neutralization of patient sera with antibodies

Patient serum samples were incubated with (A) Elspar (1.0 IU/mL) or (B) Oncaspar (1.0 IU/mL) for 1 hour. The remaining activity of the mixture was plotted against the antibody OD reading for each serum sample. Regression lines are shown.

Table 2-7. Multivariate analysis showed that low anti-Elspar antibody area-under-curve (AUC) was a risk factor of osteonecrosis (n = 360)

Patient characteristics	<i>P</i>	Hazard ratio (95% CI)
Older than 10 years	4.0×10^{-7}	5.52 (2.84 - 10.71)
Standard/high-risk arm	0.053	2.03 (0.99 - 4.16)
Log2 (anti-Elspar antibody AUC)	0.007	0.83 (0.78 - 0.89)

Association with risk of osteonecrosis was assessed using Cox proportional hazard model. Log2(anti-Elspar antibody AUC) was treated as continuous variable; e.g. a hazard ratio of 0.83 suggests 83% risk of osteonecrosis for every two-fold increase in AUC. Other characteristic features are dichotomized for presence versus absence of the indicated feature.

Figure 2-11. Cumulative incidence of osteonecrosis based on anti-asparaginase antibody AUC

Patients were stratified by low-risk (LR) versus standard/high-risk (SHR) treatment arm and age at diagnosis: (A) patients in the LR arm with age greater than 10 years; (B) patients in the SHR arm with age greater than 10 years; (C) patients in the LR arm with age under 10 years; (D) patients in the SHR arm with age under 10 years. Left: Cumulative incidence of symptomatic (grade 2 to 4) osteonecrosis (ON) in patients with anti-Elspar antibody AUC (OD × days) lower than 2.12 (blue) versus greater than 2.12 (red). Right: box plot of anti-Elspar antibody AUC in patients with (blue) versus without symptomatic ON (red). ^a*P* value based on log-rank test. ^b*P* value based on Wilcoxon rank sum test. *Fisher's exact test ($P = 0.11$) was also performed because the sample size is small.

Seven patients had pancreatitis before the end of reinduction II, and five of them were positive for antibody. Twenty-eight patients had thrombotic events before the end of reinduction II, and seven of them were positive for antibody. After adjusting for age and treatment arm, there was no apparent association between antibody positivity and pancreatitis ($P = 0.22$) or thrombosis ($P = 0.20$).

Discussion

Although the relationship between antibodies and allergic reactions to asparaginase has been observed in many studies,^{32,33,37,117} this is the first report focusing on the diagnostic utility of asparaginase antibody measures to predict or to confirm clinical allergy to asparaginase. Particularly in children, differentiating allergy from other diagnoses can be challenging. In the context of an asparaginase-intensive front-line clinical trial and by dichotomizing samples as positive versus negative, the most informative sample (at week 7 of continuation phase) had reasonably high (88%) sensitivity to predict reactions in the next 3 weeks but less favorable specificity (68%) to confirm past reactions (**Table 2-6**). In this regard, there was a relatively frequent occurrence of silent hypersensitivity during therapy (22%, 90 of 410 patients on the protocol). Based on this test performance, for every 100 patients with a reaction during weeks 1-9, 88 would be expected to have positive and 12 would have negative antibody tests during this time period (at week 7); for every 100 patients without an allergy, 32 would have positive and 68 would have negative antibody tests at that time (week 7). The diagnostic value of antibody test at this time point is similar to those of some widely used clinical laboratory tests, such as the diagnosis of venous thromboembolism with d-dimer (sensitivity 80-95% and specificity 40-70%);¹²³ the prediction of rheumatoid arthritis with rheumatoid factor (sensitivity 40-80%, specificity 70-90%);¹²⁴ and the diagnosis of systemic lupus erythematosus with antinuclear antibody (sensitivity 93%, specificity 50-80%).¹²⁵

We were unable to identify a more informative time point that yielded better antibody test performance. The early antibody test at the end of induction lacked the sensitivity to predict future clinical allergy. The later antibody tests (i.e., week 17) did not confirm as well for patients in the LR arm; for those in the SHR arm, the week 17 result had slightly reduced sensitivity (72.7%) and increased specificity (88.2%), but clinically, it would not be possible to use such a result for making therapeutic decisions. Because antibody levels tend to increase over time with successive asparaginase exposures, even in the absence of a reaction, comparisons between reacting and non-reacting patients should control for place-in-therapy. In our study, serum samples were timed uniformly relative to therapy but were not available at the exact time of the reaction in most cases (and control samples, of course, would not have been available in non-reacting patients at exactly comparable times); thus, we were not able to assess the specificity of the serum anti-Elspar antibody test at the time of clinical reaction to Elspar. However, even if we limited analysis to those reacting patients who had serum obtained 5-30 days before or after the allergy ($n = 132$ patients), the estimated sensitivity was not higher than 80% (**Figure A-7**).

We also investigated whether the predictive utility of the antibody measures was improved by leaving the measure as a continuous measure (OD of anti-Elspar antibody titers) instead of dichotomizing the results as positive versus negative. We observed a linear correlation between the antibody OD at week 7 and the probability of patients having a clinical reaction in the subsequent 3-week asparaginase course, and a correlation between week 7 antibody OD and the proportion who had clinical reactions in the previous 6-week asparaginase course (**Figure 2-7**). For the LR patients with anti-Elspar OD > 0.39 at week 7, 90% of them are predicted to have an event in the subsequent course; for the SHR patients with anti-Elspar OD > 1.06 at week 7, 80% of them would have had an event in the previous course. However, falsely positive readings (high OD readings in patients with no known reaction) remained a limitation, and thus we were not able to define an alternative universal threshold for distinguishing positive from negative antibody status that resulted in better overall balanced test performance (based on the ROC curve of the antibody tests, see **Figures 2-6 and A-5**).

In addition to the possible utility of an anti-asparaginase antibody test to facilitate diagnosis of allergy, antibodies may be an indicator of reduced serum asparaginase exposure. In samples obtained at a uniform time (6-8 days) post-dose, we found that serum asparaginase activity was indeed inversely related to antibody level (**Figure 2-9**), consistent with reports from other groups.^{32,40,42,43} The *ex vivo* experiment also showed that asparaginase activity was inhibited by adding patient sera with antibodies (**Figure 2-10**), suggesting that attenuation of serum asparaginase activity may be due to a direct neutralizing effect of antibodies. However, it is also possible that the presence of antibodies is an indicator of other immune-based mechanisms that enhance drug clearance (e.g., via the reticuloendothelial system). This may explain why a few samples had low asparaginase activity despite being low in asparaginase antibodies (**Figure 2-9**).

We explored whether the presence of antibodies, with its likely attendant reduced exposure to asparaginase over time, would reduce other adverse effects of asparaginase therapy. Even though dexamethasone is the major cause of osteonecrosis in children with ALL,^{89,96,126-128} we have shown that asparaginase can increase the risk of osteonecrosis,⁹⁶ which we hypothesize could be because asparaginase inhibits protein synthesis, decreases dexamethasone clearance,³⁰ alters lipid metabolism,^{129,130} and induces coagulopathy.^{131,132} Because osteonecrosis is a long-term complication, we used antibody AUC as a measurement of long-term exposure to antibodies against Elspar, the front-line asparaginase preparation. We observed that, among younger patients on the LR arm and all older patients on either LR or SHR arm, those with high antibody AUC were at lower risk to develop symptomatic osteonecrosis than those with low antibody AUC (**Figure 2-11**). The difference was not apparent among the younger patients on the SHR arm, possibly because younger patients are less prone to osteonecrosis, and those on the SHR arm also had lower antibodies, perhaps too low to have any effect on the development of osteonecrosis (**Figure 2-11D**, right panel). In multivariate analysis, after adjusting for age and treatment arm, antibody AUC was significantly associated with grade 2-4 osteonecrosis (**Table 2-7**). This is the first report that anti-asparaginase antibodies were inversely related to the risk of osteonecrosis, but is consistent with our hypothesis that asparaginase potentiates glucocorticoid effects.⁹⁶ We recently reported that asparaginase

antibodies were associated with a higher risk of central nervous system relapse,²¹ but did not find that asparaginase antibodies were related to pancreatitis or thrombosis, perhaps indicating that these latter two adverse events are less “dose-related” than others.

In the Total XV study, with Elspar as the front-line asparaginase preparation, the frequency of clinical hypersensitivity to Elspar was 41% (169/410) for all patients who started with Elspar, and the incidence of IgG antibodies to Elspar was 58% (236/410), similar to the average from other reported series.^{31,40,43,117} The hypersensitivity appeared to differ by treatment arm and immunophenotype, consistent with the result from our previous ALL trial, Total XIII.^{31,117} Patients on the LR arm exhibited more hypersensitivity to asparaginase than did those on the SHR arm, possibly because the LR arm included less immunosuppressive chemotherapy and included longer periods of asparaginase “holiday” followed by re-challenge, a practice noted to be associated with allergy by others.¹¹⁶

There was a very high frequency of “silent hypersensitivity” observed in patients who subsequently received Oncaspar (55% of patients with no allergy had positive antibodies against Oncaspar). However, 24% of patients who never received Oncaspar had positive antibodies at week 7 and 17, which could be due to cross reactivity between sera positive to both Elspar and Oncaspar in patients who are exposed initially to Elspar (Oncaspar is pegylated Elspar).¹³³ Consistent with that idea, only 13% of patients who received Erwinase had silent hypersensitivity, compared to 6% of patients who never received Erwinase. Allergy was also more common ($P = 0.05$) in those who received Oncaspar rather than Erwinase as their second-line agent, and serum asparaginase activity was lower in those with (compared to those without) anti-Elspar antibodies who received Oncaspar ($P = 0.005$), but not in those who received Erwinase ($P = 0.29$), consistent with recent reports.⁴² Together, these data suggest that Erwinase may be preferred over Oncaspar in those who receive primary treatment with Elspar and experience allergy, although it is possible that such a decision depends upon the dose of Oncaspar versus Erwinase and the absolute level of anti-Elspar antibodies.⁴²

Interestingly, patients with T-cell ALL were less likely to develop asparaginase allergy and had lower antibody levels at post-asparaginase time points than those with B-lineage ALL. The mechanisms underlying this difference are unclear, but suggest an intriguing added value for asparaginase in T-cell ALL.

Conclusion

In summary, we comprehensively analyzed anti-Elspar antibody in an asparaginase-intensive front-line clinical trial. In this context, in which Elspar is given as the primary form of asparaginase, serum anti-Elspar antibodies have good utility as tools to help make the diagnosis of clinical hypersensitivity. Our data show that higher antibody levels are associated with greater clinical pharmacologic effects including a higher risk of clinical allergy, and a greater attenuation of serum asparaginase enzyme

activity with associated lower risk of osteonecrosis. Thus, measures of serum antibodies to asparaginase can be useful in patients with ALL.

CHAPTER 3. POTENTIATING EFFECT OF ASPARAGINASE ON GLUCOCORTICOID-INDUCED OSTEONECROSIS

Introduction

Osteonecrosis is a common complication in patients treated for ALL, primarily due to the use of glucocorticoids (e.g. dexamethasone and prednisone).⁸⁸⁻⁹² Proposed mechanisms of glucocorticoid-induced osteonecrosis included inhibition of angiogenesis, bone marrow adipogenesis, hypercoagulation, and apoptosis of endothelial cells and osteocytes.⁹³ Our study recently suggested that damage to blood vessels supplying bone (arteriopathy) plays a primary role in the pathogenesis of osteonecrosis.⁹⁴ Clinical studies have identified several risk factors for osteonecrosis in ALL patients, including adolescent age,^{88-92,126,128,134-136} female sex,^{88,89,91,134,136} white race,^{89,126} and exposure to intensive, long-term glucocorticoids.⁸⁸⁻⁹²

The reported frequency of osteonecrosis has varied widely from 1% to 20% among different ALL protocols^{88-92,126,128,134-136} even with relatively similar glucocorticoid regimens, and interactions with accompanying antileukemic medication is suspected to contribute to this variability. Evidence is accumulating that asparaginase can increase the risk of osteonecrosis. Asparaginase exposure was associated with decreased clearance and increased exposure of dexamethasone,³⁰ and the development of an antibody response against asparaginase is associated with a decreased incidence of symptomatic osteonecrosis.⁸⁷ A recent ALL trial showed that extra doses of asparaginase during interim maintenance therapy caused more osteonecrosis in patients receiving prednisone.⁹⁵ Moreover, the hypoalbuminemia caused by asparaginase was also associated with osteonecrosis risk.⁹⁰

However, these clinical data are not definitive, in that they do not come from a trial in which patients receive identical glucocorticoid regimens with different exposures to asparaginase. Thus, our study aimed to address the impact of asparaginase on osteonecrosis in a controlled, pre-clinical model. We also evaluated the impact of host factors (e.g. age and gender), treatment factors (e.g. glucocorticoid regimen and duration), and environmental factors (mice derived from in-house breeding colonies vs. shipped from vendor) on dexamethasone tolerance and the development of osteonecrosis using this mouse model.

Methods

Chemicals

Dexamethasone sodium phosphate solution was purchased from American Pharmaceutical Partners, Inc. (Schaumburg, IL). PEG-asparaginase (Oncaspar) was a gift from Sigma Tau (Gaithersburg, MD). Tetracycline was purchased from Sigma-Aldrich

(St. Louis, MO), and sulfamethoxazole/trimethoprim oral suspension was obtained from Hi-Tech Pharmacal Co., Inc. (Amityville, NY).

Animals

Unless otherwise specified, we studied male BALB/cJ mice (age 24 or 28 days) bred in our own facilities (SJCRH, Memphis, TN; the original breeding pairs were from Jackson Laboratories). We also studied BALB/cJ mice directly shipped from Jackson Laboratories (Bar Harbor, ME), or male BALB/cAnNHsd mice (age 24 or 28 days) obtained from Harlan Laboratories (Houston, TX). The details are described in **Appendix B (Table B-1 and Figure B-1)**.

Asparaginase and dexamethasone pharmacokinetics

To determine the effect of asparaginase treatment on dexamethasone pharmacokinetics, we conducted an experiment using males at 6-8 weeks of age. Mice received dexamethasone at 4 mg/L (drinking water) and PEG-asparaginase intraperitoneally (i.p.) at 1500 IU/kg for 0 to 4 doses at an interval of 3.5 days (**Figure 3-1**). All mice were sacrificed at the end of week-2 (3.5 days after last asparaginase dose). Blood was collected via cardiac puncture with anticoagulant, and plasma was measured for the levels of dexamethasone, asparaginase, amylase, lipase, albumin, triglyceride and cortisol (**Figure 3-2 and Appendix B**).

Mouse model of dexamethasone-induced osteonecrosis

A series of experiments were conducted to refine a mouse model of osteonecrosis (see **Appendix B Tables B-1 and B-2**, and **Figures B-1 through B-7**). From postnatal day 28 (P28) or day 24 (P24), male and female BALB/cJ and BALB/cAnN mice were treated with 4 or 8 mg/L dexamethasone in drinking water, equivalent to 1.33 or 2.66 mg/kg/day, assuming that each mouse weighed 15 g and consumed 5 mL water daily.¹³⁷ Mice in dexamethasone groups were treated with dexamethasone with antibiotics, except in a pilot experiment where mice were given dexamethasone without antibiotics. The control groups were treated with only antibiotics. Dexamethasone was given at 4 or 8 mg/L in drinking water, equivalent to 1.33 or 2.66 mg/kg/day, assuming that each mouse weighed 15 g and consumed 5 mL water daily.¹³⁷ Treatment generally lasted for 6 weeks, while a subset of BALB/cAnN was treated for up to 8 weeks in an effort to increase the frequency of osteonecrosis. Antibiotic prophylaxis to prevent dexamethasone-induced infections consisted of tetracycline (1 g/L) constantly, and sulfamethoxazole (600 mg/L) and trimethoprim (120 mg/L) given 3.5 days per week. These antibiotics have no effect on osteonecrosis.⁹⁶ Water bottles were changed every 3.5 days.

Effect of asparaginase on osteonecrosis

Male P24 BALB/cJ in-house bred mice were used to determine the effect of asparaginase on dexamethasone-induced osteonecrosis. Ninety mice were divided into four groups: 35 mice were assigned to the dexamethasone-alone group, 35 mice to the dexamethasone and asparaginase group, 10 mice to the asparaginase-alone group and 10 mice to untreated control (**Figure 3-1**). Dexamethasone was given at 2 mg/L (equivalent to 0.67 mg/kg/day) in drinking water for 6 weeks, and asparaginase was injected i.p. at 1200 IU/kg twice weekly. The same antibiotic prophylaxis was used as described above.

Plasma dexamethasone concentration and asparaginase activity

At the time of sacrifice (between 9am and noon), mice were anesthetized with 2% isoflurane, and blood was collected via cardiac puncture. Plasma was frozen at -80°C until assayed. Dexamethasone concentration was quantified by high-performance liquid chromatography (HPLC). Plasma asparaginase activity was determined using a high-throughput assay by monitoring the enzymatically-coupled oxidation of reduced nicotinamide adenine dinucleotide (NADH) to NAD(+).¹³⁸ The linear range of the assay was established from 0.025 to 2.2 IU/mL; if the activity was out of the range, the plasma was diluted with serum albumin to be in range. Please see **Appendix B** for the detailed assay protocols.

Histological evaluation of osteonecrosis and arteriopathy

Osteonecrosis was determined by empty lacunae, pyknotic nuclei or ghost nuclei in osteocytes in the bone trabeculae, and necrosis of the adjacent marrow and stromal elements, as described previously.^{94,96,137} Arteriopathy was defined by the presence or absence of lesions in arteriolar branches of the medial genicular artery located along the surface of the distal femoral condyles.⁹⁴ If there was no evaluable arteriolar branch present, the case was categorized as “unknown”. Mice with osteonecrosis and/or arteriopathy in one or both legs were classified as positive for osteonecrosis and/or arteriopathy.

Statistical analysis

The Chi-square test was used to evaluate intergroup differences in categorical variables. The Mann-Whitney test was used to compare continuous variables. A P-value of less than 0.05 was considered statistically significant.

Results

Plasma dexamethasone concentration was increased by asparaginase treatment

After 2 weeks of treatment with dexamethasone at 4 mg/L and PEG-asparaginase at 1500 IU/kg for 0-4 doses, all 25 mice in the PK experiment were evaluable at the end of week 2 (“pharmacokinetics experiment”; **Figure 3-1**). There was no significant difference in plasma asparaginase activity among the three groups (15.3 ± 6.5 , 14.5 ± 7.7 and 13.2 ± 4.1 IU/mL, respectively) that received 2, 3 or 4 doses of asparaginase at 1500 IU/kg i.p. every 3.5 days. Those who received only one dose of asparaginase had slightly lower asparaginase activity (11.9 ± 2.6 IU/mL, $P = 0.02$) compared with other groups. We observed a positive association between plasma dexamethasone concentration and asparaginase activity ($P = 0.0001$; **Figure 3-2**). No mice developed osteonecrosis after only two weeks of treatment.

Asparaginase treatment potentiated osteonecrosis and arteriopathy in dexamethasone-treated mice

Based on the result of preliminary experiments, we studied the effect of asparaginase on dexamethasone-induced osteonecrosis using 24-day-old BALB/cJ male mice (“osteonecrosis experiment”; **Figure 3-1**). The mean weights of mice treated with asparaginase (14.7 ± 1.9 g with dexamethasone and 19.4 ± 2.5 g without dexamethasone) were significantly lower than those who did not receive asparaginase (17.4 ± 1.5 g with dexamethasone and 26.8 ± 2.3 g without dexamethasone; $P < 0.0001$) after 6 weeks of treatment. Thirty of 35 (86%) mice in the dexamethasone-alone group and 27 of 35 (77%; $P = 0.8$) mice in the dexamethasone plus asparaginase group survived the 6-week treatment. There were no deaths among the 10 untreated controls and the 10 asparaginase-alone mice.

Osteonecrosis developed in 12 of 27 (44.4%) mice who received dexamethasone and asparaginase, significantly higher ($P = 0.006$) than in mice who received dexamethasone alone (3 of 30 or 10.0%; **Figure 3-3**). An arteriolar branch coursing along the dorsal surface of the distal femur was detected in 47 of the 57 mice evaluated for osteonecrosis. Lesions were present in the vessels of 14 of 24 (58%) mice who received both dexamethasone and asparaginase, compared with 4 of 23 (17%) mice who received dexamethasone alone ($P = 0.007$). No mice in the control group or the asparaginase alone group developed either osteonecrosis or arteriopathy.

Consistent with the pharmacokinetic experiment, we observed an association between plasma dexamethasone concentration and asparaginase activity ($P = 0.005$; see **Appendix B, Figure B-8**) in the osteonecrosis experiment. In the 27 mice that received both dexamethasone and PEG-asparaginase, there was a significant trend towards higher dexamethasone levels ($P = 0.027$) and higher asparaginase activity ($P = 0.036$; **Figure 3-4**) in osteonecrosis-positive cases. A multivariate analysis suggested that plasma

Figure 3-2. Plasma dexamethasone concentration was positively associated with asparaginase activity in PK experiment

Mice received dexamethasone (DEX; 4 mg/L in drinking water) for 2 weeks and 0-4 doses of PEG-asparaginase (ASP; 1500 IU/kg i.p.) at 3.5 day intervals. Samples Blood samples from dexamethasone-alone mice (triangles) and those received additional asparaginase (points) were collected at the end of week 2 (3.5 days after the last asparaginase injection). Each dot represents a mouse. Linear regression line is shown.

Figure 3-3. Asparaginase treatment potentiated osteonecrosis and arteriopathy in dexamethasone-treated mice

Chi-square P values were calculated between dexamethasone (DEX)-treated mice that received and vs. those did not receive asparaginase (ASP).

Figure 3-4. Osteonecrosis was associated with higher plasma dexamethasone and asparaginase levels in mice receiving both dexamethasone and asparaginase treatment

BALB/cJ males received dexamethasone (4 mg/L) and PEG-asparaginase (1200 IU/kg i.p. twice weekly) for 6 weeks. Blood samples were collected approximately 3.5 days after last injection.

dexamethasone (OR = 1.01, $P = 0.029$) and asparaginase concentrations (OR = 1.06, $P = 0.002$) were both independently associated with osteonecrosis.

Arteriopathy was likely the initiating event of osteonecrosis

Among the 15 mice positive for osteonecrosis, one did not have an evaluable vessel present in the plane of section, and all the other 14 (100%) were positive for arteriopathy. Among the 33 mice with no signs of osteonecrosis, arteriopathy was present in 4 mice (12%; $P = 9 \times 10^{-9}$), consistent with our previous study which indicated that vascular damage may be the initiating event of osteonecrosis development.⁹⁴

The histopathological changes of bone and vessels during dexamethasone and asparaginase treatment are shown in **Figure 3-5**. When arteriopathy was present without osteonecrosis (**Figure 3-5D**), it was evident in branches of the medial genicular artery supplying the distal femoral epiphysis (**Figure 3-5E**) with signs of luminal occlusion and loss of endothelium and smooth muscle cells; the interruption of blood supply was localized, resulting in reduced hematopoietic cells in bone marrow (**Figure 3-5F**). When arteriopathy and osteonecrosis were both present, long-term poor circulation led to death of osteocytes and necrosis of marrow and hematopoietic cells (**Figure 3-5I**) and ultimately, extensive necrosis of the bone (**Figure 3-5G**).

Discussion

Clinical studies have indicated interaction between asparaginase and glucocorticoids in pediatric patients receiving the two critical agents of ALL chemotherapy.^{21,30} Herein, we used a mouse model to confirm the clinical observations that systemic exposure of glucocorticoids was increased by asparaginase treatment, and we showed definitively that asparaginase treatment contributes to the osteonecrotic effect of glucocorticoids.

In the present study, we confirmed the positive association between plasma dexamethasone level and asparaginase activity in two independent experiments (**Figure 3-2** and **Figure B-8**). Mice that received asparaginase and dexamethasone (2 - 4 mg/L in drinking water) had a higher level of plasma dexamethasone than those who received dexamethasone alone ($P = 0.001$ and $P = 0.005$ in two independent experiments respectively). Osteonecrosis was associated with higher systemic exposure to dexamethasone and asparaginase in the univariate ($P = 0.027$ and $P = 0.036$ respectively; **Figure 3-4**) and the multivariate analysis ($P = 0.029$ and $P = 0.002$, respectively).

Concurrent use of asparaginase and glucocorticoids can potentiate each other's effects. Asparaginase is associated with decreased clearance of dexamethasone,³⁰ which could be due to its hypoproteinemic effects, possibly decreasing hepatic CYP3A or transporters. Also, the immunosuppressive effects of glucocorticoids also inhibit the antibody response against asparaginase and prevent its neutralizing effect, which in turn

Figure 3-5. Histology of osteonecrosis and arteriopathy

H&E staining of representative stifle joints from mice negative for osteonecrosis (ON) and arteriopathy (Art; top panel), negative for osteonecrosis but positive for arteriopathy (middle panel), and positive for both osteonecrosis and arteriopathy (bottom panel). All mice received dexamethasone and PEG-asparaginase for 6 weeks. (A) Normal arteriole (arrow), marrow and trabecular bone. (B) Magnified cross-section of the arteriole in A. The inset shows the normal endothelial cells (dashed arrows) with elongated nuclei and smooth muscle cells (solid arrows) with round nuclei. (C) Magnification of boxed area in A, showing healthy osteocytes in lacunae (arrows) and healthy hematopoietic cells in marrow (asterisk). (D) Vessel with arteriopathy (arrow) and healthy bone with slightly decreased hematopoietic cells (asterisk). (E) Magnification of the arteriole in D, showing thickened, occluded blood vessel (arrow) and loss of endothelium and smooth muscle cells. (F) Magnification of boxed area in D, showing healthy osteocytes in lacunae (arrows) and decreased hematopoietic cells (asterisk). (G) Vessel with arteriopathy (arrow) and necrotic marrow and trabecular bone. (H) Magnification of the arteriole in G, showing thickened, occluded blood vessel (arrow) and loss of endothelium and smooth muscle cells. (I) Magnification of boxed area in G, showing empty lacunae and dead osteocytes (arrows) and necrotic marrow (asterisk). Scale bars are 500 microns in the left panel and 50 microns in the middle and right panels.

results in higher plasma asparaginase activity.⁸⁷ In a front-line ALL study, St. Jude Total XV, patients with antibodies against asparaginase had a lower risk of developing osteonecrosis than those who did not develop antibodies.⁸⁷

Asparaginase is always used in therapy which includes glucocorticoids. Hanada et al¹³¹ reported a pediatric ALL patient who developed osteonecrosis during asparaginase therapy, but the patient had also received prednisone. In the present study, we did not observe any significant change in bone or vessels of mice receiving asparaginase alone, consistent with asparaginase enhancing the osteonecrotic effect of glucocorticoids, rather than a direct impact on osteonecrosis.

