TITLE: Gender effects on cytidine analogue metabolism and myelodysplastic syndrome treatment outcomes

Running title: Gender, cytidine deaminase and 5-aza/decitabine

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ABSTRACT

In vivo, half-lives of cytidine analogues such as 5-azacytidine and decitabine, used to treat myelodysplastic syndromes (MDS), are determined largely by cytidine deaminase (CDA), an enzyme that rapidly metabolizes these drugs into inactive uridine counterparts. Genetic factors influence CDA activity, and hence, could impact 5-azacytidine/decitabine levels and efficacy, a possibility requiring evaluation. Using an HPLC assay, plasma CDA activity was confirmed to be decreased in individuals with the *CDA* SNP A79C. More interestingly, there was an even larger decrease in females. Explaining the decrease in enzyme activity, liver CDA expression was significantly lower in female versus male mice. As expected, decitabine plasma levels, measured by mass-spectrometry, were significantly higher in females. In mathematical modeling, the detrimental effect of shortening half-life of S-phase specific therapy was amplified in low S-phase fraction disease (e.g., MDS). Accordingly, in multivariate analysis of MDS patients treated with 5-azacytidine/decitabine, overall survival was significantly worse in males.

INTRODUCTION

The cytidine analogue drugs 5-azacytidine and decitabine are unique oncotherapeutics by virtue of a powerful molecular epigenetic effect, depletion of DNA methyl-transferase 1 (DNMT1) after incorporation into DNA[1]. Levels of these drugs well below 0.5 μM are sufficient to deplete DNMT1[2-8]. Hence, in contrast to the cytidine analogues cytarabine and gemcitabine, which are administered at high doses (100-3000 mg/m^2) derived from maximum tolerated levels and intended for anti-metabolite cytotoxic effects, 5-azacytidine and decitabine are administered at lower doses (5-75 mg/m^2). Another important consideration in the clinical application of 5-azacytidine/decitabine is that DNMT1 depletion is S-phase specific[5], and hence exposure time and schedule are likely to influence treatment efficacy[5,9,10]. Supporting this premise, reduction of decitabine dose to 20 mg/m^2/day from the 45 mg/m^2/day dose originally approved by the FDA, but administration on more days (5 days every 4 weeks instead of 3 days every 6 weeks), increased the overall response rate in myelodysplastic syndromes (MDS) to 32-73% from 17%[11-13]. It is possible that exposure time is even more pertinent in relatively indolent MDS than in aggressive acute myeloid leukemia (AML): even a short treatment exposure may be effective treatment for high S-phase fraction malignant disease, since the majority of cells may enter S-phase in the treatment window. Conversely, in disease with a low S-phase fraction, short exposure time may only treat a minor portion of the malignant cells.

Given the importance of treatment exposure time, genetic factors that influence the *in vivo* half-life of 5-azacytidine and decitabine could significantly influence treatment outcome. *In vivo*, cytidine, deoxycytidine, and analogues thereof are rapidly deaminated to uracil base moiety counterparts by the ubiquitously expressed enzyme cytidine deaminase (CDA)[14-17]. The clinical relevance of CDA is suggested by its effect on cytidine analogue half-life: the half-life of decitabine in buffer *in vitro* at 37^{0}C is >10 hours[18], by contrast, the half-life *in vivo* is <10 minutes[19], a drastic reduction largely attributable to CDA[15,20,21]. A pharmacogenetic factor known to decrease CDA enzyme activity is the non-synonymous *CDA* single nucleotide polymorphism (SNP) A79C (Lys27Gln, rs2072671); >60% of Caucasians are heterozygous or homozygous for this SNP which changes lysine to glutamine at amino-acid position 27 to cause as much as a 3-fold decrease in CDA enzyme

activity[22,23]. The decrease in enzyme activity caused by this SNP has been implicated in severe toxic events occurring with cytarabine and gemcitabine therapy[22-26].

However, the impact of A79C on 5-azacytidine or decitabine treatment outcomes has not previously been evaluated. Such an impact, if it exists, could be different from that reported with gemcitabine or cytarabine: since 5-azacytidine/decitabine are administered at relatively low doses, an increase in 5-azacytidine/decitabine levels from the A79C SNP might not produce a clinically significant increase in toxicity. Instead, shorter half-lives and lower drug levels due to genetic factors that increase CDA activity might decrease efficacy. In this regard, another genetic variable relevant to cytidine analogue metabolism and half-life is gender: for both gemcitabine and cytarabine, higher clearance and shorter half-life has been described in males compared to females[27,28]. However, the mechanisms underlying this gender difference have not been elucidated. Given the prominence of CDA in cytidine analogue metabolism and clearance, we looked for gender differences in CDA expression as a possible basis for gender differences in pharmacokinetics.