There are several possible mechanisms for the potentiating effect of asparaginase on glucocorticoid-induced osteonecrosis. Both asparaginase and glucocorticoids have been shown to induce a hypercoagulable state by suppression of anticoagulant factors such as antithrombin, plasminogen and d-dimer, and by elevation in F VIII/vWF complex.¹³⁹ The hypercoagulable state may lead to impaired circulation, vascular damage and subsequent osteonecrosis.^{131,132} Interestingly, patients between 11 and 16 years had more significant alteration of anticoagulant and fibrinolytic parameters than children of other ages,¹⁴⁰ consistent with the high susceptibility of adolescents to osteonecrosis. Moreover, alterations in lipid metabolism after asparaginase treatment^{129,130} may lead to formation of lipidic droplets that can be entrapped in the arterial lumen, followed by reduced blood flow and damage to the vascular endothelium. Asparaginase treatment is also associated with venous stasis and deep vein thrombosis in clinic¹⁴¹ and in animal models,¹⁴² although we did not observe such effects with asparaginase alone.

We previously showed preliminary data that native asparaginase could potentiate glucocorticoid-induced osteonecrosis.⁹⁶ However, native *E.coli* asparaginase is no longer commercially available; it has been replaced by PEG-asparaginase. In most ALL protocols, PEG-asparaginase is administered intravenously (i.v.) or intramuscularly (i.m.), and the typical doses range from 1000 IU/m² to 3500 IU/m².^{138,143-145} Plasma asparaginase activity at 3 days after receiving a usual recommended dose of PEG-asparaginase (2500 IU/m² i.v.) ranged from 2 to 4 IU/mL in patients.¹⁴⁶ Herein, we used doses of PEG-asparaginase (1200-1500 IU/kg i.p.) that have previously been shown to result in antileukemic effects in murine models.¹⁴⁷ After injection of PEG-asparaginase at 1200 IU/kg to mice, we achieved plasma asparaginase activity that was slightly higher (5.4 ± 2.9 IU/mL) than those in patients receiving standard dose (2500 IU/m²), and may be more comparable to patients on high-dose asparaginase therapies.

Despite the anatomical and genetic differences between mouse and human, this mouse model yielded similar lesions in bone and arteries as those that have been reported clinically. Biopsy specimens of the femoral head from patients with early-stage osteonecrosis showed structural damage to arteriolar walls before necrosis of trabecular bone and marrow.¹⁴⁸

We tested multiple host-related and treatment-related risk factors for osteonecrosis using this murine model (please see **Appendix B** for details). Osteonecrosis

was associated with male sex, earlier start of treatment (before the onset of puberty), and higher dose and longer treatment duration with dexamethasone. All of these predisposing factors have been reported in clinical studies;^{89-91,126,128,134,136} however, in clinical studies that do show a gender difference, females are at higher risk than males.^{88,89,91,134,136} Why male mice have a higher risk than females is not clear. Among the mice of different substrains, sources and ages, four-week-old in-house bred BALB/cJ male mice displayed high susceptibility to osteonecrosis with an acceptable survival rate; therefore they may serve as a reliable and reproducible mouse model for studying glucocorticoid-induced osteonecrosis in the future.

In the pharmacokinetics experiment, we also measured plasma level of proteins and lipids after dexamethasone (4 mg/L for 2 weeks) and asparaginase treatment (1500 IU/kg i.p. for 0-4 doses). Amylase was significantly increased in dexamethasone alone group, and was suppressed by asparaginase treatment (**Figure B-9**). Lipase was inhibited by asparaginase during the first week, but then rebound over time (**Figure B-10**), consistent with previous clinical report.⁶² Triglyceride level was directly correlated with lipase level (**Figure B-11**). There was no significant change in albumin level with increased asparaginase activity (**Figure B-12**). Plasma cortisol was increased by asparaginase treatment ($P = 0.026$) and suppressed by dexamethasone (**Figure B-13**).

We reviewed multiple clinical studies on the inhibitory effect of asparaginase treatment on protein synthesis, and summarized the data in **Table B-3**. Intriguingly, there was a strong association ($R\text{-squared} = 0.68$, $P < 0.0001$) between the percentage of inhibition and the content of asparagine residues of the protein (**Figure B-14**).

Almost all contemporary front-line ALL regimens contain glucocorticoids and asparaginase during induction therapy. The interaction between these two important antileukemic drugs can cause inter-individual variability in dexamethasone and asparaginase pharmacokinetics, which may influence the efficacy and toxicity of ALL treatment. This animal model allows us to study the optimal dosing strategy of glucocorticoids and asparaginase, and explore the utility of new biomarkers for osteonecrosis.

Conclusion

In summary, we were able to recapitulate the potentiating effect of asparaginase on glucocorticoid-induced osteonecrosis using a mouse model. Mice receiving asparaginase concomitantly with dexamethasone developed more arteriopathy and subsequent osteonecrosis than those received dexamethasone alone. The drug interaction should be considered when using glucocorticoids and asparaginase concomitantly in the treatment of ALL.

CHAPTER 4. ASPARAGINASE-INDUCED ACUTE PANCREATITIS

Introduction

Acute pancreatitis is a major life-threatening complication of ALL treatment. Asparaginase has been recognized as the major cause of pancreatitis in children, adolescents, and adults being treated with combination chemotherapy for ALL, although steroids and thiopurines can also cause the complication.¹⁴⁹⁻¹⁵¹ Acute pancreatitis occurs in 2-18% of patients receiving asparaginase^{40,47-54} and is one of the common causes of asparaginase intolerance. Severe pancreatitis is a contraindication to further asparaginase therapy, leading to discontinuation of this agent. Because discontinuation of asparaginase treatment has been associated with compromised ALL treatment outcome,^{49,53} better understanding of risk factors for pancreatitis would provide insights towards therapy modifications that could decrease pancreatitis risk while maintaining effectiveness in patients with ALL.

The pathogenesis of asparaginase-induced pancreatitis has not been elucidated. Currently known clinical risk factors include intensive asparaginase therapy and older age;^{47-50,56,65} however most studies lack sufficient power to robustly assess risk factors.⁴⁻⁸

Studies on pancreatitis of other etiologies, such as alcoholic, idiopathic and chronic pancreatitis, have identified a few genetic risk factors including cationic and anionic trypsinogen (*PRSS1* and *PRSS2*), serine protease inhibitor kazal type 1 (*SPINK1*), cystic fibrosis trans-membrane conductance regulator (*CFTR*), chymotrypsinogen C (*CTRC*), calcium sensing receptor (*CASR*), claudin-2 (*CLDN2*), carboxypeptidase 1 (*CPA1*), and *HLA-DRB1*07:01*, most of which relate to premature trypsin activation or sustained trypsin activity in the pancreas.^{66,72,75-79,152,162} However, there has not been an agnostic genome-wide analysis of asparaginase-induced acute pancreatitis.⁸² Here we report the largest study to date to determine the clinical and genetic risk factors for acute pancreatitis in 5398 children, adolescents, and young adults treated for newly diagnosed ALL with multiagent chemotherapy regimens containing asparaginase.

Methods

Patients and treatment

We investigated 6171 children and young adults (age 0 to 30 years) with newly diagnosed ALL treated on seven front-line ALL protocols: Total XIIIB (NCI-T93-0101D)¹⁵³, Total XV (NCT00137111)⁸, COG P9904 (NCT00005585), P9905 (NCT00005596), P9906 (NCT00005603)¹⁵⁴, AALL0232 (NCT00075725) and AALL0331 (NCT00103285) at St. Jude Children's Research Hospital (SJCRH) and in the Children's Oncology Group (COG) between 1994 and 2011 (**Table 4-1** and **Figure 4-1**).

Table 4-1. Comparison of asparaginase regimens

Protocol	Induction	Consolidation	Interim Maintenance (IM) and Delayed Intensification (DI)	Maintenance	Total ASP dose^b (U/m²)	Total ASP weeks^c	N (%) of patients developing pancreatitis
SJCRH Total XIIB (1994-1998)	<i>E. coli</i> 10000 U/m ² × 6	No ASP	NA	<i>E. coli</i> 10000 U/m ² × 6	120000	4	2/222 (0.9%)
SJCRH Total XV (2000-2007)	<i>E. coli</i> 10000 U/m ² × 6	No ASP	NA	Low risk: <i>E. coli</i> 10000 U/m ² × 18 Standard/high risk: <i>E. coli</i> 25000 U/m ² × 19	240000-535000	8-21	16/483 (3.3%)
COG P9904/P9905 (2000-2007)	<i>E. coli</i> 10000 U/m ² × 6 ^d or PEG 2500 U/m ² × 1 ^e	Randomized to PEG 2500 U/m ² × 1 vs. no ASP	NA	No ASP	50000-110000	2-4	12/1605 (0.7%)
COG P9906 (2000-2003)	<i>E. coli</i> 10000 U/m ² × 6	<i>E. coli</i> 6000 U/m ² × 12	<i>E. coli</i> 15000 U/m ² × 10 and 6000 U/m ² × 24	No ASP	420000	24	11/222 (5.0%)
AALL0232 (2004-2011)	PEG 2500 U/m ² × 1	PEG 2500 U/m ² × 2	DH/PH: PEG 2500 U/m ² × 2 (single IM/DI) or × 6 (double IM/DI)	No ASP	250000-450000	10-18	32/1359 (2.4%)
			DC/PC: PEG 2500 U/m ² × 4 (single IM/DI) or × 6 (double IM/DI)		350000-550000	14-22	44/1294 (3.4%)

Table 4-1. (Continued)

Protocol	Induction	Consolidation	Interim Maintenance (IM) and Delayed Intensification (DI)	Maintenance	Total ASP dose ^b (U/m ²)	Total ASP weeks ^c	N (%) of patients developing pancreatitis
AALL0331 (2005-2010)	PEG 2500 U/m ² × 1	Randomized to PEG 2500 U/m ² × 2 vs. no ASP	Low/average risk: Randomized to PEG 2500 U/m ² × 1 vs. × 4 High risk: PEG 2500 U/m ² × 8	No ASP	100000-350000 450000-550000	4-14 18-22	NA (Case-control study)

All asparaginase was given intramuscularly. ^aDepends on patients' bone marrow blast percent during induction or minimal residual disease (MRD) status at the end of Induction. ^bTotal dose and ^ctotal weeks of native *E.coli* asparaginase during therapy, excluded the extended Induction. PEG-asparaginase administration at 2500 U/m² biweekly was considered therapeutically equivalent to native *E.coli* asparaginase at 25000 U/m² weekly for two weeks. ^dPre-amendment (before 10/07/2003), native *E.coli* asparaginase was used. ^ePost-amendment (from 10/07/2003), PEG-ASP was used. Abbreviations: ASP, asparaginase; *E.coli*, native *E.coli* asparaginase; PEG, pegylated *E.coli* asparaginase; DH/PH, dexamethasone/prednisone during induction therapy and high-dose methotrexate/leucovorin during the first interim maintenance; DC/PC, dexamethasone/prednisone during induction therapy and escalating methotrexate/leucovorin during the first interim maintenance. NA, not applicable.

Figure 4-1. Study design

Study design and genotyping platforms of the initial GWAS (5398 patients) that aimed to identify the top ranked SNPs and genes from the cohort, and the SNPs or genes significantly associated with pancreatitis ($P < 0.05$) in the case-control group. The number of patients and number of cases (in parentheses) in each protocol are shown.

The studies were approved by the institutional review boards of all participating institutions, and informed consent was obtained in accordance with the Declaration of Helsinki.

We studied 5185 of 5958 patients (87%) enrolled on Total XIIIB/XV, COG P9904/P9905/P9906 and AALL0232 with available DNA, and a case-control group of 213 patients (71 cases and 142 controls) was selected from 5500 patients treated on AALL0331 (**Table 4-2**). In the case-control group, we studied every pancreatitis case on AALL0331 with available DNA and matched each case to two controls by age (± 2 years) and asparaginase treatment.

Diagnosis of acute pancreatitis

Acute pancreatitis was prospectively graded using NCI CTCAE version 3.0 for AALL0232 and AALL0331, and version 2.0 for all the other protocols. The diagnostic criteria included clinical symptoms (abdominal pain, nausea and vomiting), elevation in the serum amylase or lipase levels, and abnormal radiographic or surgical findings consistent with pancreatitis. For time-dependent analyses, time to first episode of pancreatitis was considered even if the patient had more than one episode of pancreatitis recorded.

Genotyping

Genotyping of SNPs was performed using the Illumina HumanExome Beadchips and either Affymetrix Genome-Wide Human SNP Array 6.0 or GeneChip Human Mapping 500K Array sets¹¹⁸ (**Figure 4-1**). This included a total of 5398 patients with Affymetrix Genome-Wide Human SNP 6.0 (or GeneChip Human Mapping 500K) Array data and 3469 patients with Illumina HumanExome Beadchips Array data. For those patients studied using the 500K array sets, SNPs were imputed to include those on the Affymetrix SNP 6.0 array using MACH1.0.¹⁵⁵ All patients in the cohort study ($n = 5185$) were genotyped (or imputed) using SNP 6.0 array, while only a subset of patients ($n = 3256$) were genotyped using the Exome Beadchips. All patients in the case-control study ($n = 213$) were genotyped on both arrays (**Figure 4-1**). The prediction of the potential damaging effect of SNPs was made using in silico prediction algorithms (SIFT,¹⁵⁶ PolyPhen,¹⁵⁷ LRT¹⁵⁸ and MutationTaster¹⁵⁹). Combined Annotation Dependent Depletion (CADD) score¹⁶⁰ was also used to estimate the deleteriousness of the coding SNPs (**Table 4-5** and **Figure C-1**).

Genetic ancestries were determined using STRUCTURE,¹¹⁸ and either treated as a continuous variable or used to categorize patients as white, black, Hispanics, Asian, and “other” groups based on the percentage of European, African, Native American and Asian ancestries (**Figure 4-2**).

Table 4-2. Number of patients included in this study

Study	Protocol	N of patients	N (%) with array data^a	N (%) with deep sequencing data^b
Cohort study	COG P9904/P9905	1883	1605 (85.2%)	1359 (72.2%)
	COG P9906	272	222 (81.6%)	158 (58.1%)
	AALL0232	3058	2653 (86.7%)	2030 (66.3%)
	Total XIIB	247	222 (89.9%)	213 (86.2%)
	Total XV	498	483 (96.9%)	457 (91.8%)
	Cohort study total	5958	5185 (87.0%)	4217 (70.8%)
Case-control study	AALL0331	5500	213 (3.9%)	162 (2.9%)

^aAffymetrix Genome-Wide Human SNP Array 6.0 or GeneChip Human Mapping 500K Array sets. ^b283 genes were sequenced, including 42 putative pancreatitis candidate genes based on literatures and the initial GWAS using the array data.

Figure 4-2. Race group assignments based on the ancestral composition of patients in the cohort (n = 5185) by treatment protocol

Number of patients of each ancestry (except Asian) are shown on the bars. Genetic ancestry was estimated using STRUCTURE,¹¹⁸ and patients were assigned to race groups categorized as white (European ancestry > 90%), black (African ancestry > 70%), Hispanic (Native American ancestry > 10%), Asian (Asian ancestry > 90%) and “other”.

SNP-based analysis for pancreatitis

After excluding the SNPs with a call rate < 95%, 751455 of 931947 SNPs on the SNP 6.0 array and 169521 of 174435 SNPs on the Exome Beadchip were interrogated in the initial GWAS. Each SNP was first individually tested using the Gray test and those achieving $P < 0.05$ were subsequently analyzed. Genotype calls were coded as 0, 1 or 2 for the number of B alleles (AA, AB or BB) assuming an additive genetic model. In the cohort ($n = 5185$), GWAS was performed using a Cox proportional hazard model considering the onset time of pancreatitis (time-dependent GWAS). Covariates included age (continuous variable in years), genetic ancestry (continuous variable as percentages of European, African, Asian and Native American ancestries), and treatment regimen (protocol-planned cumulative asparaginase dose ≤ 120000 U/m² vs. ≥ 240000 U/m² as a categorical variable). For patients with pancreatitis, their time-at-risk was censored at the first episode of pancreatitis; for those without pancreatitis, their time-at-risk was censored at off-study date. In the case-control group ($n = 213$), controls were matched to cases for age and treatment regimen, and GWAS was performed using logistic regression, adjusted for genetic ancestry only (time-independent GWAS).

Gene-level analysis of nonsense variants using SKAT

SNPs were assigned to genes using Refseq boundaries (UCSC Genome Browser, hg19 assembly). For each gene, sequence kernel association test¹⁶¹ (SKAT) was applied to test for gene level significance for the effects of nonsense variants. For time dependent analysis, Martingale residuals as a continuous phenotype in the SKAT analysis were determined after applying Cox proportional hazard model using demographic and clinical covariates, including age, genetic ancestry, and treatment regimen.

Deep sequencing of selected genes

To interrogate rare and low-frequency variants in genes of interest for ALL-related phenotypes, we sequenced 283 genes in 4217 children in the cohort and 162 in the case-control group (**Table 4-2**). The 283 genes were selected for sequencing because they were candidate genes for a number of ALL phenotypes (e.g. relapse, drug sensitivity, pharmacokinetics, leukemia risk, and several adverse effects); 42 of these were possible pancreatitis risk genes (**Table 4-3**). For more details of deep sequencing, please see **Appendix C**.

Gene-level analysis of all 283 genes and single variant analysis of 9229 SNPs in the 42 pancreatitis-associated genes was performed using the same methods as in the initial GWAS (see **Appendix C**).

Table 4-3. Putative genes associated with pancreatitis (n = 42) selected for deep sequencing based on GWAS result or literature review

Gene	Official full name	Function*	Source
<i>ADAMTS18</i>	ADAM metallopeptidase with thrombospondin type 1 motif, 18	Tumor suppressor	GWAS
<i>AGER</i>	Advanced glycosylation end product-specific receptor	Member of the immunoglobulin superfamily of cell surface receptors implicated in homeostasis, development and inflammation, and certain diseases, such as diabetes and Alzheimer's disease	GWAS
<i>ALS2CL</i>	ALS2 C-terminal like	Guanine nucleotide exchange factor (GEF) for Rab5 GTPase regulating the ALS2-mediated endosome dynamics	GWAS
<i>C11orf63</i>	Chromosome 11 open reading frame 63	Protein-coding gene	GWAS
<i>C12orf40</i>	Chromosome 12 open reading frame 40	Protein-coding gene	GWAS
<i>C9orf117</i>	Chromosome 9 open reading frame 117	Protein-coding gene	GWAS
<i>CASR</i>	Calcium-sensing receptor	G protein-coupled receptor that senses small changes in circulating calcium concentration and couples this information to intracellular signaling pathways	Literature ⁷²
<i>CD52</i>	Cluster of differentiation 52	Possible role in carrying and orienting carbohydrate	GWAS
<i>CFTR</i>	Cystic fibrosis transmembrane conductance regulator	cAMP-regulated epithelial cell channel involved in fluid transport	Literature ^{153,162}
<i>CLDN2</i>	Claudin 2	Major integral membrane protein localized exclusively at tight junction	Literature ^{66,69}
<i>CPA1</i>	Carboxypeptidase A1 (pancreatic)	A1 form of the pancreatic procarboxypeptidase produced in the pancreas and preferentially cleaves C-terminal branched-chain and aromatic amino acids from dietary proteins	Literature ^{80,81}

Table 4-3. (Continued)

Gene	Official full name	Function*	Source
<i>CPA2</i>	Carboxypeptidase A2 (pancreatic)	A2 form of pancreatic procarboxypeptidase produced in the pancreas and preferentially cleaves aromatic C-terminal residues from dietary proteins	GWAS
<i>CTRC</i>	Chymotrypsin C	Serum calcium-decreasing factor with chymotrypsin-like protease activity, regulating activation and degradation of trypsinogens and procarboxypeptidases by targeting specific cleavage sites within their zymogen precursors	Literature ¹⁶²⁻¹⁶⁴
<i>DDX49</i>	DEAD (Asp-Glu-Ala-Asp) box polypeptide 49	RNA binding and ATP-dependent helicase activity	GWAS
<i>DOCK5</i>	Dedicator of cytokinesis 5	Guanine nucleotide exchange factor (GEF) for Rho and Rac which regulates of actin distribution and amylase secretion in pancreatic acinar cells	GWAS
<i>FHIT</i>	Fragile histidine triad	Diadenosine 5',5'''-P1,P3-triphosphate hydrolase involved in purine metabolism	GWAS
<i>GCKR</i>	Glucokinase (hexokinase 4) regulator	Inhibiting glucokinase in liver and pancreatic islet cells by binding non-covalently to form an inactive complex with the enzyme	GWAS
<i>HOGA1</i>	4-hydroxy-2-oxoglutarate aldolase 1	Catalyzing the final step in the metabolic pathway of hydroxyproline, releasing glyoxylate and pyruvate	GWAS
<i>HSD17B2</i>	Hydroxysteroid (17-beta) dehydrogenase 2	Capable of catalyzing the interconversion of testosterone and androstenedione, as well as estradiol and estrone	GWAS
<i>IFNA5</i>	Interferon, alpha 5	Cytokine activity and antiviral activity	GWAS
<i>INMT</i>	Indolethylamine N-methyltransferase	Catalyzing N-methylation of indoles such as tryptamine	GWAS
<i>ITFG1</i>	Integrin alpha FG-GAP repeat containing 1	Modulator of T-cell function	GWAS
<i>KLHDC1</i>	Kelch domain containing 1	Protein-coding gene, paralog of <i>HCFC2</i> (Host Cell Factor C2)	GWAS

Table 4-3. (Continued)

Gene	Official full name	Function*	Source
<i>LRRC39</i>	Leucine rich repeat containing 39	Protein-coding gene, paralog of <i>LRSAMI</i> , which is a regulator of signaling pathways of cell adhesion, mediating self-ubiquitylation and cargo sorting during receptor endocytosis	GWAS
<i>MICAL2</i>	Microtubule associated monooxygenase, calponin and LIM domain containing 2	Nuclear monooxygenase that promotes depolymerization of F-actin, and interacts with small GTPase Rab1, which plays a role in vesicle trafficking from the endoplasmic reticulum to the Golgi	GWAS
<i>MPZL3</i>	Myelin protein zero-like 3	Mediator of homophilic cell-cell adhesion	GWAS
<i>NMI</i>	N-myc (and STAT) interactor	May be involved in augmenting coactivator protein recruitment to a group of sequence-specific transcription factors. Augments cytokine-mediated STAT transcription	GWAS
<i>PHKB</i>	Phosphorylase kinase, beta	Regulatory unit of phosphorylase kinase B that activates cAMP-dependent phosphorylase kinase pathway	GWAS
<i>PRSS1</i>	Protease, serine, 1 (trypsin 1)	Also known as cationic trypsinogen, a pancreatic serine protease that is active on peptide linkages involving the carboxyl group of lysine or arginine	Literature ^{66,69,162}
<i>PRSS2</i>	Protease, serine, 2 (trypsin 2)	Also known as anionic trypsinogen, a pancreatic serine protease that is active on peptide linkages involving the carboxyl group of lysine or arginine	Literature ^{66,69}
<i>RANBP10</i>	RAN binding protein 10	Guanine nucleotide exchange factor (GEF) for RAN GTPase. It plays an essential role in hemostasis and in maintaining microtubule dynamics, and enhances dexamethasone-induced transactivation activity of glucocorticoid receptor NR3C1	GWAS
<i>RUFY4</i>	RUN and FYVE domain containing 4	Protein-coding gene, paralog of <i>FYCO1</i> (FYVE And Coiled-Coil Domain Containing 1) that mediates microtubule plus end-directed vesicle transport	GWAS

Table 4-3. (Continued)

Gene	Official full name	Function*	Source
<i>SCAMP3</i>	Secretory carrier membrane protein 3	Secretory carrier membrane protein that functions as a carrier to the cell surface in post-golgi recycling pathways, and involves in protein trafficking in endosomal pathways	GWAS
<i>SERPINA9</i>	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 9	Protease inhibitor that inhibits trypsin and trypsin-like serine proteases	GWAS
<i>SLC6A18</i>	Solute carrier family 6 (neutral amino acid transporter), member 18	Renal transporter for neurotransmitters, amino acids, and osmolytes like betaine, taurine, and creatine	GWAS
<i>SNTG2</i>	Syntrophin, gamma 2	Interacting with mechanosensitive sodium channel that affects channel gating	GWAS
<i>SPAG8</i>	Sperm associated antigen 8	Involved in spermatogenesis by regulating microtubule formation and cell division	GWAS
<i>SPINK1</i>	Serine peptidase inhibitor, Kazal type 1	Prevention of trypsin-catalyzed premature activation of zymogens within the pancreas and the pancreatic duct	Literature ^{71,72,162}
<i>TRAP1</i>	TNF receptor-associated protein 1	Regulating cellular stress responses	GWAS
<i>ZNF233</i>	Zinc finger protein 233	Protein-coding gene that may be involved in transcriptional regulation	GWAS
<i>ZNF701</i>	Zinc finger protein 701	Protein-coding gene that may be involved in transcriptional regulation	GWAS
<i>ZRANB3</i>	Zinc finger, RAN-binding domain containing 3	DNA annealing helicase and endonuclease required to maintain genome stability at stalled or collapsed replication forks	GWAS

*Functional annotation obtained from NCBI Gene database (<http://www.ncbi.nlm.nih.gov/gene>), Uniprot (<http://www.uniprot.org>) and Genecards (www.genecards.org). Genes involved in cytoskeleton regulation and vesicle trafficking pathways are highlighted.

Statistical analysis

All statistical analyses were conducted using R (version 3.1.0). Demographics were compared using Chi square test or Wilcoxon rank sum test. Event-time distributions were analyzed using the Kaplan-Meier method, and compared using the log-rank test. Multivariate Cox proportional hazard model was used to determine the potential risk factors for acute pancreatitis. Classification and regression tree (CART) analysis was performed with the *ctree* function of the package *party*¹⁶⁵ of R (version 3.1.0).

Results

Pancreatitis associated with asparaginase

Among the 5185 patients in the cohort, 117 (2.3%) were diagnosed with at least one episode of acute pancreatitis during therapy. The frequency differed by protocols: Total XIIIB (0.9%, 2/222), Total XV (3.3%, 16/483), P9904 (0.7%, 5/717), P9905 (0.8%, 7/888), P9906 (5.0%, 11/222) and AALL0232 (2.9%, 76/2653; **Figure 4-3A**). The first episode of pancreatitis occurred at a median of 106 days from ALL diagnosis, and developed during remission induction (first 4-6 weeks of treatment) in 24 of 117 (20.5%) patients and during the first year of therapy (including remission induction) in 104 of 117 (88.9%) patients (**Figure 4-3A**). The great majority of the events were clustered with asparaginase administration (**Figure 4-4**).

Among the 71 pancreatitis cases treated on AALL0331 (case-control group), the first episode of pancreatitis occurred at a median of 78 days, and developed during remission induction in 24 of 71 (33.8%) cases and during the first year in 65 of 71 (91.5%) cases.

The risk of pancreatitis was related to cumulative dose (R-squared = 0.77, $P = 1.8 \times 10^{-4}$) and duration of asparaginase treatment (R-squared = 0.69, $P = 8.9 \times 10^{-4}$; **Figure 4-3B** and **C**). Because asparaginase dose and duration were highly correlated (R-squared = 0.86, $P = 1.6 \times 10^{-5}$), it was not possible to determine which was more important for pancreatitis risk.

Clinical risk factors

To identify covariates to include in the GWAS, we tested for the associations between pancreatitis and clinical characteristics in the cohort (**Figure 4-5**). Patients with pancreatitis were older (11.7 ± 5.1 years vs. 8.6 ± 5.6 years; $P = 2.2 \times 10^{-9}$), and the incidence was related to age as a continuous variable in univariate analysis ($P = 1.3 \times 10^{-7}$). Hispanic (3.9%) and black (3.1%) patients had higher risk than white (1.5%) and Asian (1.0%) patients. We categorized the protocols into low-dose (≤ 120000 U/m²) and high-dose asparaginase regimens (≥ 240000 U/m²; **Table 4-1**). High-dose regimens had

Figure 4-3. Incidence of pancreatitis differed by protocols

(A) Cumulative incidence of pancreatitis in the cohort (n = 5185) by different protocols. (B) The relationship between incidence of pancreatitis and cumulative dose of ASP and (C) the relationship between incidence and treatment duration of ASP in this study (filled circles) and in some recently reported protocols^{49,50,52,53} (empty circles). The individual points show the protocol-defined dose or treatment duration of native *E. coli*-ASP across all treatment arms for each protocol. PEG-ASP 2500 U/m² biweekly is considered equivalent to native *E. coli*-ASP at 25000 U/m² weekly for two weeks. The linear regression lines, R-squared and P values are shown. ASP, asparaginase.

Figure 4-4. Time of pancreatitis in the cohort (n = 5185) during the first year of therapy

The first episode of pancreatitis (red stars) and asparaginase administration (arrows) are shown. DI, delayed intensification; DH/PH, dexamethasone/prednisone and high-dose methotrexate arm; DC/PC, dexamethasone/prednisone and Capizzi (escalating) methotrexate arm; LR, low-risk; SHR, standard/high-risk; PEG-ASP, pegylated E.coli asparaginase; IM, interim maintenance.

Study (N of cases in the first year/N of patients)

P9904/5 (11/1605)

No DI (6/938)

DI (5/667)

P9906 (7/222)

AALL0232 (73/2653)

DH/PH (30/1359)

DC/PC (43/1294)

Total XIIIB (1/222)

Total XV (12/483)

LR (5/235)

SHR (7/248)

Weeks

0

10

20

30

40

50

★ Pancreatitis (first episode only)

Native *E.coli*-ASP

↑ 6000 U/m²

↑ 10000 U/m²

↑ 15000 U/m²

↑ 25000 U/m²

PEG-ASP

↑ 2500 U/m²

↑* Post-amendment, 10/07/2003

↑ Extended induction

(↑) Double IM/DI

Figure 4-5. The incidence of pancreatitis based on clinical characteristics

*Low-dose asparaginase ($\leq 120000 \text{ U/m}^2$, including P9904, P9905 and Total XIIIIB) and high-dose asparaginase regimens ($\geq 240000 \text{ U/m}^2$, including P9906, AALL0232 and Total XV). Chi-square P values for categorical variables and #P value from linear regression model for continuous variable (age) are shown. ASP, asparaginase.

more pancreatitis (3.1% vs. 0.8%, $P = 1.2 \times 10^{-8}$). These features remained as independent risk factors in a multivariate model (**Table 4-4**). Interestingly, there was a direct correlation between the percentage of Native American ancestry and a higher risk of pancreatitis (**Figure 4-6**). Only P9904/P9905 included some patients treated with one or the other formulation of asparaginase (**Table 4-1**), and the incidence of pancreatitis did not differ between those who received native *E.coli* asparaginase alone (0.8%, 6/723), PEG-asparaginase alone (0.9%, 3/347) or both (0.6%, 3/535).

A CART analysis was also used to depict the clinical risk factors in order of importance (**Figure 4-7**). The covariates included in the model are ancestry, age, lineage, gender, asparaginase regimen and formulation. The most important factor for pancreatitis is high exposure to asparaginase. For children on a low-dose regimen (≤ 120000 U/m²), those older than 10 years of age had a higher risk (2.2%, 6/267) than those younger than 10 years of age (0.5%, 8/1560; $P = 0.01$). For those on a high-dose regimen (≥ 240000 U/m²), Hispanic and black children had a higher risk (4.9%, 52/1054) than other race groups (2.2%, 51/2304; $P = 5.2 \times 10^{-5}$).

Thus we used age, ancestry and treatment regimen as covariates in all genomic analysis in the cohort, and ancestry in the case-control group.

Gene-level analysis of nonsense variants identified *CPA2*

Focusing on rare variants and nonsense variants, six genes were associated with pancreatitis in the cohort at the $P < 0.05$ level that were also associated with pancreatitis in the case-control group (**Table 4-5**). In the cohort, *CPA2*, with a nonsense SNP at rs199695765 showed the strongest association ($P = 2.1 \times 10^{-9}$). The C>T SNP at rs199695765 was predicted to cause early termination of the CPA2 protein (**Figure 4-8A**). Two of 3469 patients carried this variant (one in the cohort and one in the case/control group) and both developed early pancreatitis (**Figure 4-8B**); one of them had recurrent episodes after each of two asparaginase re-exposures.

GWAS of common SNPs

Analyzing all coding and non-coding common SNPs (minor allele frequency [MAF] $\geq 1\%$) in the cohort, we identified loci in a gene involved in purine metabolism, *FHIT* (rs9849262, $P = 8.4 \times 10^{-6}$ and rs9859201, $P = 1.2 \times 10^{-4}$). Among the top 20 common SNPs, 14 were assigned to 7 genes, and 7 of the 14 SNPs were in 3 genes involved in cytoskeleton regulation, including *DOCK5*, *ACTN2*, and *MICAL2* (**Table 4-6**). These SNPs were also associated with higher risk in the case-control study, although some of them did not reach $P < 0.05$ in this relatively small group. Results for top ranked variants from single SNP analyses, including nonsense SNPs, missense SNPs, and synonymous and non-coding SNPs are shown in **Appendix C** (**Tables C-1, C-2 and C-3**).

Table 4-4. Multivariate analysis for clinical risk of pancreatitis in the cohort study (n = 5185)

Patient characteristics	<i>P</i>	Odds ratio (95% CI)
Native American ancestry (+10%)	7.6×10^{-8}	1.2 (1.1 – 1.3)
Age (years)	1.2×10^{-5}	1.1 (1.0 – 1.1)
High-dose ASP regimen [§]	1.1×10^{-4}	3.3 (1.8 – 5.9)
African ancestry (+10%)	0.048	1.1 (1.0 – 1.2)
Native <i>E.coli</i> ASP only	0.14	1.4 (0.9 – 2.2)
T-cell lineage ALL	0.27	1.7 (0.7 – 4.5)
Asian ancestry (+10%)	0.60	1.0 (0.9 – 1.2)
Male gender	0.68	1.1 (0.7 – 1.6)

Association with risk of pancreatitis was assessed using Cox proportional hazard model. Age and ancestry were treated as continuous variables; e.g. an odds ratio of 1.2 suggests 1.2 fold higher risk of pancreatitis for every 10% increase in Native American ancestry. Other characteristic features are dichotomized for presence versus absence of the indicated feature. [§]Total protocol-specified *E.coli* asparaginase dose ≥ 240000 U/m² (i.e. all patients on Total XV, COG P9906 and AALL0232) compared to ≤ 120000 U/m².

Figure 4-6. Association between Native American ancestry and pancreatitis
 The distribution of patients with pancreatitis and the total number of patients by the percentage of Native American ancestry (in 10% interval). R-squared and P value were calculated using a univariate linear model.

Figure 4-7. CART analysis of clinical risk factors for pancreatitis in the cohort (n = 5185)

The covariates included in the model are ancestry, age, lineage, gender, asparaginase regimen and formulation.

Table 4-5. Six genes associated with pancreatitis from the gene-level analysis of nonsense SNPs

Gene	Chr	Nonsense SNPs (major allele/risk allele)	Population RAF% (n) ^a	CADD score ^b	Cohort (n = 3256)			<i>P</i> ^c	Case-control (n = 213)			<i>P</i> ^d	PPV/ NPV ^e (n = 3256)
					RAF% in patients with AP (n = 95)	RAF% in patients without AP (n = 3161)	Coeff-icient ^c (SE)		RAF% in cases (n = 71)	RAF% in controls (n = 142)	Coeff-icient ^d (SE)		
<i>CPA2</i>	7	rs199695765 (C/T)	0.009 (11)	18.64	0.5	0	6.5 (1.1)	2.1×10 ⁻⁹	0.8	0	15.8 (7.4)	0.03	100.0/95.6
<i>SERPINA9</i>	14	rs78283108 (G/A)	0.6 (732)	10.87	2.7	0.7	1.2 (0.4)	0.006	0.8	0	16.2 (6.7)	0.02	11.5/95.6
<i>HSD17B2</i>	16	rs8191246 (A/G)	1.4 (1557)	6.948	3.3	0.7	1.5 (0.5)	0.006	3.4	0	17.1 (5.9)	0.004	17.2/95.7
		rs200540657 (T/G)	0.001 (1)	15.63	0.1	0			0	0			0.0/94.8
<i>GCKR</i>	2	rs146053779 (C/T)	0.09 (111)	14.41	1.1	0.3	1.7 (0.7)	0.015	0.8	0	15.4 (8.5)	0.07	16.7/95.6
<i>ZNF233</i>	19	rs144014948 (G/A)	0.09 (112)	26.8	0.5	0.1	1.9 (0.8)	0.019	0.8	0	15.5 (8.3)	0.06	25.0/95.6
		rs148348903 (T/A)	0.09 (114)	34.0	1.1	0.1			0	0			20.0/95.6
<i>SLC6A18</i>	5	rs143864547 (C/A)	0.003 (4)	15.71	0.5	0.02	5.0 (2.3)	0.028	0	0	16.0 (7.4)	0.03	50.0/95.6
		rs200802505 (C/T)	0.03 (41)	16.64	0	0.08			0.8	0			20.0/95.5

Table 4-5. (Continued)

The genes were rank ordered by SKAT P value in the cohort study, and then filtered by the direction in the case-control study. Only patients with exome array data are included in this analysis of nonsense SNPs. ^aPopulation risk allele frequency and number of carriers of the nonsense SNP based on the ExAC data set¹⁶⁶ of 61486 unrelated individuals of European, African, Latino and Asian origins. ^bCADD score¹⁶⁰ was estimated for each nonsense SNP. The average CADD score of 9 SNPs is 17.7 (scores higher than 15 are considered as deleterious). ^cCoefficient, standard error (SE) and SKAT P value were estimated using Cox proportional hazard model after collapsing all nonsense SNPs. ^dCoefficient, standard error and SKAT P value were estimated using logistic regression model after collapsing all nonsense SNPs. ^ePPV (positive predictive value) = (number of patients who carried the nonsense SNP and developed pancreatitis)/(number of patients who carried the nonsense SNP). NPV (negative predictive value) = (number of patients who did not carry the nonsense SNP or develop pancreatitis)/(number of patients who did not carry the nonsense SNP). The number of patients from the cohort and the case-control group were combined (n = 3469). Chr, chromosome. AP, acute pancreatitis. RAF, risk allele frequency.

Figure 4-8. Nonsense SNP rs199695765 in *CPA2* associated with pancreatitis
 (A) Protein structure of proCPA2 (without the 16-amino-acid signal peptide) with the nonsense SNP rs199695765 (arrow) located in the propeptide domain. The unaffected region of p.Q51X variant (magenta), the affected propeptide (yellow) and the active enzyme moiety (blue) are shown. (B) Cumulative incidence of pancreatitis in patients who carried any *CPA2* nonsense or missense SNPs (variant) was higher than that in patients who are classified as “wild-type,” i.e. they carried none, or only synonymous or non-coding SNPs (* $P = 0.018$ after adjusting for clinical characteristics).

Table 4-6. Top 20 common SNPs (cohort risk allele frequency > 1%) significantly associated with pancreatitis in the cohort

SNP	Gene	Chr	Location ^a	Major allele/ risk allele	Cohort (n = 5185)				Case-control (n = 213)				Cohort RAF vs race (n = 5185)	
					RAF% in patients with AP (n = 117)	RAF% in patients without AP (n = 5068)	Coeff- icient ^b (SE)	P ^b	RAF% in cases (n = 71)	RAF% in controls (n = 142)	Coeff- icient ^c (SE)	P ^c	Hispanic +Black (n = 1527)	White +Asian (n = 3168)
rs9849262	<i>FHIT</i>	3	60937049	C/T	4.7	1.0	1.4 (0.3)	8.4×10 ⁻⁶	0.7	0	15 (11.2)	0.18	2.8*	0.2*
rs12589386		14	47223965	A/G	8.5	3.7	1.1 (0.2)	1.8×10 ⁻⁵	9.4	3.2	1.0 (0.4)	0.03	2.2	3.9
rs17377657		8	78054203	C/T	11.5	5.9	0.9 (0.2)	1.8×10 ⁻⁵	6.5	2.9	1.0 (0.5)	0.06	3.0	7.3
rs7826058	<i>DOCK5</i>	8	25095058	A/C	8.1	2.8	1.1 (0.3)	2.7×10 ⁻⁵	5.1	1.1	1.2 (0.7)	0.07	5.7*	1.3*
rs1768056	<i>ACTN2</i>	1	236890781	G/A	13.2	3.7	1.0 (0.3)	4.3×10 ⁻⁵	8.0	2.9	0.8 (0.6)	0.13	11.2*	0.6*
rs16955095	<i>ITFG1</i>	16	47333623	C/A	11.1	4.7	0.9 (0.2)	4.6×10 ⁻⁵	3.6	2.5	0.3 (0.6)	0.56	6.1*	4.0*
rs17117423		14	47008512	T/C	9.1	4.2	1.0 (0.2)	5.0×10 ⁻⁵	8.7	2.9	1.2 (0.5)	0.02	3.3	4.3
rs16892294	<i>AGPAT4</i>	6	161626177	C/T	29.1	13.1	0.7 (0.2)	1.0×10 ⁻⁴	23.2	12.9	0.5 (0.3)	0.08	28.2*	6.3*
rs9859201	<i>FHIT</i>	3	60935372	T/A	4.3	1.1	1.3 (0.3)	1.2×10 ⁻⁴	0.7	0	15 (11.2)	0.18	3.0*	0.2*
rs1034347		21	21535391	G/A	17.1	10.4	0.7 (0.2)	1.6×10 ⁻⁴	13.0	9.0	0.4 (0.4)	0.24	11.7*	9.9*
rs16953067	<i>ITFG1</i>	16	47200165	A/G	11.1	5.0	0.9 (0.2)	1.6×10 ⁻⁴	3.6	2.9	0.2 (0.6)	0.71	6.7*	4.1*
rs1564947	<i>MICAL2</i>	11	12176409	G/A	68.8	50.7	0.6 (0.2)	1.8×10 ⁻⁴	58.0	42.1	0.5 (0.2)	0.06	71.1*	40.2*
rs11961305	<i>AGPAT4</i>	6	161627211	G/A	31.2	14.9	0.6 (0.2)	1.8×10 ⁻⁴	23.9	15.5	0.4 (0.3)	0.25	32.3*	6.6*
rs16907254		11	4931083	C/T	24.1	13.0	0.6 (0.2)	1.8×10 ⁻⁴	13.8	9.0	0.4 (0.3)	0.28	18.3*	11.1*
rs11606424		11	43159756	C/A	11.6	5.4	0.8 (0.2)	1.9×10 ⁻⁴	8.0	5.8	0.2 (0.4)	0.65	6.1	5.3
rs2010463	<i>MICAL2</i>	11	12186296	C/T	61.1	55.3	0.6 (0.2)	2.0×10 ⁻⁴	54.5	34.6	0.7 (0.2)	0.003	56.4*	39.2*
rs1341861	<i>ACTN2</i>	1	236883686	T/G	16.7	6.2	0.8 (0.2)	2.6×10 ⁻⁴	9.4	3.6	0.8 (0.5)	0.10	17.2*	1.2*
rs16945382	<i>PHKB</i>	16	47521171	T/G	12.0	5.6	0.8 (0.2)	2.7×10 ⁻⁴	6.9	5.9	1.0 (0.5)	0.06	8.5*	4.0*
rs17053225	<i>DOCK5</i>	8	25097310	A/G	10.6	4.5	1.0 (0.3)	2.8×10 ⁻⁴	5.9	0.7	1.7 (0.7)	0.02	10.1*	1.6*
rs10831758	<i>MICAL2</i>	11	12218497	C/T	39.7	25.8	0.5 (0.2)	3.0×10 ⁻⁴	31.2	21.9	0.4 (0.3)	0.11	36.5*	20.3*

Table 4-6. (Continued)

The SNPs were rank ordered by P value in the cohort study, and then filtered by the direction in the case-control study. The RAFs of combined race groups (Hispanic + Black and White + Asian) in the cohort (n = 5185) are shown. *Higher RAF ($P < 0.05$) in the Hispanic and black groups than in whites and Asians. ^aPhysical location of SNP based on Feb 2009 human genome assembly (hg19). ^bCoefficient, standard error and P value were estimated using Cox proportional hazard model. ^cCoefficient, standard error and P value were estimated using logistic regression model. Chr, chromosome. AP, acute pancreatitis. RAF, risk allele frequency.

Deep sequencing detected novel variants in *CPA2* and other candidate genes

We identified additional variants by sequencing 283 genes, 42 of which were *a priori* classified as likely candidate genes ($n = 34$) for pancreatitis based on our GWAS results or based on prior links to pancreatitis in other settings ($n = 8$; *PRSSI*, *PRSS2*, *SPINK1*, *CFTR*, *CASR*, *CTRC*, *CPA1*, *CLDN2*; **Table 4-3**).

For the 4217 children in the cohort and 162 in the case-control group with available deep sequencing data, 380 variants were identified in *CPA2*, including 354 variants that were not interrogated on either the exome or SNP 6.0 array (see **Appendix C, Table C-4**). Sixteen *CPA2* SNPs were associated ($P < 0.05$) with pancreatitis, and 13 of 24 patients who carried at least one of these variants developed pancreatitis. Interestingly, two of three patients who had multiple (i.e. three) episodes carried one of these *CPA2* variants (the nonsense SNP rs199695765 and an intronic SNP chr7:129915817 G>A). Patients who carried *CPA2* coding variants had a higher risk of pancreatitis than those who did not carry any coding variants (OR = 2.4, 95% CI 1.2 – 5.1, $P = 0.018$) after adjusting for clinical features (**Figure 4-8B**). For patients who carried the nonsense SNP rs199695765, the estimated risk was uncertain but very high (OR = 587, 95% CI 66.8–5166, $P = 9.0 \times 10^{-9}$). The positive predictive value (PPV) and negative predictive value (NPV) of rs199695765 were 100% (95% CI: 19.3 – 100%) and 95.6% (95% CI: 94.8 – 96.2%). Of 61486 subjects reported in the Exome Aggregation Consortium (ExAC) data set,¹⁶⁶ a total of 11 individuals were heterozygous for this variant, yielding an allele frequency in the general population of only 0.009% (**Table 4-5**).

Similar to the initial GWAS, we conducted a gene-level analysis of nonsense variants in the 42 putative pancreatitis genes versus the 241 non-pancreatitis genes (see **Appendix C, Table C-5**). Among the 283 genes, 21 were related to pancreatitis at the $P < 0.05$ level, 16 of which were pancreatitis genes. Based on sequencing, a higher proportion of putative candidate pancreatitis genes had significant association than the non-candidate genes at $P < 0.05$ (16/42 vs 5/241, $P = 2.7 \times 10^{-11}$). The pathway analysis showed that the top networks associated with the 42 genes included endocrine system disorders, cell cycle and proliferation, and gastrointestinal disease (**Figure C-2**). When the analysis was limited to the 34 genes identified by initial GWAS (**Table 4-3**), these pathways still remained the most significant (**Figure C-3**).

In the gene-level analysis, *HOGAI* with a nonsense SNP (chr10:99361676 C>T, p.R255X) showed the strongest association in the cohort ($P = 6.4 \times 10^{-11}$). The only patient who was heterozygous for this nonsense variant developed pancreatitis very early, at day 17. *CPA2* was ranked second (for nonsense SNPs, $P = 6.7 \times 10^{-11}$) and remained significant when the gene-based analysis included any missense SNPs ($P = 5.5 \times 10^{-3}$) or any coding variants ($P = 0.045$).

Among the 8 candidate genes previously associated with pancreatitis in other settings, 1732 coding polymorphisms were identified via sequencing (**Table C-6**). Eighty-one SNPs were associated with pancreatitis at the $P < 0.05$ level in either the

cohort or the case-control group, but none of them were significant in both groups. A variant (chr7:117119516 G>A) upstream of *CFTR* showed the strongest association in the cohort ($P = 4.4 \times 10^{-9}$); this variant was carried by only one patient, who developed early pancreatitis at day 25. None of the candidate genes were significantly associated ($P < 0.05$) with pancreatitis in the gene-level analysis.

Discussion

Acute pancreatitis has long been recognized as a serious complication of asparaginase-containing regimens and can limit the ability to deliver chemotherapy. However, the pathogenesis and mechanisms remain unknown, and it is not currently possible to predict whether and when a patient will experience this toxicity. There is no definitive treatment for acute pancreatitis; only supportive care is given.⁶¹ Thus, the genetic and non-genetic risk factors we discovered in this largest study to date may have importance for understanding the mechanisms and developing targeted interventions for patients with predisposition.

Here we report that variants in *CPA2*, encoding carboxypeptidase A2, were associated with risk of acute pancreatitis in patients with ALL. This gene encodes a 417-amino-acid proenzyme (proCPA2) that comprises a 16-amino-acid signal sequence, a 94-amino-acid propeptide and an active enzyme moiety.¹⁶⁷ There are 11 exons; the nonsense SNP rs199695765 is in exon 2, resulting in an early termination in the propeptide region (**Figure 4-8A**). Among 4379 patients tested, the two who carried this variant developed severe pancreatitis within weeks of their first dose of the drug (**Figure 4-8B**), suggesting that asparaginase will not be tolerated long enough to be effective in these rare patients. Whether the MAF (0.023%) in this ALL cohort was comparable to or higher than the MAF in the general population (0.009%) is not clear, but in any case, it is rare. In addition to rs199695765, there were 15 other variants in *CPA2* associated with pancreatitis (**Table C-4**). The 16 variants were carried by 25 of 4379 patients (collective MAF = 0.6%) with sequencing data, and all 10 patients who carried top 10 *CPA2* variants developed pancreatitis. These data suggest that *CPA2* variant carriers may be at unacceptably high risk of pancreatitis if challenged with asparaginase. ALL treatment regimens vary substantially in the amount of asparaginase they include, and many “low asparaginase” regimens achieve comparable outcomes to “high asparaginase” regimens. Selecting an ALL treatment regimen to exclude asparaginase may be preferable for these rare individuals.

The underlying mechanism by which *CPA2* variants confer a higher risk of acute pancreatitis is unclear. Pancreatic carboxypeptidases are zinc metalloenzymes that hydrolyze C-terminal amino acids. Two types of carboxypeptidases, A-type (CPA) and B-type (CPB) are both secreted from pancreatic acinar cells as pro-enzymes, then cleaved and activated by trypsin in the duodenum. There are two isoforms of CPA in humans, CPA1 and CPA2, which have significant similarity in amino acid sequence and three-dimensional structure.¹⁶⁸ Interestingly, patients heterozygous for functionally impaired variants in a related isoform, *CPA1*, are at higher risk of non-alcoholic chronic

pancreatitis.⁸⁰ It was suggested that the mechanisms may involve misfolding-induced endoplasmic reticulum stress in pancreatic acinar cells, rather than premature activation of trypsin.⁸⁰ It is possible that similar mechanisms may underlie how *CPA2* variants predispose to acute pancreatitis.

The top ranked gene based on sequencing of 283 pharmacogenomic candidate genes was *HOGA1*, an aldolase that catalyzes the final step in the metabolic pathway of hydroxyproline, releasing glyoxylate and pyruvate.¹⁶⁹ Only one patient of 4379 studied carried the nonsense variant chr10:99361676 C>T (R255X). Reduced enzymatic activity of *HOGA1* may cause calcium oxalate urolithiasis;¹⁷⁰ perhaps a similar cytosolic excess of oxalate could lead to pancreatitis, as pancreas is a potential site of hydroxyproline degradation.¹⁷¹

Whereas variants in *CPA2* and *HOGA1* were rare, the genome-wide analysis of common variants (**Table 4-6**) also provided some insights into other possible predisposing mechanisms. The lead gene *FHIT* (fragile histidine triad protein) is involved in purine metabolism.¹⁶⁹ Because thiopurines are essential to ALL therapy and can also induce acute pancreatitis,^{150,151} this finding may link thiopurines to some cases of pancreatitis in our study.

Other top genes (*DOCK5*, *ACTN2* and *MICAL2*) clustered in cytoskeleton function, supporting the hypothesis that the cytoskeleton may be involved in the pathogenesis of pancreatitis.^{172 173} Interruption of cytoskeleton and pancreatic enzyme secretion has been seen in animal models of drug-induced pancreatitis¹⁷² and in patients with acute pancreatitis.¹⁷⁴ It has been well established that exocytosis highly depends on vesicle trafficking via cytoskeleton in all types of secretory cells including pancreatic acinar cells.^{175,176} There were also case reports of clinical pancreatitis following cytoskeleton inhibitors such as brentuximab vedotin¹⁷⁷ and taxanes.¹⁷⁸ Indeed cerulein, used as the primary chemical tool to initiate pancreatitis in animal models, causes cytoskeleton disorganization.¹⁷⁹ *DOCK5* (dedicator of cytokinesis 5) encodes a guanine nucleotide exchange (GEF) factor that activates small GTPases including Rho and Rac, which are key regulators of actin distribution and amylase secretion in pancreatic acinar cells.^{180,181} *ACTN2* (actinin-2) encodes an actin-binding protein that bundles actin filaments in multiple cell types and cytoskeleton frameworks.¹⁸² It has been reported that α -actinin played a putative role in regulated exocytosis by connecting the actin filaments to secretory vesicles.¹⁸³ Intriguingly, the interaction between actin and actinin can be affected by increased level of intracellular Ca^{2+} , which was also observed concurrently with secretion blockade has been implicated in the early pathogenesis of acute pancreatitis. *MICAL2* (molecule interacting with CasL protein 2) encodes a member of MICAL family that interacts with small GTPase Rab1, which plays a role in vesicle trafficking from the endoplasmic reticulum to the Golgi.¹⁸⁴ MICAL proteins also bind to actin, regulate cytoskeletal rearrangements and play important regulatory functions in membrane trafficking.¹⁸⁵ In addition, among the 42 genes that were putatively associated with pancreatitis or ranked the top in the GWAS (**Table 4-6**), 10 (24%) have been shown to be involved in cytoskeleton regulation and vesicle transportation. Together, these findings suggest that the addition to a role for highly penetrant rare genetic variants in

some cases, common variants affecting purines or cytoskeleton function may affect risk of pancreatitis.

Eight genes (e.g. *PRSSI*, *PRSS2*, *SPINK1*, *CTRC*, *CFTR*, *CASR*, *CPA1*, *CLDN2*) have been identified as risk factors for non-drug-induced pancreatitis. Most SNPs in these genes were not as strongly associated as the variants discovered using a genome-wide agnostic approach (**Table C-6**). Using gene-based approaches, none of these eight genes were among these with $P < 0.05$, using deep sequencing data (**Table C-5**). *HLA-DRB1*07:01* (tagged by SNP rs17885382) has been associated with thiopurine-induced pancreatitis⁷⁸ but we found no association with pancreatitis in our study ($P = 0.1$). These findings suggest different underlying mechanisms of asparaginase-induced versus other forms of pancreatitis. The fact that asparaginase-induced pancreatitis was dose-dependent (**Figure 4-3**), whereas thiopurine-induced pancreatitis was not,⁷⁸ also suggests different pathogenesis of pancreatitis caused by these two important antileukemic drugs.

Consistent with reports from other groups,^{47-49,65,186} older age was associated with higher risk of pancreatitis. The relationship was also observed in children who had pancreatitis with other etiologies, e.g. biliary, traumatic, hereditary or idiopathic pancreatitis,¹⁸⁷⁻¹⁸⁹ indicating an intrinsic pancreatic susceptibility that increases with age. It has been shown that serum amylase levels were positively associated with age in healthy children¹⁹⁰ and those who developed acute pancreatitis.¹⁹¹

Similar to previous studies,^{48,50} gender and ALL lineage did not seem to influence the risk of pancreatitis in this study. We observed similar pancreatitis rates using two different asparaginase formulations, which is consistent with some studies,^{27,49} but different from several earlier ones^{40,47,65} that PEG-asparaginase induced more pancreatitis than native *E.coli* asparaginase did. However, in those studies^{40,47,65} native *E. coli*-asparaginase was usually given at a lower dose (e.g. 6000 U/m² thrice weekly) comparing to that used in current practice (10000 U/m² thrice weekly or 25000 U/m² weekly) so the patients on native *E. coli* arm might have been exposed to less asparaginase than the equivalent dose given to those on PEG-asparaginase arm (typically at 2500 U/m² biweekly).

Although other agents (e.g. glucocorticoids and thiopurines) can cause pancreatitis, asparaginase is mostly likely the cause in most of our patients, due to its early onset (coinciding with asparaginase; **Figure 4-4**), and its relationship to asparaginase cumulative dose and duration^{49,50,52,53} (**Figure 4-3B and C**). The association with genetically defined ancestry is consistent with an important contribution of genetics in the development of pancreatitis (**Table 4-4, Figures 4-6 and 4-7**); for example, risk alleles for 16 of the top 20 common variants (**Table 4-6**) were present at a higher frequency (at $P < 0.05$) in the Hispanic and black groups than in whites and Asians. Notably, of the two carriers of the *CPA2* nonsense SNP at rs199695765, one was of European and one had high Native American ancestry.

Conclusion

In summary, this comprehensive study showed that older age, higher exposure to asparaginase, higher Native American ancestry or African ancestry were independent risk factors for pancreatitis in children and young adults treated for ALL. Rare nonsynonymous variants in *CPA2*, especially a nonsense variant rs199695765, were highly penetrant in their effect on risk of pancreatitis, and more common variants in genes critical to purine metabolism and cytoskeleton function were also associated with development of pancreatitis. Our findings are consistent with a mixed genetic architecture underlying serious adverse drug effects, wherein a combination of rare but highly penetrant and common but weakly penetrant genetic risk factors contribute to genetic risk. For the small number of patients carrying the highly penetrant *CPA2* nonsense variants, consideration should be given to treatment with a non-asparaginase containing ALL chemotherapy regimens.

CHAPTER 5. SUMMARY

In this study, we have discussed the major adverse effects of asparaginase in patients with pediatric ALL, including hypersensitivity, acute pancreatitis, and the potentiating effect on osteonecrosis.

Hypersensitivity is the most common dose-limiting event of asparaginase. We showed that measurements of serum antibodies against Elspar may be useful in clinical practice. Higher antibody levels were associated with higher risk of clinical allergy, and a greater attenuation of serum asparaginase activity. Osteonecrosis, an adverse event associated with glucocorticoids, was associated with lower anti-Elspar antibody levels (i.e. higher serum asparaginase activity).

Thus, we studied the effect of asparaginase on glucocorticoid-induced osteonecrosis using a murine model. We were able to recapitulate the clinical observation that asparaginase treatment added to dexamethasone was associated with more arteriopathy and osteonecrosis in mice than dexamethasone treatment alone. The drug interaction confirmed in this preclinical study may be clinically important, as glucocorticoids and asparaginase are concomitantly used in the treatment ALL.

Acute pancreatitis is one of the serious adverse effects that may cause temporary or permanent withdrawal of asparaginase treatment. However, only a few large studies focused on clinical risk factors of pancreatitis, and there was previously no study of genetic risk factors. Here we performed a comprehensive study using a large cohort of pediatric patient with ALL and an independent case-control group. Age, asparaginase exposure, Native American ancestry or African ancestry were independent clinical risk factors. Rare nonsynonymous variants in *CPA2*, especially a nonsense variant rs199695765, were highly penetrant genetic risk factors. These findings may have implications for modifying therapy decisions in the future.

In summary, our studies have identified risk factors for adverse effects of asparaginase, yielded insight into the underlying mechanisms of drug response, and provided important guidance for precision medicine. Future work in our laboratory that builds on my work will include evaluating diagnostic tests to predict allergy to Oncaspar (the PEGylated form), further analyses of the mechanism by which Oncaspar contributes to osteonecrosis, and creation of transgenic mice bearing the *CPA2* variant to test for effects on pancreatitis preclinically.

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APPENDIX A. SUPPLEMENTARY INFORMATION FOR CHAPTER 2

Anti-asparaginase Antibody ELISA

Positive and negative controls

An initial ELISA was achieved by using one plasma sample from an ALL patient who recently had an allergic reaction. The antibody level of this sample had an OD value of 1.0 at a 1:10000 dilution. Using this plasma sample as a positive control, and normal human plasma as a negative control, we then tested 65 samples from six ALL patients who received Elspar, Erwinase or both formulations, and of whom one received Oncaspar. Subsequently, 21 samples (from six patients) with OD values greater than 1.0 at a 1:3200 dilution were selected for establishing an anti-asparaginase antibody reference pool. After combining these 21 samples, the antibody reference was re-analyzed and then aliquoted and stored at -80°C. Normal human plasma (NHP) samples were obtained from young individuals (students at UTHSC) who had never received asparaginase. Informed consent was obtained from patients according to Institutional Review Board guidelines and from students according to University of Tennessee Review Board guidelines.

Asparaginase antigens

Using this anti-asparaginase antibody reference pool, the optimal concentrations of Elspar (Merck & Co., Inc. Kenilworth, NJ), Erwinase (MRA/CAMR, Salisbury, UK) and Oncaspar (Enzon, Inc., Piscataway, NJ) commercial asparaginase preparations were standardized as distinct antigens. The ELISA was performed with these three antigens using separate well-coating concentrations ranging from 0.3125 to 40 µg/ml in the same plate. The antibody reference plasma was diluted 1:3200 and aliquoted to each well. NHP at 1:3200 (v/v) was used as a negative control. To ensure that the antigen completely saturated the plate, the optimal concentrations of the three coating antigens were chosen as the second concentration above that defining the plateau absorbance value.

Measurement of anti-asparaginase IgG antibodies by ELISA

Microplates (Nunc-Immuno plate, purchased from Thomas Scientific Product, Swedesboro, NJ) were coated with 100 µl of Elspar, Erwinase or Oncaspar commercial preparations diluted in 0.05 M carbonate/bicarbonate buffer, pH 9.5 (Sigma, St. Louis, MO) and incubated overnight at 2–8°C. The wells were drained without washing and blocked for at least 90 min at room temperature with 300 µl/well of a solution of 0.1 M phosphate-buffered saline (PBS), pH 7.2, containing 0.1% bovine serum albumin (Sigma, St. Louis, MO) and 0.05% Tween-20 (Sigma, St. Louis, MO). The plates were then washed three times with 0.05% Tween-20 in saline. For each asparaginase preparation, 100 µl of two-fold serial dilutions of the antibody reference, NHP, or plasma samples

with 0.05% Tween-20 in PBS were added, then incubated in appropriate wells for 1 h at room temperature. After incubation, the plates were again washed as described above. A polyclonal goat anti-human IgG horseradish peroxidase conjugate (specific for human IgG, but not for IgA, IgM, Bence Jones Kappa and Lambda myeloma proteins; purchased from Sigma, St. Louis, MO) was freshly diluted to 1:10000 (v/v). A 100- μ l volume of diluted conjugate was added to each well and incubated for 1 h at room temperature. The wells were drained, washed with Tween-20–saline four times, and incubated with 100 μ l of a freshly made substrate solution containing 20 mg of *o*-phenylenediamine dihydrochloride (OPD; purchased from Sigma, St. Louis, MO) in 50 ml of 0.1 M citrate buffer, pH 6.0, and 167 μ l of 3% H₂O₂ for 30 min at room temperature in the dark. After exactly 30 min, a 100- μ l aliquot of 1.0 M phosphoric acid solution was added to stop the reaction. Anti-asparaginase antibody concentrations were measured at 490 nm for the enzymatic product (subtracting the absorbance at 650 nm for non-specific absorbance) using a microplate reader (Model: THERMO max; purchased from Molecular Devices, LLC, Sunnyvale, CA). The cut-offs for positivity in plasma were determined as the mean plus two standard deviations of the OD values determined for plasma from healthy volunteers. Assays were performed in duplicates.

Normalization of Antibody Readings

Among the 2010 serum samples for anti-asparaginase antibodies measured by ELISA, 471 samples were assayed at UTHSC, 1345 samples were assayed at SJCRH, and 194 serum samples measured for antibody levels at both. The magnitudes of the scale of antibody OD readings obtained at UTHSC was significantly higher than those obtained at SJCRH (**Table A-1** and **Figure A-1**). Therefore, we normalized the OD readings based on a comparison of the antibody OD readings of the 194 serum samples measured at both facilities.

A nonlinear mixed model approach was used to find the correlation between SJCRH and UTHSC readings 194 serum samples. The correlation can be described by an exponential growth curve as **Equation A-1** (y is the SJCRH OD reading and x is the relative UTHSC OD reading), or **Equation A-2** (y' , x' and A_0' are natural logarithms of y , x and A_0).

$$y = A_0 \cdot \exp(A_1 \cdot x^k) \quad (\text{Eq. A-1})$$

$$y' = A_0' + A_1 \cdot \exp(kx') \quad (\text{Eq. A-2})$$

Figure A-2 shows the fitted curves for antibodies against three formulations (Elspar, Erwinase and Oncaspar). To maintain the consistency in range and threshold of the OD readings from two different facilities, the positive cut-off and the maximum OD reading were given more weight when adjusting the curve. The optimization of parameters was performed by R 2.8.0 using a nonlinear mixed model.

Table A-1. Comparison between antibody OD readings obtained at SJCRH (n = 1539) and UTHSC (n = 665)

Antigen	Facility	Positive cut-off ^a	Median (range)	Mean (SD) of positive readings	<i>P</i> ^b	Mean (SD) of negative readings	<i>P</i> ^b
Elspar	SJCRH	0.036	0.012 (0.003 – 1.512)	0.358 (0.312)	-	0.011 (0.007)	-
	UTHSC	0.275	0.017 (0.001 – 2.349)	1.109 (0.616)	2×10^{-16}	0.032 (0.058)	0.11
	UTHSC (norm)	0.037	0.010 (0.007 – 1.541)	0.377 (0.352)	0.99	0.011 (0.006)	0.15
Erwinase	SJCRH	0.086	0.017 (0.003 – 1.242)	0.210 (0.216)	-	0.022 (0.015)	-
	UTHSC	0.454	0.016 (0.001 – 1.387)	0.756 (0.362)	2×10^{-7}	0.031 (0.049)	5×10^{-5}
	UTHSC (norm)	0.083	0.015 (0.013 – 1.245)	0.326 (0.319)	0.23	0.016 (0.006)	3×10^{-8}
Oncaspar	SJCRH	0.018	0.007 (0.001 – 0.560)	0.103 (0.102)	-	0.007 (0.003)	-
	UTHSC	0.125	0.006 (0.001 – 1.596)	0.501 (0.363)	2×10^{-16}	0.012 (0.021)	4×10^{-6}
	UTHSC (norm)	0.018	0.007 (0.005 – 0.559)	0.091 (0.113)	0.16	0.007 (0.002)	5×10^{-5}

^aA sample assayed at SJCRH was considered positive if the natural log of its 1:400 OD reading was greater than 2.58 standard deviations above the negative control process mean. A sample assayed at UTHSC was considered positive if the natural log of its 1:3200 OD reading was greater than 2 standard deviations above the negative control process mean. Samples that were not positive were considered negative. The negative control reference pool was prepared from healthy volunteers who had never received asparaginase treatment. ^bP-values compared with SJCRH OD readings using the Wilcoxon rank sum test. SJCRH, St. Jude Children's Research Hospital. UTHSC, University of Tennessee Health Science Center. Norm, normalized.

Figure A-1. Anti-Elspar OD readings obtained at SJCRH (n = 1539) and UTHSC (n = 665) relative to the time of clinical reaction

Natural logarithm of anti-Elspar OD level in patient samples obtained at SJCRH (blue triangles and red circles) and UTHSC (black triangles and green circles) relative to the number of days elapsed before (negative) or after (positive) between sample collection and clinical reaction to Elspar. SJ, St. Jude Children's Research Hospital. UT, University of Tennessee Health Science Center. Pos, positive. Neg, negative.

Figure A-2. Fitted curves for (A) anti-Elspar, (B) anti-Erwinase and (C) anti-Oncaspar OD readings of the samples (n = 194) measured at both SJCRH and UTHSC

The positive cut-off (blue circle with cross and blue arrow), the maximum OD value (red circle and red arrow) and the fitted line are shown.

Figure A-2. (Continued)

For anti-Elspar antibody, the (y', x') for the positive cut-off is $(-1.297, -3.326)$, and the (y', x') for the maximum OD reading is $(0.854, 0.414)$. $A_0' = -5.066$, $A_1 = 3.509$, $k = 0.526$ (**Figure A-2A**).

For anti-Erwinase antibody, the (y', x') for the positive cut-off is $(-0.786, -2.448)$; and the (y', x') for the maximum OD reading is $(0.327, 0.216)$. $A_0' = -4.360$, $A_1 = 3.518$, $k = 0.806$ (**Figure A-2B**).

For anti-Oncaspar antibody, the (y', x') for the positive cut-off is $(-2.080, -4.046)$; and the (y', x') for the maximum OD reading is $(0.468, -0.580)$. $A_0' = -5.280$, $A_1 = 3.684$, $k = 0.520$ (**Figure A-2C**).

After normalization, the thresholds of antibody positivity and the ranges (especially for the positive OD readings) are consistent between two facilities (**Table A-1** and **Figure A-3**).

Figure A-3. UTHSC OD readings before and after normalization

The OD readings of anti-Elspar (top row), anti-Erwinase (middle row) and anti-Oncaspar (bottom row) are plotted by antibody positivity calls made by UTHSC (n = 665) or SJCRH (n = 1539). The antibody positivity thresholds are shown. Left column: OD readings obtained at UTHSC before normalization. Middle column: OD readings obtained at UTHSC after normalization. Right column: OD readings obtained at SJCRH.

Figure A-4. Determination of AUC threshold

The AUC threshold was greater than 2.12 (OD value $\times$ days), which corresponds to 99% confidence interval (CI) or 2.58 standard deviations above the mean value of negative cases (i.e., the antibody AUCs of the patients negative for anti-Elspar antibody consistently during therapy).

Table A-2. Sensitivity, specificity and predictive values of anti-Elspar antibody test at week 7 for predicting or confirming clinical reactions to Elspar using current positivity cutoff and optimized cutoff by recursive partitioning

Risk arm	N of patients	Time of Ab test	Time of Rxn	Time between test and rxn (weeks)	Cutoff OD value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Current positivity cut-off									
LR	185	Week 7	Weeks 7-9	0-3	0.037	87	68	73	85
SHR	190	Week 7	Weeks 1-6	0-6	0.037	88	69	51	94
Optimized cut-off by recursive partitioning									
LR	189	Week 7	Weeks 7-9	0-3	0.055	83	74	74	83
SHR	174	Week 7	Weeks 1-6	0-6	0.050	89	71	50	95

SHR, standard/high-risk; LR, low-risk; PPV, positive predictive value; NPV, negative predictive value; Ab, anti-Elspar antibody; Rxn, clinical allergic reaction; Week 7, week 7 of continuation therapy (23 weeks from diagnosis of ALL).

Figure A-5. Week-7 optimal threshold of OD value to predict or to confirm clinical reactions

Left: anti-Elspar OD readings of patients who had allergy (red circles) and those who did not (black circles) at week 7 were plotted against the days from start of therapy. Current antibody positivity threshold (black line) and optimized threshold (blue line) are shown. Right: the thresholds on ROC curves. The optimal thresholds to predict week 7 reactions (A and C) or to confirm week 1-6 reactions (B and D) were determined by recursive partitioning. SHR, standard/high-risk; LR, low-risk; PPV, positive predictive value; NPV, negative predictive value; Ab, anti-Elspar antibody; Rxn, clinical allergic reaction; Week 7, week 7 of continuation therapy (about 23 weeks from diagnosis of ALL).

Figure A-6. Elspar has stronger cross-reactivity with Oncaspar than with Erwinase

OD readings of anti-Elspar were plotted against (A) anti-Erwinase and (B) anti-Oncaspar for each sample (n = 3597). Regression lines and R-squared values are shown.

Figure A-7. Sensitivity of antibody tests within 30 days of reactions

Sensitivity of antibody tests to (A) predict future reactions (n = 108) or (B) confirm past reactions (n = 59) were plotted against the number of days within which clinical reactions occurred. Sensitivity was calculated as (number of patients who were positive for antibody within n days of the reaction)/(number of patients who had reactions in n days).

APPENDIX B. SUPPLEMENTARY INFORMATION FOR CHAPTER 3

Mouse Model of Dexamethasone-induced Osteonecrosis

We previously showed that BALB/cJ had a higher frequency of osteonecrosis with acceptable survival rates than BALB/cAnN.¹³⁷ Multiple risk factors of osteonecrosis were evaluated using BALB/cAnNHsd and BALB/cJ mice, including host-related factors such as age, gender, substrain and source of mice, and treatment-related factors such as dexamethasone dose and treatment duration.

Methods

Chemicals

The chemicals used for treatment have been described in Chapter 3. For dexamethasone and corticosterone measurements, corticosterone and ammonium acetate (98%, v/v in water) were purchased from Sigma (St. Louis, MO); d4_dexamethasone was from CDN Isotopes Inc. (Pointe-Claire, Quebec, Canada); acetonitrile was of HPLC grade and obtained from Burdick & Jackson (Muskegon, MI).

Animals

All animal work conducted for this study was approved by the Institutional Animal Care and Use Committee (IACUC) at SJCRH and performed according to NIH guidelines. Male and female BALB/cJ mice (age 21 or 24 days) were shipped from Jackson Laboratories (Bar Harbor, ME), or obtained from our own breeding facilities (SJCRH, Memphis, TN). Male BALB/cAnNHsd mice (age 21 or 24 days) were obtained from Harlan Laboratories (Houston, TX). Vendor-derived mice that were 21 days old were shipped with foster mothers during transport due to the young age of the mice.

Before starting the experiment, both in-house bred and vendor-derived mice were transferred into a separate experimental room where they were maintained in sterile micro-isolator cages (Micro Vent System 75 JAG, Allentown, Allentown, NJ) and housed in ventilated racks, with up to 5 mice in a cage with corncob bedding (Andersons Bed-O'cobs, Pharmaserv, Framingham, MA). Irradiated folic-acid deficient diet (purchased from TestDiet, Richmond, IN) that contained less than 0.05 ppm folic acid was used⁹⁶. The mice had access to food and water ad libitum. Indirect health surveillance was accomplished with ICR sentinel mice (from Jackson Lab) by dirty-bedding exchange weekly. The sentinels are tested quarterly and were found to be free of Sendai virus, mouse parvovirus, minute virus of mice, mouse hepatitis virus, Theiler murine encephalomyelitis virus, epizootic diarrhea of infant mice, pneumonia virus of mice, reovirus, K virus, polyoma virus, *Mycoplasma pulmonis*, lymphocytic

choriomeningitis virus, mouse adenovirus, ectromelia virus, and ecto- and endoparasites. Health checks were done twice a day at a minimum and those animals that became moribund or lost 20% of their maximum body weight were immediately euthanized according to IACUC-approved procedures.

Treatments

Treatment started at postnatal day 28 (P28) or day 24 (P24). Dexamethasone was given at 4 or 8 mg/L in drinking water, equivalent to 1.33 or 2.66 mg/kg/day, assuming that each mouse weighed 15 g and consumed 5 mL water daily.¹³⁷ Water bottles were changed every 3.5 days. Treatment generally lasted for 6 weeks, while a subset of BALB/cAnN was treated for up to 8 weeks in an effort to increase the frequency of osteonecrosis.

Antibiotic prophylaxis to prevent dexamethasone-induced infections consisted of tetracycline (1 g/L) constantly, and sulfamethoxazole (600 mg/L) and trimethoprim (120 mg/L) given 3.5 days per week. These antibiotics have no effect on osteonecrosis.⁹⁶ Mice in dexamethasone groups were treated with dexamethasone with antibiotics, except in a pilot experiment where mice were given dexamethasone without antibiotics. The control groups were treated with only antibiotics.

Enteric and blood cultures

Enteric aerobic cultures were done on a single mouse per cage at the beginning and the end of experiments, and blood aerobic cultures were performed on moribund mice to determine the pathogens causing sepsis. The procedures have been described previously.¹³⁷

Measurement of plasma concentration of dexamethasone and corticosterone

An assay was developed and validated to simultaneously quantify dexamethasone and corticosterone in mouse plasma by using high-performance liquid chromatography (HPLC) with tandem mass spectrometric (MS) detection.

To 10 μ L of sample, 50 μ L of internal standard (IS; 50 ng/mL isotopically labeled d₄-dexamethasone in acetonitrile) was added and vortexed for 15 min at 1700rpm using a DVX-2500 multi-tube vortexer (purchased from VWR, Radnor, PA). After centrifugation at 16000g for 8 min at 4°C, the supernatant was transferred to an autosampler vial and 3 μ L of the solution was injected onto the HPLC-MS system. The chromatographic separation was achieved by using a Waters (Milford, MA) UPLC with Waters ACQUITY BEHC₁₈ 1.7 μ m, 100 x 2.1 mm column, and ACQUITY in-line filter as guard column. The column was maintained at 40°C $\pm$ 5 °C and autosampler at 15 ° $\pm$ 5 °C. The mobile phase was generated by mixing 10mM ammonium acetate in H₂O with acetonitrile. The

flow rate was 0.7 mL/min. The linear gradient started at time zero from 30% acetonitrile and 70% buffer, then changed to 70% acetonitrile and 30% buffer in 1.2 min, and returned to the starting condition in a 0.01-minute step, followed by a 1.79 min held for equilibrium. The injection interval was 3.2 min. Detection of analytes was performed with a Waters TQD mass spectrometer operated in the positive multiple reaction monitoring (MRM) mode using Masslynx 4.1 software. The following mass ions (m/z) were used for detection: m/z 393.10>373.03 for dexamethasone; m/z 347.10>120.96 for corticosterone, and m/z 397.10>377.09 for IS. The following tune parameters were retained for the optimal detections: capillary voltage, 0.5 kV; cone voltage, 20 V for dexamethasone, 34 V for corticosterone, and 22 V for IS; source temperature, 150°C; desolvation temperature, 450°C; cone gas flow, 5 L/h; desolvation gas flow, 800 L/h and collision energy, 8 eV for Dexamethasone, 30 eV for corticosterone, and 10 eV for IS.

Standards were prepared in stripped mouse plasma over the linear ranges of 2 - 500 ng/ml and 4 - 1000 ng/ml for dexamethasone and corticosterone respectively. The interday and intraday imprecision of dexamethasone at 6, 80 and 400 ng/mL and corticosterone at 15, 80 and 800 ng/mL was within 8.9% and the accuracy was between 98.3% and 114%. The limit of quantitation (LOQ) was 5nM (2 ng/mL) for dexamethasone and 11.5 nM (4 ng/mL) for corticosterone. At the LOQ, the interday and intraday imprecision was within 14% and accuracy was greater than 88%. No interference from endogenous compounds was observed.

Results

Body weight differed by substrains

On P28, the mean weight of vendor-derived BALB/cAnNHsd males (16.3 ± 1.2 g) was significantly higher than those of vendor-derived BALB/cJ males (13.6 ± 1.5 g, $P < 0.0001$; **Figure B-1**), as we reported.¹³⁷ Vendor-derived BALB/cJ males were also slightly smaller ($P = 0.07$) than those raised in-house (14.7 ± 1.9 g). After 6-weeks of dexamethasone treatment, there was no difference in body weight of the three colonies (17.5 ± 1.4 g, 17.3 ± 1.8 g and 17.4 ± 1.3 g, respectively; $P = 0.95$). As expected, weights of dexamethasone-treated mice (17.4 ± 1.5 g) were significantly lower than those of untreated controls (26.8 ± 2.3 g; $P < 0.0001$) at the end of treatment.

Effect of substrain, source, age and treatment regimens on survival and development of osteonecrosis

Osteonecrosis was observed as early as 4 weeks on therapy in BALB/cJ mice, and 6 weeks in BALB/cAnNHsd mice, at all tested dexamethasone doses and ages of mice at the start of therapy. The incidence of osteonecrosis tended to increase with longer treatment duration in both strains (**Figure B-2**). Among all the mice with osteonecrosis,

Figure B-1. Body weight of mice before and after treatment

Body weight of BALB/cAnNHsd and BALB/cJ (vendor-derived and in-house bred) male mice at the beginning of treatment (P28, Wk-0) and after completion of 6-week therapy (Wk-6) with dexamethasone at 4 mg/L (DEX) and their untreated controls (CTRL). # $P < 0.001$, ## $P < 0.0001$.

Figure B-2. Incidence of osteonecrosis increased with longer treatment duration BALB/cAnNHsd and BALB/cJ male mice from 6 experiments combined in which they were treated beginning on postnatal day 24 or 28 with either 4 or 8 mg/L dexamethasone. Evaluation of osteonecrosis at 6 weeks or later indicates that a cohort of mice had completed therapy, whereas evaluation of osteonecrosis before 6 weeks occurred because mice had to be euthanized due to morbidity (usually sepsis), and bones were evaluated at the time of sacrifice. Number of mice positive for osteonecrosis and total number mice sacrificed at each time point are shown if at least 5 mice were sacrificed at the same time point.

the severity of osteonecrotic lesions was similar in mice of different substrain, age and treatment groups (**Figure B-3**).

After 6 weeks of treatment with a low dose of dexamethasone (4 mg/L), 28-days-old BALB/cAnNHsd mice did not develop any osteonecrosis. However, BALB/cJ mice of the same age had a higher incidence of osteonecrosis (23.7% vs. 0%, $P = 0.029$; **Table B-1**) and significantly lower survival rate (55% vs. 100%, $P = 0.025$; **Figure B-4A**) on the same dexamethasone regimen. These results are consistent with our prior report that the BALB/cAnNHsd substrain was more resistant to dexamethasone toxicity than the BALB/cJ substrain.¹³⁷

We also tested whether earlier start of therapy, higher dexamethasone dose and longer treatment duration increased dexamethasone toxicity in mice, as indicated by higher incidence of osteonecrosis and lower survival rate. Earlier start of treatment (P24 vs. P28) led to a minor increase in the incidence of osteonecrosis and a significant lower survival rate in both strains (**Table B-1** and **Figure B-4B**). Dexamethasone showed a dose-dependent toxicity in both strains. When the dose of dexamethasone was increased from 4 mg/L to 8 mg/L, the incidence of osteonecrosis increased from 6.7% to 35% at 6 weeks of therapy in P24 BALB/cAnNHsd mice. Similarly, a high loading dose of 8 mg/L in the first week increased the incidence of osteonecrosis from 23.7% to 40.9% in P28 vendor-derived BALB/cJ mice at 6 weeks, and also significantly reduced the survival (28% vs. 55%, $P = 0.016$; **Figure B-4A**), compared to those treated with 4 mg/L without a loading dose. Finally, longer treatment duration seems to slightly increase the incidence of osteonecrosis in P24 BALB/cAnNHsd mice (45% at 8 weeks vs. 35% at 6 weeks).

Blood cultures on moribund mice were positive for intestinal bacteria (e.g. *Enterococcus faecalis*), indicating that translocation of intestinal bacteria was responsible for sepsis-related morbidity in these dexamethasone-induced immunosuppressed mice. Antibiotics were necessary to decrease the incidence of sepsis, as a small group of BALB/cJ mice receiving dexamethasone without prophylactic antibiotics all had to be sacrificed due to sepsis within two weeks (**Figure B-4A**).

An interesting observation was that BALB/cJ mice derived from in-house breeding had significantly better survival (75% vs. 55%, $P = 0.016$; **Figure B-4A**) and higher incidence of osteonecrosis (49.5% vs. 23.7%, $P = 0.025$; **Table B-1**) than did those shipped from the vendor. To determine whether the difference in sepsis-related morbidity was associated with different gut flora in the two colonies, we identified bacterial isolates by enteric aerobic culture at the start and at the end of therapy, and tested their antimicrobial sensitivity (**Table B-2**). At the start of therapy, isolates from both colonies (e.g. *E. faecalis*, *Staphylococcus xylosus* and *Lactococcus lactis*) were sensitive to the antibiotics (tetracycline and trimethoprim) used in our experiments. At the end of therapy, vendor-derived mice developed resistant *E. faecalis* at the end of therapy, while all isolates cultured from the in-house bred mice before and after therapy were sensitive to trimethoprim. Resistant *E. faecalis* was also present in the blood of moribund vendor-derived BALB/cJ mice.

Figure B-3. Similar histologic appearance of osteonecrosis in mice of different substrain, age and treatment groups

Normal stifle joint from (A) an untreated vendor-derived BALB/cJ mouse, and osteonecrotic joints from (B) P24 BALB/cAnNHsd on 8 mg/L; (C) P28 vendor-derived BALB/cJ on 4 mg/L; (D) P28 vendor-derived BALB/cJ on 8 mg/L for the first week and 4 mg/L thereafter; (E) P24 vendor-derived BALB/cJ on 8 mg/L for the first week and 4 mg/L thereafter; (F) P28 in-house bred BALB/cJ on 4 mg/L dexamethasone. All mice were treated for 6 weeks. Magnification: 4 $\times$.

Table B-1. Incidence of ON in male BALB/c mice from different experiments

Substrain	Source	Age	DEX	Treatment duration (weeks)	Frequency of ON^a (n positive/n analyzed)
BALB/cAnNHsd	Vendor	P28	4 mg/L	6	0% (0/20)
BALB/cAnNHsd	Vendor	P24	4 mg/L	6	6.7% (1/15)
BALB/cAnNHsd	Vendor	P24	8 mg/L	6	35.0% (7/20)
BALB/cAnNHsd	Vendor	P24	8 mg/L	8	45.0% (9/20)
BALB/cJ	Vendor	P28	4 mg/L	6	23.7% (6/26)
BALB/cJ	Vendor	P28	8/4 mg/L ^b	6	40.9% (9/22)
BALB/cJ	Vendor	P24	8/4 mg/L	6	45.4% (5/11)
BALB/cJ	In-house	P28	4 mg/L	6	49.5% (48/97)

BALB/cAnNHsd mice were shipped from vendor (Harlan Lab). BALB/cJ mice were shipped from vendor (Jackson Lab) or bred in-house (SJCRH). All mice received dexamethasone alone (DEX; 4 or 8 mg/L in drinking water). ^aOsteonecrosis (ON) was evaluated in mice treated for at least 4 weeks. ^bDexamethasone was given at 8 mg/L for the first week and 4 mg/L thereafter. P28, postnatal day 28. P24, postnatal day 24.

Figure B-4. Survival differed by substrains, sources, regimens and age of mice
(A) Kaplan-Meier curves of vendor-derived BALB/cJ, in-house bred BALB/cJ, and vendor-derived BALB/cAnNHsd male mice treated with different dexamethasone regimens beginning on postnatal day 28. BALB/cJ mice were given either 4 mg/L throughout the 6-week treatment period (low-dose), or 8 mg/L for the first week and 4 mg/L thereafter (high-dose). All BALB/cAnNHsd mice were on the low-dose regimen. Note that all mice except a small group ($n = 4$) received prophylactic antimicrobials to prevent infection. (B) Kaplan-Meier curves of vendor-derived BALB/cJ and BALB/cAnNHsd males with dexamethasone treatment beginning on postnatal day 28 (solid lines) or 24 (dashed lines). BALB/cJ mice received high-dose regimen, and BALB/cAnNHsd mice received low-dose regimen.

Table B-2. Enteric culture isolates from BALB/cJ mice from vendor and in-house breeding colony

Time	Vendor-derived			In-house bred		
	Isolate	TCN sensitivity	TMP sensitivity	Isolate	TCN sensitivity	TMP sensitivity
Week 0	<i>Enterococcus faecalis</i>	R	S	<i>Enterococcus faecalis</i>	S	S
	<i>Staphylococcus xylosus</i>	S	S	<i>Lactococcus lactis</i>	S	R
	<i>Staphylococcus epidermidis</i>	S	R	<i>Escherichia coli</i>	S	S
Week 6	<i>Enterococcus faecalis</i>	R	R	<i>Enterococcus faecalis</i>	R	S
	<i>Enterococcus gallinarum</i>	R	S	<i>Enterococcus gallinarum</i>	R	S

The isolates and their sensitivity to tetracycline (TCN) and trimethoprim (TMP) before the start of therapy (Week 0) and after completion of therapy (Week 6) are shown. R, resistant; S, sensitive.

Dexamethasone pharmacokinetics and pharmacodynamics

To investigate the pharmacokinetics and pharmacodynamics of dexamethasone in mice, we measured plasma concentration of dexamethasone and corticosterone in BALB/cAnNHsd and BALB/cJ males on the same dexamethasone regimen (4 mg/L). Plasma dexamethasone levels were significantly higher in in-house bred BALB/cJ mice than in vendor-derived BALB/cJ ($P = 0.04$) and BALB/cAnNHsd mice ($P = 0.0002$; **Figure B-5A**). This is consistent with the high incidence of osteonecrosis in in-house bred BALB/cJ mice (**Table B-1**). Furthermore, dexamethasone levels were higher in mice with osteonecrosis than those without osteonecrosis ($P = 0.013$; **Figure B-5B**). These plasma levels are comparable to pediatric ALL patients receiving 8-12 mg/m² dexamethasone.³⁰ As a marker of dexamethasone pharmacodynamic effect, plasma corticosterone was suppressed in all dexamethasone-treated mice compared with untreated controls (**Figure B-6**).

Gender-dependent difference in susceptibility to osteonecrosis

After 6 weeks of treatment with dexamethasone (4 mg/L), the survival of in-house bred BALB/cJ mice did not differ significantly between males and females, but tended to be lower in males (75% vs. 80%, $P = 0.3$; **Figure B-7A**). However, only 5 of 78 (6.4%) females developed osteonecrosis, whereas 48 of 97 (49.5%) males developed osteonecrosis ($P < 0.0001$; **Figure B-7B**). Plasma concentrations of dexamethasone were comparable between male mice (61.4 ± 37.5 nM) and female mice (69.3 ± 39.7 nM, $P = 0.1$; **Figure B-7C**), and corticosterone was completely suppressed (<11.5 nM) in both male and female mice. Thus, the gender-dependent difference in susceptibility of osteonecrosis cannot be attributed to different pharmacokinetics or pharmacodynamics of dexamethasone.

Discussion

This is the first study to evaluate the effect of multiple risk factors on development of glucocorticoid-induced osteonecrosis in a mouse model. We report that earlier age of treatment (P24 vs. P28), male sex, higher dose of dexamethasone, longer treatment duration and higher plasma dexamethasone levels were associated with osteonecrosis in BALB/c mice, an osteonecrosis -susceptible strain we reported previously.⁹⁶ With phenotypes such as osteonecrosis that require prolonged treatment with immunosuppressive medications, it is a challenge to derive regimens that induce the target phenotype (osteonecrosis) without causing undue “off-target” toxicities (such as sepsis). Understanding the myriad factors that contribute to desired and undesired drug effects in this mouse model is thus critical.

Age has been known to be a critical risk factor for osteonecrosis in ALL patients. Clinical studies reported that adolescents (10-20 years old) were at higher risk than

Figure B-5. Plasma concentrations of dexamethasone

Dexamethasone concentrations measured at the time of euthanasia in BALB/cJ males (vendor-derived vs. in-house bred) treated with dexamethasone (DEX) administered at 4 mg/L.

Figure B-6. Inverse association between plasma corticosterone and dexamethasone levels

Individual data points are shown for all male BALB/cAnNHsd and BALB/cJ mice treated with 0 or 4 mg/L dexamethasone at the time of euthanasia (n = 167).

Figure B-7. Gender-dependent differences in susceptibility to osteonecrosis
 (A) Kaplan-Meier curve of in-house bred BALB/cJ males and females treated with dexamethasone at 4 mg/L. (B) Frequency of osteonecrosis in male and female mice treated with dexamethasone at 4 mg/L for 4-6 weeks. (C) Plasma dexamethasone concentration in in-house bred BALB/cJ males and females treated with dexamethasone at 4 mg/L.

children less than 10 years old^{88-92,126,128,134-136} and adults over 20 years old.⁹² It is hypothesized that hormonal and physiologic changes during puberty, and the skeletal maturation, may contribute to their susceptibility to osteonecrosis¹⁹². The impact of age on the development of osteonecrosis was recapitulated in our mouse model. We have determined that the onset of puberty in BALB/c males occurs at postnatal 28 ± 1 days (data not published). In a pilot study, osteonecrosis was mostly observed in mice that started treatment between 4 and 8 weeks of age, with a decreasing incidence in older mice, regardless of dexamethasone dose and treatment duration (data not published). In the present study we showed that younger mice (24 days old) were slightly more susceptible to osteonecrosis than older mice (28 days old, **Table B-1**). We hypothesize that starting treatment right before onset of puberty probably maintained plasma dexamethasone at high levels for the whole pubertal period, resulting in an increased incidence of osteonecrosis in mice. However, earlier treatment significantly increased dexamethasone toxicity, and resulted in high mortality of BALB/cJ mice due to sepsis (**Figure B-4B**).

Strain- and substrain-specific differences in susceptibility to glucocorticoid-induced osteonecrosis are observed in mice and rats and can be attributed to different genetic backgrounds.^{96,137,193} An earlier study showed substrain-specific differences in survival and osteonecrosis incidence in a small group of vendor-derived BALB/cJ and BALB/cAnNHsd mice.¹³⁷ In this experiment we replicated these findings using a larger group of mice treated on multiple regimens. We found that BALB/cAnNHsd mice had higher body weight and better survival, and were also less susceptible to osteonecrosis than BALB/cJ, suggesting that BALB/cJ substrain, rather than BALB/cAnNHsd, may serve as an appropriate mouse model for studying glucocorticoid-induced osteonecrosis.

Here, for the first time, we showed that BALB/cJ mice shipped from a vendor (at 21-24 days of age) had different responses to dexamethasone compared with those from in-house breeding colonies. When treated on the same regimen, we observed higher mortality and lower incidence of osteonecrosis in BALB/cJ mice shipped from a vendor than those bred in our own facility. Our first hypothesis for the high mortality of vendor-derived mice is the stress caused by transportation and unfamiliar environment. The relationship between stress and disease susceptibility has been demonstrated in many animal models,^{194,195} and BALB/c was relatively stress-vulnerable among several strains.¹⁹⁶⁻¹⁹⁸ Olfe et al¹⁹⁹ also showed that transportation during the juvenile life caused a long-term stress-induced lymphocytopenia in vendor-derived BALB/c mice compared with those derived from in-house breeding. This is consistent with our observation that vendor-derived BALB/c mice had more sepsis-related morbidity. The other hypothesis is that mice shipped from a vendor vs. bred in-house would have different gastrointestinal flora, and differing microbial sensitivity to prophylactic antibiotics may also have contributed to the difference in survival rates. Lethal gut-derived sepsis was likely caused by resistant *E. faecalis*, which was isolated from enteric cultures from healthy vendor-derived BALB/cJ mice, and blood cultures from moribund vendor-derived BALB/cJ mice after dexamethasone. Our experiments suggested that in-house bred BALB/cJ males were most susceptible to osteonecrosis with acceptable toxicity in response to

dexamethasone treatment, and therefore may serve as an appropriate model for glucocorticoid-induced osteonecrosis.

As we expected, osteonecrosis was associated with higher dexamethasone exposure, including higher dose, longer treatment duration, and higher plasma concentration of dexamethasone (**Table B-1** and **Figure B-5B**). This is consistent with clinical observations that osteonecrosis was associated with intense glucocorticoid treatment and lower dexamethasone clearance,⁸⁸⁻⁹² As we reported previously,¹³⁷ the resistance to dexamethasone-induced osteonecrosis in vendor-derived BALB/cAnNHsd mice compared to vendor-derived BALB/cJ mice was not due to dexamethasone pharmacokinetics (**Figure B-5A**). However, in-house bred BALB/cJ mice had higher ($P = 0.04$) plasma dexamethasone levels than did either group of vendor-derived mice. The lower plasma dexamethasone concentration in vendor-derived mice was consistent with the lower incidence of osteonecrosis compared with those raised in-house (23.7% vs. 49.5%; **Table B-1**).

There are conflicting clinical data regarding the gender-dependent difference in risk of glucocorticoid-induced osteonecrosis in patients. Some studies reported that girls had higher risk than boys,^{88,89,91,134,136} but the difference was absent in several other studies.^{90,92,128} The incidence of osteonecrosis in mice, unlike the patients, was significantly higher among males than females after 6 weeks of treatment with 4 mg/L dexamethasone ($P < 0.0001$; **Figure B-7B**). There were no significant difference between males and females in the survival rates and plasma levels of dexamethasone and corticosterone, suggesting some intrinsic sensitivity to osteonecrosis (e.g. sex hormones, bone or vasculature properties) may predispose male mice to osteonecrosis.

Conclusion

In summary, we report that development of dexamethasone-induced osteonecrosis in mice was affected by multiple factors, including age, gender, dexamethasone dose, treatment duration, substrain and sources of mice. Four-week-old in-house bred BALB/cJ male mice displayed high susceptibility to osteonecrosis with an acceptable survival rate; therefore they may serve as a reliable and reproducible mouse model for studying glucocorticoid-induced osteonecrosis in the future.

Effect of Asparaginase Treatment on Dexamethasone Pharmacokinetics

Figure B-8. Plasma dexamethasone concentration was positively associated with asparaginase activity in osteonecrosis experiment

Mice received dexamethasone (DEX; 4 mg/L in drinking water) for 6 weeks (triangles) or dexamethasone and PEG-asparaginase (ASP; 1200 IU/kg i.p.) at 3.5 day intervals (12 doses in total) for 6 weeks (points). Samples were collected at the end of week 6. Each dot represents a mouse. Linear regression line is shown.

Effect of asparaginase and dexamethasone on plasma protein and lipid levels

Figure B-9. Mouse plasma amylase level after treatment with dexamethasone and asparaginase

Plasma amylase level was induced by dexamethasone treatment and suppressed by asparaginase treatment. Mice received dexamethasone (Dex; 4 mg/L in drinking water) for 2 weeks, or dexamethasone and PEG-asparaginase (Asp; 1500 IU/kg i.p.) at 3.5 day intervals for 1-4 doses, or PEG-asparaginase alone for 1 or 4 doses. Samples were collected at the end of week 2 (3.5 days from last asparaginase administration). The reference range of plasma amylase level (salivary and pancreatic) is 602-2311 U/L in mouse.

Figure B-10. Mouse plasma lipase level after treatment with dexamethasone and asparaginase

(A) Plasma lipase level was initially inhibited after asparaginase treatment, but then rebound over time. Mice received dexamethasone (Dex; 4 mg/L in drinking water) for 2 weeks, or dexamethasone and PEG-asparaginase (Asp; 1500 IU/kg i.p.) at 3.5 day intervals for 1-4 doses, or PEG-asparaginase alone for 1 or 4 doses. Samples were collected at the end of week 2 (3.5 days from last asparaginase administration). (B) Lipase was directly associated with triglyceride ($P = 9 \times 10^{-5}$).

Figure B-11. Mouse plasma triglyceride level after treatment with dexamethasone and asparaginase

Plasma triglyceride level was initially inhibited by asparaginase during the first week, but then increased to a level higher than normal range (reference range 55-144 mg/dl). Mice received dexamethasone (Dex; 4 mg/L in drinking water) for 2 weeks, or dexamethasone and PEG-asparaginase (Asp; 1500 IU/kg i.p.) at 3.5 day intervals for 1-4 doses, or PEG-asparaginase alone for 1 or 4 doses. Samples were collected at the end of week 2 (3.5 days from last asparaginase administration).

Figure B-12. Mouse plasma albumin level after treatment with dexamethasone and asparaginase

(A) Plasma albumin level was similar in all groups (Kruskal-Wallis $P = 0.10$). Mice received dexamethasone (Dex; 4 mg/L in drinking water) for 2 weeks, or dexamethasone and PEG-asparaginase (Asp; 1500 IU/kg i.p.) at 3.5 day intervals for 1-4 doses, or PEG-asparaginase alone for 1 or 4 doses. Samples were collected at the end of week 2 (3.5 days from last asparaginase administration). (B) No significant change in plasma albumin level with increased asparaginase activity ($P = 0.9$).

Figure B-13. Mouse plasma cortisol level after treatment with dexamethasone and asparaginase

Plasma cortisol level was induced by asparaginase treatment ($P = 0.026$ after 4 injections) and suppressed by dexamethasone. Mice received dexamethasone (Dex; 4 mg/L in drinking water) for 2 weeks, or dexamethasone and PEG-asparaginase (Asp; 1500 IU/kg i.p.) at 3.5 day intervals for 1-4 doses, or PEG-asparaginase alone for 1 or 4 doses. Samples were collected at the end of week 2 (3.5 days from last asparaginase administration).

Table B-3. Inhibition of plasma protein level in human and mouse by asparaginase treatment

Protein	Data source	Inhibition%	N of ASN/ total AA	ASN%	N of GLN/ total AA	GLN%	ASN+ GLN%
Human							
Prealbumin	Minowa et al ⁶²	45%	4/176	2.3%	4/176	2.3%	4.6%
Albumin	Haskell et al ²⁰⁰	27%	17/609	2.8%	20/609	3.3%	6.1%
Protein C	Saito et al ¹⁴⁸ Appel et al ²⁰¹	20% 44%	13/461	2.8%	15/461	3.3%	6.1%
Retinol-binding protein	Minowa et al ⁶²	40%	9/201	4.5%	8/201	4.0%	8.5%
Transferrin	Minowa et al ⁶²	48%	34/698	4.9%	17/698	2.4%	7.3%
Plasminogen	Appel et al ²⁰¹ Saito et al ¹⁴⁸	39% 58%	41/810	5.1%	31/810	3.8%	8.9%
Antithrombin	Merlen et al ²⁰² Hunault-Berger et al ²⁰³ Saito et al ¹⁴⁸	43% 50% 51%	24/464	5.2%	13/464	2.8%	8.0%
Fibrinogen	Merlen et al ²⁰² Hunault-Berger et al ²⁰³ Saito et al ¹⁴⁸	51% 52% 63%	11/211	5.2%	6/211	2.8%	8.0%
Protein S	Appel et al ²⁰¹	55%	44/676	6.5%	25/676	3.7%	10.2%

Table B-3. (Continued)

Protein	Data source	Inhibition%	N of ASN/ total AA	ASN%	N of GLN/ total AA	GLN%	ASN+ GLN%
Mouse							
Albumin	Mouse PK experiment	18%*	19/608	3.1%	23/608	3.8%	6.9%
Lipase	Mouse PK experiment	56%*	32/465	6.9%	18/465	3.9%	10.8%
Amylase	Mouse PK experiment	89%*	47/508	9.3%	13/508	2.6%	11.9%

Data were taken from mouse PK experiment (**Figure 3-1**) and literatures^{62,148,200-203} and were sorted by the content of asparagine (ASN) residues in the protein. The percentage of inhibition was calculated using the median or mean values of mice who did vs. did not receive asparaginase or of patients pre- vs. post-asparaginase treatment. Glucocorticoids were given concurrently. *Comparing the protein level of dexamethasone alone (4 mg/L for 2 weeks) group vs. the lowest protein level of any dexamethasone and asparaginase (1200 IU/kg i.p. at day 10.5) group. GLN, glutamine; AA, amino acid.

Figure B-14. Correlation between asparagine content in the protein and the extent of inhibition by asparaginase treatment

Data were taken from mouse PK experiment (**Figure 3-1**) and literatures^{62,148,200-203} (see **Table B-3**). The percentage of inhibition was plotted against the percentage of asparagine (ASN) residues in the protein. Median, range and linear regression line are shown.

APPENDIX C. SUPPLEMENTARY INFORMATION FOR CHAPTER 4

Deep Sequencing of Selected Genes

To identify rare and low-frequency variants in genes of interest for ALL-related phenotypes, we sequenced 283 genes. The genes were selected for sequencing based on their prior association with ALL phenotypes of interest (e.g. relapse, drug sensitivity, pharmacokinetics, leukemia risk, and adverse effects). Of these, 42 were putatively pancreatitis-associated genes: 34 were top genes from preliminary results of the current GWAS, and 8 were candidate genes associated with pancreatitis in other settings; **Table 4-3**). Illumina dual-indexed libraries were constructed using germline DNA from 4217 children in the cohort (including 97 cases with pancreatitis) and 162 in the case-control group (including 47 cases). These libraries were pooled in sets of 96 and hybridized with the Roche NimbleGen SeqCap EZ custom probes to capture the selected gene regions. Targeted regions for sequencing include all coding regions and putative functional regions within 10K base pairs of the transcripts including promoters and enhancers as predicted according to ENCODE/Roadmap Epigenomics data.²⁰⁴ In addition, we also sequenced the putative functional regions within the linkage disequilibrium block defined by the top GWAS SNPs. The titer of samples was determined by PCR, and the flow cell was sequenced on the Illumina HiSeq 2000 as a paired-end, multiplexed, 2×101 bp run. The pooled sample was then demultiplexed with coverage of $> 20\times$ depth across $> 90\%$ of the targeted regions. Burrows-Wheeler Alignment was performed on the sequence reads and GATK pipeline version 3.1 was used to call the genetic variants. The concordance between deep sequencing and arrays was greater than 98% for 3635 of the 3639 SNPs on both platforms.

SNP-based and Gene-based Analysis of Variants Identified by Deep Sequencing

After excluding the SNPs with a call rate less than 95% and a Variant Quality Score Recalibration (VQSR) score greater than 99.5%, we interrogated 9229 SNPs in 283 genes. Genotype calls were coded as 0, 1 or 2 for the number of B alleles (AA, AB or BB) assuming an additive genetic model. For patients with deep sequencing data in the cohort ($n = 4217$), GWAS was performed using a Cox proportional hazard model considering the onset time of pancreatitis (time-dependent GWAS). Covariates included age (continuous variable in years) and genetic ancestry (continuous variable as percentages of European, African, Asian and Native American ancestries) and treatment regimen (asparaginase dose ≤ 120000 U/m² vs. ≥ 240000 U/m²) coded as a categorical variable. For patients with pancreatitis, their time-at-risk was censored at the date of first episode of pancreatitis; for those without pancreatitis, their time-at-risk was censored at off-study date. In the case-control group ($n = 162$), cases and controls were matched for age and treatment regimen, and GWAS was performed using logistic regression, adjusted for genetic ancestry only (time-independent GWAS).

SNPs were assigned to genes according to Refseq boundaries downloaded from UCSC Genome Browser (hg19 assembly). For the 283 genes sequenced, we applied sequence kernel association test¹⁶¹ (SKAT) to test for gene level significance for the effects of nonsense variants. For time dependent analysis, we first obtained Martingale residuals as a continuous phenotype in the SKAT analysis after applying Cox proportional hazard model using demographic and clinical covariates, including age, genetic ancestry, and treatment regimen.

Figure C-1. CADD score in all SNPs on exome array (n = 162586) and pancreatitis-associated SNPs ($P < 0.05$; n = 8018)

Distribution of CADD score of SNPs associated ($P < 0.05$) with pancreatitis (n = 8018, gray bar) in the cohort vs. all SNPs on exome array with a CADD score (n = 162586, clear bar). The range, median, mean and quartile values are shown. Inset graph shows similar CADD score of pancreatitis-associated vs. non-pancreatitis SNPs. NS, not significant.

Table C-1. Top 10 SNPs from genome-wide analysis of nonsense SNPs; n = 2,357 SNPs

SNP	Gene	Chr	Location ^a	Major allele/ risk allele	Population RAF% (n) ^b	Cohort (n = 3256)				Case/control (n = 213)			
						RAF% in patients with AP (n = 95)	RAF% in patients without AP (n = 3161)	Coeff- icient ^c	P ^c	RAF% in cases (n = 71)	RAF% in controls (n = 142)	Coeff- icient ^d	P ^d
rs199695765	<i>CPA2</i>	7	129696084	C/T	0.009 (11)	0.5	0	6.6	1.5×10^{-8}	0.8	0	15.8	0.08
rs144556038	<i>ZNF701</i>	19	57777659	C/T	0.04 (46)	1.1	0.03	3.6	2.0×10^{-6}	0.8	0	15.1	0.17
rs116191233	<i>C17orf66</i>	17	31215928	G/A	0.2 (227)	0.5	0.02	3.3	0.001	0.8	0	15.5	0.12
rs79537388	<i>C9orf117</i>	9	129515133	C/T	0.2 (171)	1.6	0.2	1.7	0.005	0.8	0	15.3	0.17
rs113207856	<i>SLC26A10</i>	12	56304127	G/A	0.4 (515)	1.6	0.4	1.6	0.006	1.7	0	16.3	0.04
rs78283108	<i>SERPINA9</i>	14	94005381	G/A	0.6 (732)	2.7	0.7	1.2	0.015	0.8	0	15.1	0.17
rs1023840	<i>HEATR7B2</i>	5	41097472	C/T	22.6 (23995)	31.5	21.4	0.4	0.016	24.6	23.6	0.03	0.90
rs41463245	<i>CCL26</i>	7	75239199	C/T	0.9 (1053)	2.2	0.8	1.2	0.019	1.7	0	0.09	0.92
rs67047829	<i>ERV3-1</i>	7	64090173	G/A	10.8 (12129)	15.8	9.4	0.5	0.022	14.4	9.4	4.7	0.21
rs146053779	<i>GCKR</i>	2	27598876	C/T	0.09 (111)	1.1	0.3	1.6	0.024	0.8	0	15.5	0.75

The SNPs were rank ordered by P value in the cohort study, and then filtered by the direction in the case-control study.

^aPhysical location of SNP based on Feb 2009 human genome assembly (hg19). ^bPopulation risk allele frequency and number of carriers of the SNP based on the ExAC data set¹⁶⁶ of 61486 unrelated individuals of European, African, Latino and Asian origins. ^cCoefficient and P value of risk alleles were estimated using Cox proportional hazard model. ^dCoefficient and P value of risk alleles were estimated using logistic regression model. Chr, chromosome. AP, acute pancreatitis. RAF, risk allele frequency.

Table C-2. Top 10 SNPs from genome-wide analysis of missense SNPs (excluding nonsense SNPs); n = 144,349 SNPs

SNP	Gene	Chr	Location ^a	Major allele/ risk allele	Population RAF% (n) ^b	Cohort (n = 3256)				Case/control (n = 213)			
						RAF% in patients with AP (n = 95)	RAF% in patients without AP (n = 3161)	Coeff- icient ^c	P ^c	RAF% in cases (n = 71)	RAF% in controls (n = 142)	Coeff- icient ^d	P ^d
rs112141546	<i>ADAMTS18</i>	16	77401515	C/T	0.2 (225)	0.2	0.03	3.3	4.2×10 ⁻¹⁰	0.8	0	14.9	0.28
rs45461499	<i>CDC20</i>	1	43828736	G/A	0.05 (65)	1.1	0.2	4.6	3.6×10 ⁻⁹	0.8	0.4	1.1	0.46
rs200495769	<i>PDLIM5</i>	4	95494528	C/T	0.01 (12)	0.5	0.02	5.7	2.9×10 ⁻⁷	0.8	0.4	1.0	0.51
rs141708090	<i>C3orf20</i>	3	14803109	G/T	0.2 (243)	1.1	0.02	3.6	5.7×10 ⁻⁷	0.8	0	15.3	0.14
19:49564629 C/T	<i>NTF4</i>	19	49564629	C/T	0.01 (16)	0.5	0	5.1	7.0×10 ⁻⁷	0.8	0	15.3	0.14
rs201734206	<i>C8orf84</i>	8	73984100	C/T	0.04 (46)	0.5	0	4.9	2.0×10 ⁻⁶	0.8	0	15.3	0.14
rs202124287	<i>NEB</i>	2	152534216	C/T	0.06 (74)	0.5	0	4.8	3.8×10 ⁻⁶	0.8	0	15.4	0.13
rs143348702	<i>MICAL1</i>	6	109770636	T/C	0.02 (27)	0.5	0	4.6	9.9×10 ⁻⁶	0.8	0	16.0	0.07
rs147501499	<i>MYOM2</i>	8	2092718	C/T	0.2 (266)	0.5	0	4.5	1.2×10 ⁻⁵	0.8	0	14.6	0.24
rs141452852	<i>TAS2R16</i>	7	122635645	A/G	0.02 (24)	1.1	0.05	3.1	1.5×10 ⁻⁵	0.8	0	15.2	0.15

The SNPs were rank ordered by P value in the cohort study, and then filtered by the direction in the case-control study.

^aPhysical location of SNP based on Feb 2009 human genome assembly (hg19). ^bPopulation risk allele frequency and number of carriers of the SNP based on the ExAC data set¹⁶⁶ of 61486 unrelated individuals of European, African, Latino and Asian origins. ^cCoefficient and P value of risk alleles were estimated using Cox proportional hazard model. ^dCoefficient and P value of risk alleles were estimated using logistic regression model. Chr, chromosome. AP, acute pancreatitis. RAF, risk allele frequency.

Table C-3. Top 10 SNPs from genome-wide analysis of synonymous and non-coding SNPs; n = 774,238 SNPs

SNP	Gene	Chr	Location ^a	Function	Major allele/ risk allele	Population RAF% (n) ^b	Cohort (n = 3256)				Case/ctrl (n = 213)			
							RAF% in patients with AP (n = 95)	RAF% in patients without AP (n = 3161)	Coeff- icient ^c	P ^c	RAF% in cases (n = 71)	RAF% in controls (n = 142)	Coeff- icient ^d	P ^d
rs112141546	<i>HOGAI</i>	10	99349783	Syn	C/T	0.2 (242)	2.2	0.03	3.5	1.1×10 ⁻⁶	0.8	0	14.9	0.15
rs9849262	<i>FHIT</i>	3	60912089	Intron	C/T	2.7* (132)	4.7	1.0	1.4	8.4×10 ⁻⁶	0.7	0	13.7	0.18
rs12589386	-	14	46293715	-	A/G	8.3* (416)	8.5	3.7	2.8	1.8×10 ⁻⁵	9.4	3.2	1.0	0.03
rs142728074	<i>SPATA21</i>	1	16727294	Syn	G/A	0.07 (91)	1.1	0.05	3.3	2.5×10 ⁻⁵	0.8	0	14.7	0.23
rs6721376	-	2	240102585	-	C/T	2.9* (146)	3.8	0.9	0.2	5.0×10 ⁻⁵	2.2	0	16.7	0.02
rs17117423	-	14	46078262	-	C/T	9.5* (476)	9.1	4.2	0.4	5.0×10 ⁻⁵	8.7	2.9	1.3	0.02
rs10979693	<i>C9orf5</i>	9	110916644	Intron	A/C	2.6* (127)	2.1	0.4	5.8	6.4×10 ⁻⁵	0.7	0	13.7	0.39
rs9859201	<i>FHIT</i>	3	60910412	Intron	A/T	2.9* (145)	4.3	1.1	3.6	1.2×10 ⁻⁴	0.7	0	13.7	0.18
rs6724701	-	2	180968192	-	G/A	0.9* (46)	3.1	0.6	5.1	1.4×10 ⁻⁴	2.3	0	17.0	0.01
rs2010463	<i>MICAL2</i>	11	12142872	Intron	C/T	55.8* (2213)	61.1	44.7	0.6	2.0×10 ⁻⁴	54.5	34.6	0.6	0.003

The SNPs were rank ordered by P value in the cohort study, and then filtered by the direction in the case-control study. If the risk allele frequency is greater than 1% in the case-controls study, the respective P value should be less than 0.05. Population risk allele frequency and number of carriers of the SNP based on the ExAC data set¹⁶⁶ of 61486 unrelated individuals of European, African, Latino and Asian origins, and *population risk allele frequency and number of alleles based on 1000Genome phase 1 population (<http://www.1000genomes.org/node/506> [May 2011]) of 1088 individuals. ^cCoefficient and P value of risk alleles were estimated using Cox proportional hazard model. ^dCoefficient and P value of risk alleles were estimated using logistic regression model. AP, acute pancreatitis. Chr, chromosome. RAF, risk allele frequency. Syn, synonymous.

Table C-4. All 380 CPA2 variants identified in the cohort (n = 4217) and the case-control group (n = 162)

Chr:Loc ^a	RSID	Location	Function	Cohort (n = 4217)				Case-control (n = 162)			
				MAF% in patients with AP (n = 97)	MAF% in patients without AP (n = 3220)	Coefficient ^b	P ^b	MAF% in cases (n = 47)	MAF% in controls (n = 115)	Coefficient ^c	P ^c
7:129912621		Intron		0.52	0.00	6.1	5.00×10^{-10}	0.00	0.00	NA	NA
7:129917351	rs19187532	Intron		0.52	0.00	6.7	6.03×10^{-10}	0.00	0.00	NA	NA
7:129908848	rs199695765	Exon	Stop-gain damaging	0.52	0.00	6.1	1.38×10^{-8}	1.06	0.00	15.8	0.986
7:129908689		Intron		0.52	0.00	6.1	1.42×10^{-8}	0.00	0.00	NA	NA
7:129929042	rs7544262	Intron		0.52	0.00	4.7	4.65×10^{-6}	0.00	0.00	NA	NA
7:129916139		Intron		0.52	0.00	4.6	7.17×10^{-6}	0.00	0.00	NA	NA
7:129908835		Exon	Syn	0.52	0.00	3.9	1.12×10^{-4}	0.00	0.00	NA	NA
7:129915385		Intron		0.52	0.00	4.0	1.58×10^{-4}	0.00	0.00	NA	NA
7:129915649		Intron		0.52	0.00	3.5	9.56×10^{-4}	0.00	0.00	NA	NA
7:129915690		Intron		0.52	0.00	3.3	1.28×10^{-3}	0.00	0.00	NA	NA
7:129915313	rs18247836	Intron		0.52	0.01	2.9	4.21×10^{-3}	0.00	0.00	NA	NA
7:129915817		Intron		0.52	0.04	2.5	1.37×10^{-2}	0.00	0.00	NA	NA
7:129913443		Intron		0.52	0.01	2.3	2.05×10^{-2}	0.00	0.00	NA	NA
7:129929468	rs146796996	Exon	Nonsyn damaging	0.52	0.06	2.2	2.68×10^{-2}	0.00	0.00	NA	NA
7:129912224	rs15176341	Intron		0.52	0.02	2.1	3.68×10^{-2}	0.00	0.00	NA	NA
7:129917748	rs139875564	Exon	Nonsyn damaging	0.52	0.02	2.1	3.68×10^{-2}	0.00	0.00	NA	NA
7:129916325	rs4127417	Intron		21.35	19.88	0.3	5.24×10^{-2}	11.70	18.26	-0.4	0.342
7:129916008	rs73146775	Intron		21.13	19.81	0.3	5.74×10^{-2}	11.70	18.26	-0.4	0.342
7:129916960	rs1198355	Intron		21.13	19.82	0.3	5.77×10^{-2}	11.70	18.26	-0.4	0.342
7:129915538	rs11971847	Intron		21.13	19.83	0.3	5.89×10^{-2}	11.70	18.26	-0.4	0.342
7:129919105	rs12536877	Intron		21.13	19.83	0.3	5.95×10^{-2}	11.70	18.26	-0.4	0.342
7:129913198	rs1197851	Intron		20.10	18.81	0.3	6.43×10^{-2}	10.64	18.26	-0.5	0.243

Table C-4. (Continued)

Chr:Loc ^a	RSID	Location	Function	Cohort (n = 4217)				Case-control (n = 162)			
				MAF% in patients with AP (n = 97)	MAF% in patients without AP (n = 3220)	Coefficient ^b	P ^b	MAF% in cases (n = 47)	MAF% in controls (n = 115)	Coefficient ^c	P ^c
7:129912349	rs115716317	Intron		1.03	0.22	1.4	6.87×10^{-2}	0.00	0.00	NA	NA
7:129913993	rs14977797	Intron		1.03	0.41	1.3	7.05×10^{-2}	0.00	0.43	-13.3	0.988
7:129915816		Intron		0.52	0.11	1.8	7.20×10^{-2}	0.00	0.00	NA	NA
7:129917148	rs14223236	Intron		1.03	0.39	1.2	1.07×10^{-1}	0.00	0.43	-13.3	0.988
7:129929673	rs73146781	Downstream		20.10	19.71	0.3	1.28×10^{-1}	12.77	16.96	-0.2	0.656
7:129913520	rs125753	Intron		28.80	31.15	-0.3	1.31×10^{-1}	41.11	37.16	0.3	0.539
7:129913510		Intron		0.62	0.16	1.4	1.77×10^{-1}	0.00	0.00	NA	NA
7:129912168	rs6467292	Intron		28.87	26.58	0.2	2.18×10^{-1}	13.83	23.48	-0.5	0.147
7:129912145	rs111673838	Intron		28.87	26.60	0.2	2.22×10^{-1}	13.83	23.48	-0.5	0.147
7:129915016	rs61731658	Exon	Nonsyn	2.06	0.68	0.7	2.23×10^{-1}	0.00	0.87	-15.2	0.988
7:129912783	rs111374282	Intron		2.06	0.68	0.7	2.24×10^{-1}	0.00	0.87	-15.2	0.988
7:129912602	rs2242337	Intron		28.87	26.67	0.2	2.28×10^{-1}	13.83	23.48	-0.5	0.147
7:129921282	rs79737155	Intron		2.06	0.69	0.6	2.32×10^{-1}	0.00	0.87	-15.2	0.988
7:129916792	rs772521	Intron		2.06	0.70	0.6	2.37×10^{-1}	0.00	0.87	-15.2	0.988
7:129913193		Intron		0.52	0.33	1.1	2.68×10^{-1}	0.00	0.00	NA	NA
7:129916515	rs1759	Exon	Syn	67.01	62.79	0.2	2.87×10^{-1}	67.02	66.09	0	0.944
7:129916738	rs11537129	Intron		0.52	0.13	1.1	2.91×10^{-1}	0.00	0.00	NA	NA
7:129918302	rs139699694	Intron		0.52	0.13	1.1	2.91×10^{-1}	0.00	0.00	NA	NA
7:129913367	rs18269827	Intron		0.52	0.12	1.0	3.34×10^{-1}	0.00	0.00	NA	NA
7:129910463	rs2291457	Intron		32.47	33.99	-0.2	3.36×10^{-1}	51.06	40.79	0.4	0.179
7:129913535	rs376231	Intron		35.71	38.36	-0.5	3.94×10^{-1}	44.44	47.06	-1.1	0.522
7:129913113	rs14822868	Intron		0.52	0.18	0.8	4.32×10^{-1}	0.00	0.43	-14	0.987
7:129916709	rs13891471	Intron		0.52	0.18	0.8	4.48×10^{-1}	0.00	0.00	NA	NA
7:129918022	rs142361564	Intron		0.52	0.18	0.8	4.48×10^{-1}	0.00	0.00	NA	NA

Table C-4. (Continued)

Chr:Loc ^a	RSID	Location	Function	Cohort (n = 4217)				Case-control (n = 162)			
				MAF% in patients with AP (n = 97)	MAF% in patients without AP (n = 3220)	Coefficient ^b	P ^b	MAF% in cases (n = 47)	MAF% in controls (n = 115)	Coefficient ^c	P ^c
7:129917130	rs729332	Intron		64.43	61.89	0.1	4.51×10^{-1}	67.02	65.22	0.1	0.773
7:129921236	rs138455311	Intron		0.52	0.61	-0.6	5.70×10^{-1}	0.00	0.00	NA	NA
7:129915384	rs14829691	Intron		0.52	0.20	0.6	5.78×10^{-1}	0.00	0.00	NA	NA
7:129913297	rs1241681	Intron		6.19	6.50	-0.2	5.93×10^{-1}	3.19	4.78	-0.4	0.578
7:129912437	rs7778449	Intron		6.19	6.49	-0.2	5.97×10^{-1}	3.19	4.78	-0.4	0.578
7:129913519		Intron		3.25	2.19	0.2	6.50×10^{-1}	3.95	2.13	0.5	0.573
7:129917763	rs6943732	Intron		0.52	0.28	0.4	7.05×10^{-1}	0.00	0.00	NA	NA
7:129916390	rs5973876	Intron		24.74	21.64	-0.1	7.06×10^{-1}	28.72	23.04	0.1	0.76
7:129916030	rs9641869	Intron		24.74	21.64	-0.1	7.08×10^{-1}	28.72	23.04	0.1	0.76
7:129913187		Intron		0.52	0.63	0.3	7.62×10^{-1}	0.00	0.00	NA	NA
7:129913521		Intron		5.81	5.83	-0.1	7.67×10^{-1}	11.63	4.46	1.3	0.014
7:129918947	rs1244258	Intron		0.52	0.22	0.3	7.78×10^{-1}	0.00	0.00	NA	NA
7:129929090	rs38772	Intron		19.07	20.63	0.0	8.06×10^{-1}	24.47	25.22	0	0.97
7:129921366	rs73146777	Intron		0.52	0.55	0.2	8.12×10^{-1}	0.00	0.87	-14.4	0.989
7:129915931	rs12612	Intron		2.06	1.19	0.1	8.38×10^{-1}	0.00	0.00	NA	NA
7:129929715	rs28537519	Downstream		18.23	19.88	0.0	8.68×10^{-1}	24.47	25.22	0	0.97
7:129917678	rs144237288	Exon	Nonsyn damaging	0.52	0.18	0.2	8.77×10^{-1}	1.06	0.00	14.8	0.987
7:129917142	rs38734	Intron		7.22	6.52	0.0	9.05×10^{-1}	3.19	5.22	-0.5	0.497
7:129909554	rs6199765	Exon	Nonsyn	1.03	0.29	0.1	9.08×10^{-1}	0.00	0.43	-14	0.987
7:129913569	rs112953454	Intron		1.03	0.29	0.1	9.08×10^{-1}	0.00	0.43	-14	0.987
7:129913522	rs191671331	Intron		0.52	0.44	0.1	9.44×10^{-1}	0.00	0.00	NA	NA
7:129921360	rs1436277	Intron		0.52	0.24	0.1	9.52×10^{-1}	0.00	0.00	NA	NA
7:129913207	rs7988728	Intron		1.03	0.61	0.0	9.84×10^{-1}	0.00	0.00	NA	NA

Table C-4. (Continued)

Chr:Loc ^a	RSID	Location	Function	Cohort (n = 4217)				Case-control (n = 162)			
				MAF% in patients with AP (n = 97)	MAF% in patients without AP (n = 3220)	Coefficient ^b	P ^b	MAF% in cases (n = 47)	MAF% in controls (n = 115)	Coefficient ^c	P ^c
7:129928960	rs14872586	Intron		0.00	0.12	-13.1	9.93×10^{-1}	0.00	0.00	NA	NA
7:129915959	rs116884893	Intron		0.00	0.64	-14.8	9.93×10^{-1}	1.06	0.00	15.5	0.986
7:129918160		Intron		0.00	0.04	-12.8	9.93×10^{-1}	0.00	0.00	NA	NA
7:129929298	rs143283211	Intron		0.00	0.23	-14.7	9.93×10^{-1}	0.00	0.43	-15.3	0.986
7:129913534		Intron		0.00	0.66	-15.0	9.93×10^{-1}	0.00	0.47	-14.1	0.987
7:129919517	rs2291458	Intron		0.00	0.11	-13.3	9.94×10^{-1}	0.00	0.00	NA	NA
7:129929649	rs6953189	Downstream		0.00	0.10	-13.1	9.94×10^{-1}	0.00	0.00	NA	NA
7:129917612		Intron		0.00	0.02	-13.2	9.94×10^{-1}	0.00	0.00	NA	NA
7:129913337		Intron		0.00	0.05	-11.7	9.94×10^{-1}	0.00	0.00	NA	NA
7:129913125		Intron		0.52	0.09	-13.6	9.94×10^{-1}	0.00	0.00	NA	NA
7:129918369	rs187321656	Intron		0.00	0.47	-14.9	9.94×10^{-1}	0.00	0.43	-13.3	0.988
7:129913181		Intron		0.00	0.45	-14.6	9.94×10^{-1}	0.00	0.00	NA	NA
7:129917256		Intron		0.00	0.01	-11.4	9.94×10^{-1}	0.00	0.00	NA	NA
7:129918306		Intron		0.00	0.04	-13	9.94×10^{-1}	0.00	0.00	NA	NA
7:129912250		Intron		0.00	0.04	-12.8	9.94×10^{-1}	0.00	0.00	NA	NA
7:129916927	rs181854161	Intron		0.00	0.18	-14.0	9.94×10^{-1}	0.00	0.43	-13.3	0.988
7:129913498		Intron		0.00	0.04	-13.0	9.94×10^{-1}	0.00	0.00	NA	NA
7:129912859		Intron		0.00	0.01	-11.7	9.94×10^{-1}	0.00	0.00	NA	NA
7:129916607	rs373726453	Intron		0.00	0.04	-12.7	9.94×10^{-1}	0.00	0.00	NA	NA
7:129913512		Intron		0.00	0.02	-12.2	9.94×10^{-1}	0.00	0.00	NA	NA
7:129916836	rs73476856	Intron		0.52	0.18	-14.7	9.94×10^{-1}	0.00	0.00	NA	NA
7:129913894		Intron		0.00	0.01	-10.9	9.94×10^{-1}	0.00	0.00	NA	NA
7:129917161		Intron		0.00	0.01	-10.9	9.94×10^{-1}	0.00	0.00	NA	NA
7:129909598	rs146466435	Exon	Syn	0.00	0.07	-12.8	9.94×10^{-1}	0.00	0.00	NA	NA

Table C-4. (Continued)

Chr:Loc ^a	RSID	Location	Function	Cohort (n = 4217)				Case-control (n = 162)			
				MAF% in patients with AP (n = 97)	MAF% in patients without AP (n = 3220)	Coefficient ^b	P ^b	MAF% in cases (n = 47)	MAF% in controls (n = 115)	Coefficient ^c	P ^c
7:129909672		Intron		0.00	0.02	-12.2	9.94×10^{-1}	0.00	0.00	NA	NA
7:129921924	rs11583336	Intron		0.00	0.41	-15.7	9.94×10^{-1}	0.00	0.00	NA	NA
7:129912748		Intron		0.00	0.04	-12.4	9.94×10^{-1}	0.00	0.00	NA	NA
7:129917991		Intron		0.00	0.02	-12.6	9.94×10^{-1}	0.00	0.00	NA	NA
7:129916068	rs116555283	Intron		0.00	0.40	-15.7	9.94×10^{-1}	0.00	0.00	NA	NA
7:129918337		Intron		0.00	0.02	-12.7	9.94×10^{-1}	0.00	0.00	NA	NA
7:129928692		Intron		0.00	0.01	-13.2	9.94×10^{-1}	0.00	0.00	NA	NA
7:129914855		Intron		0.00	0.09	-13.2	9.94×10^{-1}	0.00	0.00	NA	NA
7:129910456	rs11474756	Intron		0.00	0.39	-15.7	9.95×10^{-1}	0.00	0.00	NA	NA
7:129916445	rs1523838	Intron		0.00	0.39	-15.7	9.95×10^{-1}	0.00	0.00	NA	NA
7:129917844	rs18987654	Intron		0.00	0.21	-13.8	9.95×10^{-1}	1.09	0.43	1.3	0.353
7:129909727		Intron		0.00	0.11	-12.7	9.95×10^{-1}	0.00	0.00	NA	NA
7:129913397		Intron		0.00	0.35	-15.0	9.95×10^{-1}	0.00	0.00	NA	NA
7:129913635	rs115348366	Intron		0.00	0.38	-15.7	9.95×10^{-1}	0.00	0.00	NA	NA
7:129906649		Up-stream		0.00	0.01	-10.8	9.95×10^{-1}	0.00	0.00	NA	NA
7:129916273		Intron		0.00	0.01	-12.7	9.95×10^{-1}	0.00	0.00	NA	NA
7:129913206		Intron		0.00	0.04	-12	9.95×10^{-1}	0.00	0.00	NA	NA
7:129928650		Intron		0.00	0.02	-10.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129918673		Intron		0.00	0.01	-12.7	9.95×10^{-1}	0.00	0.00	NA	NA
7:129929366	rs3737297	Intron		0.00	0.02	-10.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129915582		Intron		0.00	0.01	-12.7	9.95×10^{-1}	0.00	0.00	NA	NA
7:129917273		Intron		0.00	0.01	-10.6	9.95×10^{-1}	0.00	0.00	NA	NA
7:129914105		Intron		0.00	0.02	-11.8	9.95×10^{-1}	0.00	0.00	NA	NA
7:129921337		Intron		0.00	0.01	-10.7	9.95×10^{-1}	0.00	0.43	-13.3	0.988

Table C-4. (Continued)

Chr:Loc ^a	RSID	Location	Function	Cohort (n = 4217)				Case-control (n = 162)			
				MAF% in patients with AP (n = 97)	MAF% in patients without AP (n = 3220)	Coefficient ^b	P ^b	MAF% in cases (n = 47)	MAF% in controls (n = 115)	Coefficient ^c	P ^c
7:129928834		Intron		0.00	0.01	-13.1	9.95×10^{-1}	0.00	0.00	NA	NA
7:129914996		Exon	Nonsyn damaging	0.00	0.02	-10.2	9.95×10^{-1}	0.00	0.00	NA	NA
7:129929488	rs1415849	Exon	Syn	0.00	0.04	-11.3	9.95×10^{-1}	0.00	0.00	NA	NA
7:129929694		Downstream		0.00	0.01	-10.7	9.95×10^{-1}	0.00	0.00	NA	NA
7:129916375		Intron		0.00	0.04	-11.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129913426		Intron		0.00	0.02	-11.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129918886		Intron		0.00	0.04	-11.2	9.95×10^{-1}	0.00	0.00	NA	NA
7:129906657		Upstream		0.00	0.01	-12.4	9.95×10^{-1}	0.00	0.43	-13.5	0.988
7:129913339		Intron		0.00	0.02	-12.4	9.95×10^{-1}	0.00	0.00	NA	NA
7:129915314		Intron		0.00	0.01	-12.5	9.95×10^{-1}	0.00	0.43	-13.4	0.988
7:129919091		Intron		0.00	0.01	-12.4	9.95×10^{-1}	0.00	0.00	NA	NA
7:129929682	rs373156748	Downstream		0.00	0.02	-12.6	9.95×10^{-1}	0.00	0.43	-13.8	0.988
7:129908963		Intron		0.00	0.01	-12.4	9.95×10^{-1}	0.00	0.00	NA	NA
7:129919373	rs151137985	Exon	Syn	0.00	0.15	-14.1	9.95×10^{-1}	0.00	0.43	-13.3	0.988
7:129915643		Intron		0.00	0.01	-13.1	9.95×10^{-1}	0.00	0.00	NA	NA
7:129917292		Intron		0.00	0.04	-13.7	9.95×10^{-1}	0.00	0.00	NA	NA
7:129917909		Intron		0.00	0.01	-10.1	9.95×10^{-1}	0.00	0.00	NA	NA
7:129916653		Intron		0.00	0.01	-12.2	9.95×10^{-1}	0.00	0.00	NA	NA
7:129921096		Intron		0.00	0.02	-11.8	9.95×10^{-1}	0.00	0.00	NA	NA
7:129921822		Intron		0.00	0.02	-12.0	9.95×10^{-1}	0.00	0.00	NA	NA
7:129919472	rs6199762	Exon	Syn	0.00	0.01	-12.1	9.95×10^{-1}	0.00	0.00	NA	NA
7:129912195		Intron		0.00	0.01	-12.6	9.95×10^{-1}	0.00	0.00	NA	NA

Table C-4. (Continued)

Chr:Loc ^a	RSID	Location	Function	Cohort (n = 4217)				Case-control (n = 162)			
				MAF% in patients with AP (n = 97)	MAF% in patients without AP (n = 3220)	Coefficient ^b	P ^b	MAF% in cases (n = 47)	MAF% in controls (n = 115)	Coefficient ^c	P ^c
7:129915203		Intron		0.00	0.07	-13.3	9.95×10^{-1}	0.00	0.00	NA	NA
7:129917640	rs36936412	Intron		0.00	0.01	-12.5	9.95×10^{-1}	0.00	0.00	NA	NA
7:129913227		Intron		0.00	0.01	-12	9.95×10^{-1}	0.00	0.00	NA	NA
7:129909574	rs37757816	Exon	Syn	0.00	0.01	-12.5	9.95×10^{-1}	0.00	0.00	NA	NA
7:129918877		Intron		0.00	0.08	-13.4	9.95×10^{-1}	0.00	0.00	NA	NA
7:129913064		Intron		0.00	0.01	-10.6	9.95×10^{-1}	0.00	0.00	NA	NA
7:129919025		Intron		0.00	0.01	-10.6	9.95×10^{-1}	0.00	0.00	NA	NA
7:129915432		Intron		0.00	0.02	-12.0	9.95×10^{-1}	0.00	0.00	NA	NA
7:129922036		Intron		0.00	0.01	-11.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129922130		Intron		0.00	0.01	-11.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129916138		Intron		0.00	0.01	-11.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129928818		Intron		0.00	0.01	-11.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129913210		Intron		0.00	0.01	-11.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129929484	rs14662328	Exon	Nonsyn	0.00	0.07	-12.8	9.95×10^{-1}	0.00	0.00	NA	NA
7:129917796	rs14873587	Intron		0.00	0.13	-14.5	9.95×10^{-1}	0.00	0.00	NA	NA
7:129917035		Intron		0.00	0.01	-12.2	9.95×10^{-1}	0.00	0.00	NA	NA
7:129915677		Intron		0.00	0.01	-11.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129928707		Intron		0.00	0.01	-11.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129921120		Intron		0.00	0.01	-11.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129914143		Intron		0.00	0.01	-11.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129913542		Intron		0.00	0.05	-12.7	9.95×10^{-1}	0.00	0.00	NA	NA
7:129928912		Intron		0.00	0.01	-12.3	9.95×10^{-1}	0.00	0.00	NA	NA
7:129918717		Intron		0.00	0.01	-11.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129917225		Intron		0.00	0.01	-11.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129915294		Intron		0.00	0.01	-12.4	9.95×10^{-1}	0.00	0.00	NA	NA

Table C-4. (Continued)

Chr:Loc ^a	RSID	Location	Function	Cohort (n = 4217)				Case-control (n = 162)			
				MAF% in patients with AP (n = 97)	MAF% in patients without AP (n = 3220)	Coefficient ^b	P ^b	MAF% in cases (n = 47)	MAF% in controls (n = 115)	Coefficient ^c	P ^c
7:129915471		Intron		0.00	0.01	-11.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129906630		Up-stream		0.00	0.01	-12.2	9.95×10^{-1}	0.00	0.00	NA	NA
7:129929706		Down-stream		0.00	0.01	-12.4	9.95×10^{-1}	0.00	0.00	NA	NA
7:129929716		Down-stream		0.00	0.01	-11.8	9.95×10^{-1}	0.00	0.00	NA	NA
7:129913920		Intron		0.00	0.02	-12.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129916729		Intron		0.00	0.01	-11.8	9.95×10^{-1}	0.00	0.00	NA	NA
7:129919020		Intron		0.00	0.01	-11.8	9.95×10^{-1}	0.00	0.00	NA	NA
7:129912816		Intron		0.00	0.01	-11.7	9.95×10^{-1}	0.00	0.00	NA	NA
7:129909434		Intron		0.00	0.01	-11.7	9.95×10^{-1}	0.00	0.00	NA	NA
7:129912305		Intron		0.00	0.02	-12.0	9.95×10^{-1}	0.00	0.00	NA	NA
7:129909575	rs142582879	Exon	Nonsyn	0.00	0.01	-11.6	9.95×10^{-1}	0.00	0.00	NA	NA
7:129906791	rs376131992	Exon	Nonsyn damaging	0.00	0.01	-11.6	9.95×10^{-1}	0.00	0.00	NA	NA
7:129908878		Intron		0.00	0.01	-12.7	9.95×10^{-1}	0.00	0.00	NA	NA
7:129912704		Intron		0.00	0.01	-11.6	9.95×10^{-1}	0.00	0.00	NA	NA
7:129918402		Intron		0.00	0.01	-12.4	9.95×10^{-1}	0.00	0.00	NA	NA
7:129918422		Intron		0.00	0.01	-11.5	9.95×10^{-1}	0.00	0.00	NA	NA
7:129915570		Intron		0.00	0.02	-11.6	9.95×10^{-1}	0.00	0.00	NA	NA
7:129916824		Intron		0.00	0.01	-12.0	9.95×10^{-1}	0.00	0.00	NA	NA
7:129917027		Intron		0.00	0.01	-11.5	9.95×10^{-1}	0.00	0.00	NA	NA
7:129919529		Intron		0.00	0.01	-11.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129913523		Intron		0.00	0.06	-13	9.95×10^{-1}	0.00	0.00	NA	NA
7:129915355		Intron		0.00	0.01	-11.5	9.95×10^{-1}	0.00	0.00	NA	NA

Table C-4. (Continued)

Chr:Loc ^a	RSID	Location	Function	Cohort (n = 4217)				Case-control (n = 162)			
				MAF% in patients with AP (n = 97)	MAF% in patients without AP (n = 3220)	Coefficient ^b	P ^b	MAF% in cases (n = 47)	MAF% in controls (n = 115)	Coefficient ^c	P ^c
7:129906595		Up-stream		0.00	0.02	-13.6	9.95×10^{-1}	0.00	0.00	NA	NA
7:129913127		Intron		0.00	0.01	-11.8	9.95×10^{-1}	0.00	0.00	NA	NA
7:129929035		Intron		0.00	0.01	-11.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129921169		Intron		0.00	0.01	-11.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129917832		Intron		0.00	0.04	-12.5	9.95×10^{-1}	1.06	0.00	15.9	0.986
7:129919343	rs17591	Exon	Syn	0.00	0.32	-15.0	9.95×10^{-1}	1.06	0.00	15.9	0.986
7:129915955		Intron		0.00	0.01	-11.5	9.95×10^{-1}	0.00	0.00	NA	NA
7:129917249		Intron		0.00	0.01	-9.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129921087	rs183914994	Intron		0.00	0.01	-11.7	9.95×10^{-1}	0.00	0.00	NA	NA
7:129917339	rs18727464	Intron		0.00	0.06	-12.8	9.95×10^{-1}	0.00	0.00	NA	NA
7:129917368		Intron		0.00	0.01	-11.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129912413		Intron		0.00	0.01	-11.3	9.95×10^{-1}	0.00	0.00	NA	NA
7:129918387		Intron		0.00	0.01	-11.3	9.95×10^{-1}	0.00	0.00	NA	NA
7:129919244		Intron		0.00	0.01	-9.8	9.95×10^{-1}	0.00	0.00	NA	NA
7:129918064		Intron		0.00	0.01	-9.8	9.95×10^{-1}	0.00	0.00	NA	NA
7:129922154		Intron		0.00	0.01	-9.6	9.95×10^{-1}	0.00	0.00	NA	NA
7:129915511	rs18355629	Intron		0.00	0.13	-13.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129915411		Intron		0.00	0.01	-11.5	9.95×10^{-1}	0.00	0.00	NA	NA
7:129914008		Intron		0.00	0.01	-11.2	9.95×10^{-1}	0.00	0.00	NA	NA
7:129914011		Intron		0.00	0.01	-11.2	9.95×10^{-1}	0.00	0.00	NA	NA
7:129915197		Intron		0.00	0.01	-11.5	9.95×10^{-1}	0.00	0.00	NA	NA
7:129916627		Intron		0.00	0.01	-11.4	9.95×10^{-1}	0.00	0.00	NA	NA
7:129915103		Intron		0.00	0.02	-12.7	9.95×10^{-1}	0.00	0.00	NA	NA
7:129929316		Intron		0.00	0.01	-11.1	9.95×10^{-1}	0.00	0.00	NA	NA

Table C-4. (Continued)

Chr:Loc ^a	RSID	Location	Function	Cohort (n = 4217)				Case-control (n = 162)			
				MAF% in patients with AP (n = 97)	MAF% in patients without AP (n = 3220)	Coefficient ^b	P ^b	MAF% in cases (n = 47)	MAF% in controls (n = 115)	Coefficient ^c	P ^c
7:129919002		Intron		0.00	0.06	-13.0	9.95×10^{-1}	0.00	0.00	NA	NA
7:129913784		Intron		0.00	0.01	-11.8	9.95×10^{-1}	0.00	0.00	NA	NA
7:129929604	rs373858339	UTR3		0.00	0.01	-11.8	9.95×10^{-1}	0.00	0.00	NA	NA
7:129912430		Intron		0.00	0.15	-14	9.95×10^{-1}	0.00	0.00	NA	NA
7:129917141		Intron		0.00	0.01	-11.0	9.95×10^{-1}	0.00	0.00	NA	NA
7:129918804		Intron		0.00	0.01	-11.0	9.95×10^{-1}	0.00	0.00	NA	NA
7:129912807		Intron		0.00	0.01	-11.0	9.95×10^{-1}	0.00	0.00	NA	NA
7:129916431	rs199873336	Intron		0.00	0.01	-11.0	9.95×10^{-1}	0.00	0.00	NA	NA
7:129914923		Intron		0.00	0.01	-11.0	9.95×10^{-1}	0.00	0.00	NA	NA
7:129928939		Intron		0.00	0.01	-11.0	9.95×10^{-1}	0.00	0.00	NA	NA
7:129913992	rs18736757	Intron		0.00	0.01	-11.5	9.95×10^{-1}	0.00	0.00	NA	NA
7:129917111		Intron		0.00	0.01	-11.0	9.95×10^{-1}	0.00	0.00	NA	NA
7:129917623	rs3762143	Intron		0.00	0.04	-11.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129913067		Intron		0.00	0.01	-10.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129929713		Downstream		0.00	0.01	-11.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129919169		Intron		0.00	0.01	-11.6	9.95×10^{-1}	0.00	0.00	NA	NA
7:129917811		Intron		0.00	0.02	-13	9.95×10^{-1}	0.00	0.00	NA	NA
7:129913994		Intron		0.00	0.04	-11.5	9.95×10^{-1}	0.00	0.00	NA	NA
7:129918027		Intron		0.00	0.01	-11.3	9.95×10^{-1}	0.00	0.00	NA	NA
7:129915200		Intron		0.00	0.01	-10.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129929571	rs377236326	Exon	Nonsyn	0.00	0.01	-10.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129918048		Intron		0.00	0.02	-12.3	9.95×10^{-1}	0.00	0.00	NA	NA
7:129929365	rs377343685	Intron		0.00	0.01	-10.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129921879		Intron		0.00	0.01	-9.6	9.95×10^{-1}	0.00	0.00	NA	NA

Table C-4. (Continued)

Chr:Loc ^a	RSID	Location	Function	Cohort (n = 4217)				Case-control (n = 162)			
				MAF% in patients with AP (n = 97)	MAF% in patients without AP (n = 3220)	Coefficient ^b	P ^b	MAF% in cases (n = 47)	MAF% in controls (n = 115)	Coefficient ^c	P ^c
7:129919250		Intron		0.00	0.01	-11.6	9.95×10^{-1}	0.00	0.00	NA	NA
7:129929127		Intron		0.00	0.01	-11.5	9.95×10^{-1}	0.00	0.00	NA	NA
7:129918102		Intron		0.00	0.01	-11.6	9.95×10^{-1}	0.00	0.00	NA	NA
7:129928953		Intron		0.00	0.01	-10.8	9.95×10^{-1}	0.00	0.00	NA	NA
7:129914304		Intron		0.00	0.01	-10.8	9.95×10^{-1}	0.00	0.00	NA	NA
7:129909748		Intron		0.00	0.01	-10.7	9.95×10^{-1}	0.00	0.00	NA	NA
7:129928717		Intron		0.00	0.01	-10.8	9.95×10^{-1}	0.00	0.00	NA	NA
7:129918631		Intron		0.00	0.01	-11.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129913666		Intron		0.00	0.01	-11.0	9.95×10^{-1}	0.00	0.00	NA	NA
7:129916523		Exon	Nonsyn damaging	0.00	0.01	-10.8	9.95×10^{-1}	0.00	0.00	NA	NA
7:129912564		Intron		0.00	0.01	-10.5	9.95×10^{-1}	0.00	0.00	NA	NA
7:129916747		Intron		0.00	0.02	-12.3	9.95×10^{-1}	0.00	0.00	NA	NA
7:129913097		Intron		0.00	0.01	-10.8	9.95×10^{-1}	0.00	0.00	NA	NA
7:129914879		Intron		0.00	0.01	-10.7	9.95×10^{-1}	0.00	0.00	NA	NA
7:129930548		Downstream		0.00	12.50	-16.7	9.95×10^{-1}	NA	NA	NA	NA
7:129930566		Downstream		0.00	0.06	-12.7	9.95×10^{-1}	0.00	0.00	NA	NA
7:129930598		Downstream		0.00	0.01	-9.9	9.95×10^{-1}	0.00	0.00	NA	NA
7:129918332		Intron		0.00	0.01	-10.8	9.96×10^{-1}	0.00	0.00	NA	NA
7:129915065		Exon	Nonsyn damaging	0.00	0.01	-10.8	9.96×10^{-1}	0.00	0.00	NA	NA
7:129914022		Intron		0.00	0.01	-10.9	9.96×10^{-1}	0.00	0.00	NA	NA
7:129913438		Intron		0.00	0.01	-10.8	9.96×10^{-1}	0.00	0.00	NA	NA

Table C-4. (Continued)

Chr:Loc ^a	RSID	Location	Function	Cohort (n = 4217)				Case-control (n = 162)			
				MAF% in patients with AP (n = 97)	MAF% in patients without AP (n = 3220)	Coefficient ^b	P ^b	MAF% in cases (n = 47)	MAF% in controls (n = 115)	Coefficient ^c	P ^c
7:129916260		Intron		0.00	0.01	-10.6	9.96×10^{-1}	0.00	0.00	NA	NA
7:129921118		Intron		0.00	0.01	-11.8	9.96×10^{-1}	0.00	0.00	NA	NA
7:129916148		Intron		0.00	0.01	-11.6	9.96×10^{-1}	0.00	0.00	NA	NA
7:129912591		Intron		0.00	0.01	-10.9	9.96×10^{-1}	0.00	0.00	NA	NA
7:129909733	rs3744533	Intron		0.00	0.02	-12.1	9.96×10^{-1}	0.00	0.00	NA	NA
7:129919380	rs369196272	Exon	Nonsyn	0.00	0.02	-12.3	9.96×10^{-1}	0.00	0.00	NA	NA
7:129914926		Intron		0.00	0.01	-10.9	9.96×10^{-1}	0.00	0.00	NA	NA
7:129917261		Intron		0.00	0.01	-11.2	9.96×10^{-1}	0.00	0.00	NA	NA
7:129916891		Intron		0.00	0.01	-10.6	9.96×10^{-1}	0.00	0.00	NA	NA
7:129917257	rs11485895	Intron		0.00	0.01	-10.6	9.96×10^{-1}	0.00	0.00	NA	NA
7:129914335		Intron		0.00	0.04	-13.8	9.96×10^{-1}	0.00	0.00	NA	NA
7:129910659		Intron		0.00	0.01	-10.5	9.96×10^{-1}	0.00	0.00	NA	NA
7:129906881		Intron		0.00	0.01	-10.5	9.96×10^{-1}	0.00	0.00	NA	NA
7:129928904		Intron		0.00	0.01	-11.9	9.96×10^{-1}	0.00	0.00	NA	NA
7:129919383		Exon	Nonsyn damaging	0.00	0.02	-13.1	9.96×10^{-1}	0.00	0.00	NA	NA
7:129922108	rs139699182	Intron		0.00	0.01	-9.3	9.96×10^{-1}	0.00	0.00	NA	NA
7:129909634		Exon	Nonsyn	0.00	0.01	-10.5	9.96×10^{-1}	0.00	0.00	NA	NA
7:129909459		Intron		0.00	0.01	-10.4	9.96×10^{-1}	0.00	0.00	NA	NA
7:129913672		Intron		0.00	0.01	-10.7	9.96×10^{-1}	0.00	0.00	NA	NA
7:129913034		Intron		0.00	0.01	-10.4	9.96×10^{-1}	0.00	0.00	NA	NA
7:129915808		Intron		0.00	0.01	-10.6	9.96×10^{-1}	0.00	0.00	NA	NA
7:129921430		Intron		0.00	0.02	-12.1	9.96×10^{-1}	0.00	0.00	NA	NA
7:129917371		Intron		0.00	0.01	-10.3	9.96×10^{-1}	0.00	0.00	NA	NA
7:129913346		Intron		0.00	0.01	-10.3	9.96×10^{-1}	0.00	0.00	NA	NA

Table C-4. (Continued)

Chr:Loc ^a	RSID	Location	Function	Cohort (n = 4217)				Case-control (n = 162)			
				MAF% in patients with AP (n = 97)	MAF% in patients without AP (n = 3220)	Coefficient ^b	P ^b	MAF% in cases (n = 47)	MAF% in controls (n = 115)	Coefficient ^c	P ^c
7:129913524		Intron		0.00	0.01	-10.3	9.96×10^{-1}	0.00	0.00	NA	NA
7:129912495		Intron		0.00	0.01	-10.3	9.96×10^{-1}	0.00	0.00	NA	NA
7:129918785		Intron		0.00	0.01	-10.3	9.96×10^{-1}	0.00	0.00	NA	NA
7:129906818	rs112731688	Intron		0.00	0.01	-10.3	9.96×10^{-1}	0.00	0.00	NA	NA
7:129917401		Intron		0.00	0.01	-10.3	9.96×10^{-1}	0.00	0.00	NA	NA
7:129929719		Downstream		0.00	0.01	-10.7	9.96×10^{-1}	0.00	0.00	NA	NA
7:129917255		Intron		0.00	0.02	-13.0	9.96×10^{-1}	0.00	0.00	NA	NA
7:129917279		Intron		0.00	0.02	-13.0	9.96×10^{-1}	0.00	0.00	NA	NA
7:129918659		Intron		0.00	0.01	-11.0	9.96×10^{-1}	0.00	0.00	NA	NA
7:129917317		Intron		0.00	0.01	-11.9	9.96×10^{-1}	0.00	0.00	NA	NA
7:129921307		Intron		0.00	0.01	-10.6	9.96×10^{-1}	0.00	0.00	NA	NA
7:129916538	rs2188138	Exon	Nonsyn damaging	0.00	0.02	-12.4	9.96×10^{-1}	0.00	0.00	NA	NA
7:129929119		Intron		0.00	0.01	-11.2	9.96×10^{-1}	0.00	0.00	NA	NA
7:129916215		Intron		0.00	0.01	-10.6	9.96×10^{-1}	0.00	0.00	NA	NA
7:129928968		Intron		0.00	0.02	-12.6	9.96×10^{-1}	0.00	0.00	NA	NA
7:129913793		Intron		0.00	0.01	-11.9	9.96×10^{-1}	0.00	0.00	NA	NA
7:129906635		Upstream		0.00	0.01	-10.1	9.96×10^{-1}	0.00	0.00	NA	NA
7:129913545		Intron		0.00	0.03	-12.2	9.96×10^{-1}	0.00	0.45	-13.4	0.988
7:129908764		Intron		0.00	0.01	-10.1	9.96×10^{-1}	0.00	0.00	NA	NA
7:129918370		Intron		0.00	0.01	-10.1	9.96×10^{-1}	0.00	0.00	NA	NA
7:129917990		Intron		0.00	0.01	-10.1	9.96×10^{-1}	0.00	0.00	NA	NA
7:129913518		Intron		0.00	0.06	-13.3	9.96×10^{-1}	0.00	1.69	-15.9	0.993

Table C-4. (Continued)

Chr:Loc ^a	RSID	Location	Function	Cohort (n = 4217)				Case-control (n = 162)			
				MAF% in patients with AP (n = 97)	MAF% in patients without AP (n = 3220)	Coefficient ^b	P ^b	MAF% in cases (n = 47)	MAF% in controls (n = 115)	Coefficient ^c	P ^c
7:129913972		Intron		0.00	0.01	-10.8	9.96×10^{-1}	0.00	0.00	NA	NA
7:129914066		Intron		0.00	0.01	-10.0	9.96×10^{-1}	0.00	0.00	NA	NA
7:129914076		Intron		0.00	0.01	-8.6	9.96×10^{-1}	0.00	0.00	NA	NA
7:129916234		Intron		0.00	0.01	-9.9	9.96×10^{-1}	0.00	0.00	NA	NA
7:129913835		Intron		0.00	0.02	-11.5	9.96×10^{-1}	0.00	0.00	NA	NA
7:129913836		Intron		0.00	0.02	-11.5	9.96×10^{-1}	0.00	0.00	NA	NA
7:129912302		Intron		0.00	0.01	-8.8	9.96×10^{-1}	0.00	0.00	NA	NA
7:129928839		Intron		0.00	0.01	-9.9	9.96×10^{-1}	0.00	0.00	NA	NA
7:129919065		Intron		0.00	0.01	-10.8	9.96×10^{-1}	0.00	0.00	NA	NA
7:129912112		Intron		0.00	0.01	-10.5	9.96×10^{-1}	0.00	0.00	NA	NA
7:129921820		Intron		0.00	0.01	-9.9	9.96×10^{-1}	0.00	0.00	NA	NA
7:129919344	rs373354928	Exon	Nonsyn damaging	0.00	0.01	-9.9	9.96×10^{-1}	0.00	0.00	NA	NA
7:129928846		Intron		0.00	0.01	-12.8	9.96×10^{-1}	0.00	0.00	NA	NA
7:129916807		Intron		0.00	0.01	-10.0	9.96×10^{-1}	0.00	0.00	NA	NA
7:129919464		Exon	Nonsyn damaging	0.00	0.01	-9.8	9.96×10^{-1}	0.00	0.00	NA	NA
7:129906897		Intron		0.00	0.01	-9.8	9.96×10^{-1}	0.00	0.00	NA	NA
7:129913430		Intron		0.00	0.01	-9.8	9.96×10^{-1}	0.00	0.00	NA	NA
7:129916835		Intron		0.00	0.01	-9.8	9.96×10^{-1}	0.00	0.00	NA	NA
7:129913613		Intron		0.00	0.01	-10.7	9.96×10^{-1}	0.00	0.00	NA	NA
7:129919063		Intron		0.00	0.01	-9.8	9.96×10^{-1}	0.00	0.00	NA	NA
7:129912247		Intron		0.00	0.01	-11.6	9.96×10^{-1}	0.00	0.00	NA	NA
7:129921304		Intron		0.00	0.01	-9.8	9.96×10^{-1}	0.00	0.00	NA	NA
7:129910453		Intron		0.00	0.01	-9.8	9.96×10^{-1}	0.00	0.00	NA	NA

Table C-4. (Continued)

Chr:Loc ^a	RSID	Location	Function	Cohort (n = 4217)				Case-control (n = 162)			
				MAF% in patients with AP (n = 97)	MAF% in patients without AP (n = 3220)	Coef-ficient ^b	P ^b	MAF% in cases (n = 47)	MAF% in controls (n = 115)	Coef-ficient ^c	P ^c
7:129929093	rs3748345	Intron		0.00	0.01	-9.8	9.96×10^{-1}	0.00	0.00	NA	NA
7:129915269		Intron		0.00	0.01	-9.9	9.96×10^{-1}	0.00	0.00	NA	NA
7:129912403		Intron		0.00	0.01	-9.8	9.96×10^{-1}	0.00	0.00	NA	NA
7:129913332		Intron		0.00	0.01	-10.1	9.96×10^{-1}	0.00	0.00	NA	NA
7:129919202		Intron		0.00	0.02	-11.4	9.96×10^{-1}	0.00	0.00	NA	NA
7:129929387		Intron		0.00	0.01	-9.9	9.96×10^{-1}	0.00	0.00	NA	NA
7:129913096		Intron		0.00	0.01	-9.7	9.96×10^{-1}	0.00	0.00	NA	NA
7:129910480		Intron		0.00	0.01	-9.7	9.96×10^{-1}	0.00	0.00	NA	NA
7:129915155		Intron		0.00	0.01	-10.6	9.96×10^{-1}	0.00	0.00	NA	NA
7:129919227		Intron		0.00	0.01	-9.7	9.96×10^{-1}	0.00	0.00	NA	NA
7:129912293		Intron		0.00	0.01	-9.6	9.96×10^{-1}	0.00	0.00	NA	NA
7:129918006		Intron		0.00	0.01	-9.8	9.96×10^{-1}	0.00	0.00	NA	NA
7:129909638		Exon	Nonsyn damaging	0.00	0.01	-9.7	9.96×10^{-1}	0.00	0.00	NA	NA
7:129912645		Intron		0.00	0.01	-9.7	9.96×10^{-1}	0.00	0.00	NA	NA
7:129909569	rs6974812	Exon	Nonsyn	0.00	0.01	-9.7	9.96×10^{-1}	0.00	0.00	NA	NA
7:129916448		Intron		0.00	0.01	-9.9	9.96×10^{-1}	0.00	0.00	NA	NA
7:129908748		Intron		0.00	0.01	-11.9	9.96×10^{-1}	0.00	0.00	NA	NA
7:129917688		Exon	Nonsyn damaging	0.00	0.02	-10.9	9.96×10^{-1}	0.00	0.00	NA	NA
7:129918230		Intron		0.00	0.01	-9.6	9.96×10^{-1}	0.00	0.00	NA	NA
7:129914081		Intron		0.00	0.01	-11.0	9.96×10^{-1}	0.00	0.00	NA	NA
7:129914082		Intron		0.00	0.01	-11.0	9.96×10^{-1}	0.00	0.00	NA	NA
7:129914204		Intron		0.00	0.01	-11.0	9.96×10^{-1}	0.00	0.00	NA	NA
7:129913687		Intron		0.00	0.01	-9.7	9.96×10^{-1}	0.00	0.00	NA	NA

Table C-4. (Continued)

Chr:Loc ^a	RSID	Location	Function	Cohort (n = 4217)				Case-control (n = 162)			
				MAF% in patients with AP (n = 97)	MAF% in patients without AP (n = 3220)	Coefficient ^b	P ^b	MAF% in cases (n = 47)	MAF% in controls (n = 115)	Coefficient ^c	P ^c
7:129913525		Intron		0.00	0.01	-9.4	9.96×10 ⁻¹	0.00	0.00	NA	NA
7:129915520		Intron		0.00	0.01	-9.5	9.96×10 ⁻¹	0.00	0.00	NA	NA
7:129930622		Downstream		0.00	0.01	-10.7	9.96×10 ⁻¹	0.00	0.00	NA	NA
7:129930565		Downstream		0.00	0.11	-13.5	9.97×10 ⁻¹	0.00	0.00	NA	NA
7:129922141		Intron		0.00	0.01	-10.5	9.97×10 ⁻¹	0.00	0.00	NA	NA
7:129906671		Upstream		0.00	0.00	NA	NA	0.00	0.00	NA	NA
7:129906765		Exon	Nonsyn	0.00	0.00	NA	NA	0.00	0.43	-13.4	0.988
7:129906905		Intron		0.00	0.00	NA	NA	0.00	0.00	NA	NA
7:129908688		Intron		0.00	0.00	NA	NA	0.00	0.43	-14.1	0.987
7:129908801		Exon	Nonsyn damaging	0.00	0.00	NA	NA	0.00	0.00	NA	NA
7:129910414		Intron		0.00	0.00	NA	NA	0.00	0.00	NA	NA
7:129912126		Intron		0.00	0.00	NA	NA	0.00	0.00	NA	NA
7:129912376		Intron		0.00	0.00	NA	NA	0.00	0.00	NA	NA
7:129912718		Intron		0.00	0.00	NA	NA	1.06	0.00	15	0.986
7:129912837		Intron		0.00	0.00	NA	NA	0.00	0.00	NA	NA
7:129912877		Intron		0.00	0.00	NA	NA	0.00	0.43	-13.3	0.988
7:129913382		Intron		0.00	0.00	NA	NA	0.00	0.00	NA	NA
7:129913440		Intron		0.00	0.00	NA	NA	0.00	0.00	NA	NA
7:129913596		Intron		0.00	0.00	NA	NA	0.00	0.43	-13.4	0.988
7:129914063		Intron		0.00	0.00	NA	NA	0.00	0.00	NA	NA
7:129915322		Intron		0.00	0.00	NA	NA	0.00	0.00	NA	NA
7:129915498		Intron		0.00	0.00	NA	NA	0.00	0.43	-14.1	0.987

Table C-4. (Continued)

Chr:Loc ^a	RSID	Location	Function	Cohort (n = 4217)			<i>P</i> ^b	Case-control (n = 162)			<i>P</i> ^c
				MAF% in patients with AP (n = 97)	MAF% in patients without AP (n = 3220)	Coefficient ^b		MAF% in cases (n = 47)	MAF% in controls (n = 115)	Coefficient ^c	
7:129915905		Intron		0.00	0.00	NA	NA	0.00	0.00	NA	NA
7:129916210		Intron		0.00	0.00	NA	NA	0.00	0.00	NA	NA
7:129916765		Intron		0.00	0.00	NA	NA	0.00	0.00	NA	NA
7:129917055		Intron		0.00	0.01	NA	NA	0.00	0.00	NA	NA
7:129917213		Intron		0.00	0.00	NA	NA	0.00	0.00	NA	NA
7:129918695		Intron		0.00	0.00	NA	NA	0.00	0.00	NA	NA
7:129918843		Intron		0.00	0.00	NA	NA	0.00	0.00	NA	NA
7:129919122		Intron		0.00	0.00	NA	NA	0.00	0.00	NA	NA
7:129921333		Intron		0.00	0.00	NA	NA	0.00	0.00	NA	NA
7:129921880		Intron		0.52	0.00	NA	NA	0.00	0.00	NA	NA
7:129929508		Exon	Nonsyn	0.00	0.00	NA	NA	0.00	0.43	-13.4	0.988

Variants in *CPA2* were identified by deep sequencing and ranked by P values in the cohort. ^aPhysical location of SNP based on Feb 2009 human genome assembly (hg19). ^bCoefficient and P value of minor alleles were estimated using Cox proportional hazard model. ^cCoefficient and P value of minor alleles were estimated using logistic regression model. P values less than 0.05 were highlighted. MAF, minor allele frequency. Chr, chromosome. Syn, synonymous. Nonsyn, nonsynonymous. UTR, untranslated region. NA, not available.

Table C-5. Top genes ($P < 0.05$) in the gene-level analysis of nonsense variants identified in putative pancreatitis (n = 42) and non-pancreatitis genes (n = 241) based on variants identified by deep sequencing

Rank ^a	Gene	N of SNPs ^b	Cohort SKAT P (n = 4217)	Case-control SKAT P (n = 162)	Pancreatitis-associated ^c
1	<i>HOGA1</i>	1	6.37×10^{-11}	-	Yes
2	<i>CPA2</i>	1	6.69×10^{-11}	0.03	Yes
3	<i>ZNF233</i>	4	9.90×10^{-9}	0.05	Yes
4	<i>C9orf117</i>	2	4.83×10^{-8}	-	Yes
5	<i>LRRC39</i>	1	2.48×10^{-6}	-	Yes
6	<i>XDH</i>	1	6.40×10^{-6}	-	No
7	<i>XRCC1</i>	5	1.41×10^{-5}	-	No
8	<i>IFNA5</i>	1	2.28×10^{-5}	-	Yes
9	<i>QPCT</i>	3	3.87×10^{-4}	0.73	No
10	<i>C11orf63</i>	1	4.72×10^{-4}	-	Yes
11	<i>ZRANB3</i>	1	6.79×10^{-4}	-	Yes
12	<i>C12orf40</i>	1	2.67×10^{-3}	-	Yes
13	<i>DDX49</i>	3	3.85×10^{-3}	0.72	Yes
14	<i>GCKR</i>	2	4.24×10^{-3}	0.07	Yes
15	<i>SNTG2</i>	1	5.10×10^{-3}	-	Yes
16	<i>RUFY4</i>	2	9.16×10^{-3}	-	Yes
17	<i>MPZL3</i>	1	9.40×10^{-3}	-	Yes
18	<i>DAPL1</i>	2	1.36×10^{-2}	-	No
19	<i>ALS2CL</i>	1	1.43×10^{-2}	-	Yes
20	<i>CENPQ</i>	1	1.55×10^{-2}	0.73	No
21	<i>NMI</i>	3	2.51×10^{-2}	-	Yes

^aRank of gene based on the cohort SKAT P-value. ^bNumber of SNPs in this category. ^cGenes associated with pancreatitis (based on GWAS or literature). All in 10K, all SNPs within 10K region of the gene.

Figure C-2. Top pathways and functions of pancreatitis-associated genes (n = 42)

Pathway analysis of 42 pancreatitis-associated genes (see **Table 4-3**) was performed using Ingenuity.²⁰⁵ Scores of the networks indicated the relevance between networks and genes, and P values of the top diseases or functions were calculated using Fisher's exact test to determine the probability that the association between the genes in the data set and the function is due to chance alone. The numbers of genes in each functional category are shown.

Figure C-3. Top pathways and functions of pancreatitis-associated genes identified by GWAS (n = 34)

Pathway analysis of 34 pancreatitis-associated genes identified by GWAS (see **Table 4-3**) was performed using Ingenuity.²⁰⁵ Scores of the networks indicated the relevance between networks and genes, and P values of the top diseases or functions were calculated using Fisher's exact test to determine the probability that the association between the genes in the data set and the function is due to chance alone. The numbers of genes in each functional category are shown.

Table C-6. SNPs (n = 81) identified by deep sequencing in previously reported pancreatitis-associated candidate genes (n = 8) at the $P < 0.05$ level (highlighted) in either the cohort or the case-control group

Gene (n of SNPs at $P < 0.05$ /n of all SNPs) ^a	Chr:Loc ^b	RSID	Function	Major allele/ minor allele	Cohort (n = 4217)				Case-control (n = 162)				
					MAF% in patients with AP (n = 95)	MAF% in patients without AP (n = 3161)	Coeff- icient ^c	P^c	MAF% in cases (n = 47)	MAF% in con- trols (n = 115)	Coeff- icient ^d	P^d	Dire- ction ^e
<i>CTRC</i> ¹⁶²⁻¹⁶⁴ (8/159)	1:15766660		Intronic	C/T	0.63	0.03	2.219	3.01×10^{-2}	0.00	0.00	NA	NA	NA
	1:15766722		Intronic	-/C	1.05	0.08	2.991	4.48×10^{-5}	0.00	0.00	NA	NA	NA
	1:15766730		Intronic	T/C	3.45	1.57	0.862	4.19×10^{-2}	0.00	0.00	NA	NA	NA
	1:15766836		Syn	C/T	0.52	0.00	3.386	1.53×10^{-3}	0.00	0.00	NA	NA	NA
	1:15767036	rs49778	Syn	C/T	7.37	9.99	-0.385	1.81×10^{-1}	11.70	6.52	0.93	0.044	-
	1:15768845		Intronic	C/A	0.52	0.01	3.147	2.26×10^{-3}	0.00	0.00	NA	NA	NA
	1:15770102	rs545634	Intronic	G/A	6.70	9.69	-0.343	2.36×10^{-1}	11.70	6.52	0.93	0.044	-
	1:15772962		Intronic	G/A	0.54	0.01	3.545	5.11×10^{-4}	0.00	0.00	NA	NA	NA
<i>CASR</i> ⁷² (7/255)	3:121902632	rs188613418	Upstream	A/G	0.51	0.00	3.316	1.33×10^{-3}	0.00	0.00	NA	NA	NA
	3:121952299		Intronic	C/T	0.51	0.00	2.104	4.34×10^{-2}	0.00	0.00	NA	NA	NA
	3:121952353	rs145416265	Intronic	T/C	0.51	0.02	2.718	9.32×10^{-3}	0.00	0.00	NA	NA	NA
	3:121952524		Intronic	A/G	0.51	0.02	2.318	2.46×10^{-2}	0.00	0.00	NA	NA	NA
	3:121980909		Nonsyn	G/A	0.51	0.00	5.092	9.01×10^{-7}	0.00	0.00	NA	NA	NA
	3:122000815		Intronic	C/T	0.51	0.00	4.559	1.12×10^{-5}	0.00	0.00	NA	NA	NA
<i>SPINK1</i> ^{71,153,162} (2/104)	3:122000822	rs113610526	Intronic	T/G	0.51	0.01	3.576	4.81×10^{-4}	0.00	0.00	NA	NA	NA
	5:147207668		Syn	A/G	0.52	0.01	3.217	1.50×10^{-3}	0.00	0.00	NA	NA	NA
	5:147210229		Intronic	T/C	0.52	0.00	4.064	9.97×10^{-5}	0.00	0.00	NA	NA	NA

Table C-6. (Continued)

Gene (n of SNPs at $P < 0.05$ /n of all SNPs) ^a	Chr:Loc ^b	RSID	Function	Major allele/ minor allele	Cohort (n = 4217)				Case-control (n = 162)				
					MAF% in patients with AP (n = 95)	MAF% in patients without AP (n = 3,161)	Coeff- icient ^c	P^c	MAF % in cases (n = 47)	MAF % in con- trols (n = 115)	Coeff- icient ^d	P^d	Dire- ction ^e
<i>CFTR</i> ^{153,162} (32/535)	7:117119516		Upstream	G/A	0.52	0.00	6.333	4.40×10^{-9}	0.00	0.00	NA	NA	NA
	7:117119599		Upstream	G/C	0.52	0.00	4.070	1.11×10^{-4}	0.00	0.00	NA	NA	NA
	7:117120323		Intronic	T/C	0.52	0.00	5.175	1.35×10^{-6}	0.00	0.00	NA	NA	NA
	7:117120586	rs1876659	Intronic	A/G	0.52	0.01	3.273	1.64×10^{-3}	0.00	0.00	NA	NA	NA
	7:117120672		Intronic	A/G	0.52	0.04	2.174	3.55×10^{-2}	0.00	0.00	NA	NA	NA
	7:117144420		Intronic	A/G	0.52	0.00	3.601	6.92×10^{-4}	0.00	0.00	NA	NA	NA
	7:117171012		Syn	G/A	0.52	0.00	3.601	6.92×10^{-4}	0.00	0.00	NA	NA	NA
	7:117174527	rs15757811	Intronic	C/G	0.54	0.01	3.626	4.34×10^{-4}	1.11	0.00	15.65	0.986	+
	7:117175372	rs1219946	Nonsyn damaging	A/G	0.52	0.01	3.179	2.63×10^{-3}	0.00	0.00	NA	NA	NA
	7:117180324	rs77932196	Nonsyn damaging	G/C	0.52	0.00	3.944	1.12×10^{-4}	0.00	0.00	NA	NA	NA
	7:117180338	rs193922497	Nonsyn damaging	C/T	1.03	0.04	2.525	5.13×10^{-4}	0.00	0.00	NA	NA	NA
	7:117182242		Intronic	G/A	0.54	0.00	2.782	5.92×10^{-3}	0.00	0.00	NA	NA	NA
	7:117188684		Intronic	-/TT	1.11	1.02	-0.531	5.98×10^{-1}	4.88	0.48	2.69	0.019	-
	7:117188736	rs4727853	Nonsyn damaging	C/A	39.13	40.22	-0.130	6.59×10^{-1}	31.94	41.46	-1.01	0.032	+
	7:117188750	rs2188593	Nonsyn damaging	C/T	12.96	14.23	-0.131	6.12×10^{-1}	3.85	14.20	-1.59	0.016	+
	7:117188896		Intronic	A/G	0.52	0.00	4.070	1.11×10^{-4}	0.00	0.00	NA	NA	NA

Table C-6. (Continued)

Gene (n of SNPs at $P < 0.05$ /n of all SNPs) ^a	Chr:Loc ^b	RSID	Function	Major allele/ minor allele	Cohort (n = 4217)				Case-control (n = 162)				
					MAF% in patients with AP (n = 95)	MAF% in patients without AP (n = 3,161)	Coeff- icient ^c	P^c	MAF % in cases (n = 47)	MAF % in con- trols (n = 115)	Coeff- icient ^d	P^d	Dire- ction ^e
<i>CFTR</i> ^{153,162} (32/535)	7:117199641	rs1891	Nonsyn damaging	A/G	0.52	0.02	3.483	5.85×10^{-4}	0.00	0.00	NA	NA	NA
	7:117199648	rs7457153	Nonsyn damaging	T/G	0.54	0.07	2.365	1.97×10^{-2}	0.00	0.88	-14.32	0.989	-
	7:117230283	rs1714229	Intronic	T/C	9.24	3.16	0.819	1.12×10^{-2}	2.22	0.45	1.37	0.330	+
	7:117234884		Intronic	A/G	0.53	0.00	3.909	1.25×10^{-4}	0.00	0.00	NA	NA	NA
	7:117242735		Intronic	G/A	0.52	0.00	3.583	6.37×10^{-4}	0.00	0.00	NA	NA	NA
	7:117242753		Intronic	G/A	0.52	0.00	5.333	1.24×10^{-4}	0.00	0.00	NA	NA	NA
	7:117243663	rs1219934	Nonsyn	C/T	0.52	0.02	2.852	5.08×10^{-3}	0.00	0.00	NA	NA	NA
	7:117243784		Nonsyn damaging	G/A	0.52	0.00	2.214	3.41×10^{-2}	0.00	0.00	NA	NA	NA
	7:117250493		Intronic	G/A	4.35	1.57	0.999	2.01×10^{-2}	0.00	2.33	-15.34	0.990	-
	7:117250824		Intronic	C/G	0.52	0.01	2.598	1.01×10^{-2}	0.00	0.00	NA	NA	NA
	7:117251794		Nonsyn damaging	A/C	0.52	0.00	3.696	3.48×10^{-4}	0.00	0.00	NA	NA	NA
	7:117254753		Nonsyn damaging	G/C	0.52	0.05	2.065	4.02×10^{-2}	0.00	0.00	NA	NA	NA
	7:117282413		Intronic	T/C	0.52	0.04	2.111	3.62×10^{-2}	0.00	0.00	NA	NA	NA
	7:117282716		Intronic	A/C	0.52	0.00	4.264	4.50×10^{-5}	0.00	0.00	NA	NA	NA
	7:117307164	rs1591472	Down-stream	G/A	0.52	0.01	3.180	2.21×10^{-3}	0.00	0.00	NA	NA	NA
	7:117308226		Down-stream	T/A	0.52	0.00	4.271	2.91×10^{-5}	0.00	0.00	NA	NA	NA

Table C-6. (Continued)

Gene (n of SNPs at $P < 0.05$ /n of all SNPs) ^a	Chr:Loc ^b	RSID	Function	Majora lle/ minor allele	Cohort (n = 4217)				Case-control (n = 162)				Dire- ction ^e
					MAF% in patients with AP (n = 95)	MAF% in patients without AP (n = 3,161)	Coeff- icient ^c	P^c	MAF % in cases (n = 47)	MAF % in con- trols (n = 115)	Coeff- icient ^d	P^d	
<i>CPAI</i> ^{80,81} (1/161)	7:130020234		Upstream	C/T	0.52	0.00	4.070	1.11×10^{-4}	0.00	0.00	NA	NA	NA
<i>PRSSI</i> ^{67- 69,74,162} (22/283)	7:142457184		Upstream	C/T	0.52	0.01	4.064	6.24×10^{-5}	0.00	0.00	NA	NA	NA
	7:142458285		Intronic	-/A	1.79	0.28	1.926	9.29×10^{-3}	1.56	0.00	16.28	0.991	+
	7:142458357	rs19942214	Intronic	C/T	0.52	0.05	2.515	1.27×10^{-2}	0.00	0.00	NA	NA	NA
	7:142458582	rs2688125	Intronic	C/T	2.81	0.93	1.208	8.99×10^{-3}	0.00	2.31	-16.23	0.988	-
	7:142458604	rs375924181	Intronic	T/G	1.60	0.59	1.162	4.85×10^{-2}	0.00	1.40	-16.45	0.990	-
	7:142458638	rs369272152	Intronic	C/G	1.28	0.33	1.517	3.55×10^{-2}	1.11	0.00	15.47	0.986	+
	7:142459524		Intronic	C/A	22.08	15.56	0.464	4.82×10^{-2}	16.67	20.45	-0.30	0.497	-
	7:142459525		Intronic	A/G	22.37	15.67	0.491	3.75×10^{-2}	17.74	19.78	-0.15	0.745	-
	7:142459543		Intronic	A/G	35.71	25.92	0.826	3.48×10^{-3}	33.33	31.55	0.18	0.697	+
	7:142459547		Intronic	C/G	20.39	13.84	0.522	2.81×10^{-2}	12.20	19.68	-0.70	0.109	-
	7:142459550		Intronic	G/A	6.90	6.62	0.085	7.85×10^{-1}	8.75	2.53	1.43	0.024	+
	7:142459558		Intronic	T/-	6.47	5.30	0.263	4.17×10^{-1}	8.75	2.58	1.40	0.026	+
	7:142459771	rs199769221	Nonsyn	G/C	0.53	0.08	2.434	1.62×10^{-2}	0.00	0.00	NA	NA	NA
	7:142459778		Syn	A/C	0.53	0.04	2.602	1.01×10^{-2}	0.00	0.00	NA	NA	NA
	7:142459784		Syn	C/T	0.52	0.05	2.491	1.38×10^{-2}	0.00	0.00	NA	NA	NA
	7:142459785		Nonsyn	G/T	0.52	0.02	3.417	8.34×10^{-4}	0.00	0.00	NA	NA	NA

Table C-6. (Continued)

Gene (n of SNPs at $P < 0.05$ /n of all SNPs) ^a	Chr:Loc ^b	RSID	Function	Major allele/ minor allele	Cohort (n = 4217)				Case-control (n = 162)				Dire- ction ^e
					MAF% in patients with AP (n = 95)	MAF% in patients without AP (n = 3,161)	Coeff- icient ^c	P^c	MAF% in cases (n = 47)	MAF% in con- trols (n = 115)	Coeff- icient ^d	P^d	
<i>PRSSI</i> ^{67- 69,74,162} (22/283)	7:142460163	rs111626281	Intronic	T/C	1.03	0.10	1.938	9.30×10^{-3}	0.00	0.00	NA	NA	NA
	7:142460323		Syn	C/T	0.52	0.00	3.952	1.44×10^{-4}	0.00	0.00	NA	NA	NA
	7:142460432		Intronic	C/A	2.78	0.00	2.850	1.12×10^{-2}	0.00	2.50	-17.58	0.994	-
	7:142460495		Intronic	A/C	41.28	41.98	-0.130	6.49×10^{-1}	40.24	45.71	-1.07	0.046	+
	7:142460503		Intronic	T/C	38.10	37.95	0.021	9.36×10^{-1}	36.59	43.43	-0.95	0.045	-
<i>PRSS2</i> ^{66,69} (8/43)	7:142460556	rs374121411	Intronic	C/T	17.11	18.90	-0.151	5.41×10^{-1}	28.57	17.82	0.95	0.025	-
	7:142479912	rs248489	Unknown	C/G	46.28	41.94	0.811	4.46×10^{-2}	38.89	43.75	-0.57	0.252	-
	7:142479917	rs36959197	Unknown	C/T	46.77	41.47	1.059	1.43×10^{-2}	37.78	43.36	-0.64	0.187	-
	7:142479940	rs58649169	Unknown	C/T	45.83	40.96	0.742	3.66×10^{-2}	38.30	43.04	-0.53	0.267	-
	7:142479954	rs21779716	Unknown	C/T	45.83	40.74	0.839	2.62×10^{-2}	38.30	43.04	-0.53	0.267	-
	7:142480002	rs199512312	Unknown	C/A	45.88	40.63	0.860	2.37×10^{-2}	38.30	43.04	-0.53	0.267	-
	7:142480012	rs56146961	Unknown	C/T	44.85	40.07	0.699	4.16×10^{-2}	38.30	42.61	-0.44	0.354	-
	7:142480053	rs2815151	Unknown	G/C	4.40	2.39	0.776	3.73×10^{-2}	0.00	3.92	-16.93	0.990	-
<i>CLDN2</i> ^{66,69} (1/67)	7:142480063	rs2216921	Unknown	C/A	1.11	0.13	2.280	1.50×10^{-3}	0.00	0.47	-14.24	0.987	-
	X:106163851		Intronic	T/A	0.53	0.00	3.885	1.45×10^{-4}	0.00	0.00	NA	NA	NA

Table C-6. (Continued)

^a(Number of SNPs at the $P < 0.05$ level)/(number of SNPs identified in the gene). ^bPhysical location of SNP based on Feb 2009 human genome assembly (hg19). ^cCoefficient and P value of minor alleles were estimated using Cox proportional hazard model. ^dCoefficient and P value of minor alleles were estimated using logistic regression model. P values less than 0.05 were highlighted. ^eThe SNP goes in the same (+) or different (-) direction in the cohort and the case-control group, or is absent (NA) in the case-control group. MAF, minor allele frequency. RSID, reference SNP identification number. Syn, synonymous. Nonsyn, nonsynonymous. NA, not applicable.

VITA

Chengcheng Liu was born 1985 in Dalian, China. She received her B.S. degree in Chemistry and Biology at Tsinghua University in 2008. She joined in the Integrated Program of Biomedical Sciences at University of Tennessee Health Science Center in 2009, and anticipated to complete her Ph.D. study in 2015.