Hence, we examined if genetic factors known to affect cytidine analogue metabolism, A79C SNP status and gender, impact overall survival of MDS patients treated with 5-azacytidine or decitabine. To explore the mechanistic basis for gender differences in cytidine analogue pharmacokinetics, we examined connections between gender, CDA enzyme activity, CDA expression, and decitabine pharmacokinetics. To better understand if treatment exposure time could be a more important consideration in some types of malignant disease than in others, we mathematically evaluated the relation between disease S-phase fraction and treatment exposure time. The results of these investigations improve understanding and can hopefully improve clinical application of these important oncotherapeutics.

MATERIALS AND METHODS

Patients and treatment. The analysis of MDS and AML patient data and samples was approved by the Cleveland Clinic/Case Comprehensive Cancer Center Institutional Review Board (IRB). Bone marrow or blood samples for research were obtained with written informed consent per IRB approved procedures. Patients were diagnosed with MDS (n=90) and AML (n=76) per WHO criteria, and initiated on treatment between January 2002 and December 2007, with tissue-banked samples available for SNP analysis by sequencing, and with verifiable follow-up and survival annotation. Date of diagnosis was obtained from the medical record. Date of death was based on physician documentation in the medical record, or on the social security death index and a phone call to the emergency contact. Other variables analyzed were those known to have major prognostic importance in patients with MDS and AML (bone marrow myeloblast%, karyotype, age)[29]. Patients were analyzed in two groups: **(i)** 90 MDS patients treated either with 5-azacytidine 75 mg/m^2/day D1-7 (sub-cutaneous or intravenous [IV]) or decitabine 20 mg/m^2/day D1-5 (IV over 1 hour) in 28 day cycles; and **(ii)** 76 AML patients treated with cytarabine-based induction chemotherapy: cytarabine dose 100–200 mg/m^2/day days 1-7 continuous infusion and an anthracycline days 1-3.

Measurement of CDA enzyme activity by an HPLC assay. Based on previously published methods [30], conversion of cytidine into uridine by plasma at 37^{0}C was measured by high performance liquid chromatography (HPLC). Reaction buffer of 0.1 M Tris/HCL pH 7.5 (265µl) was added to 25µl of human plasma followed by addition of cytidine to a final concentration of 4.1 mM and 5-flourouridine (0.381 mM) as an internal control (5-fluoruridine is not metabolized by CDA). This reaction mixture was incubated at 37^{0}C for 60 minutes and then terminated by addition of 50 µl of 1N hypochloric acid. Blanks used in calculations (described below) consisted of the above but with cytidine substrate added at the end of the 60 minute incubation period. After termination of reactions, protein was precipitated from the mixture by addition of trichloroacetic acid (TCA, 2%). 20 µl of supernatant was injected for HPLC using ammonium acetate (15 mM) as the mobile phase with a flow rate of 0.35 mL/min through Xbridge™ OST C18, 2.5 µm, 4.6x50mm column on Dionex UltiMate® 3000 µ-HPLC system supported with *Chromeleon*® 7.1 chromatography data system (Dionex Corporation, Sunnyvale, CA). Retention time and peak area of uridine at 260 nm were compared to the internal control for each

injection. The average net uridine peak area of test minus blank was calculated for each test sample. Known concentrations of uridine (0.0 to 95.8 μM) were used to construct a standard curve to calculate uridine amount based on net uridine peak area. One unit (U) of CDA enzyme activity is defined as the amount of enzyme needed to produce 1 μmole of uridine in 1 minute. The specific activity of purified CDA (A79A) was 308.9 U/mg. Purified CDA was used for calibration and for quality control (gift of Professor Silvia Vincenzetti, Universita di Camerino, Italy). Multiple runs with known concentrations of uridine were used to confirm accuracy and precision: between run variability was < 5%.

Sequencing for the A79C SNP in CDA (Lys27Gln, rs2072671) *and real time quantitative PCR (QRT-PCR) measurement of CDA mRNA*. DNA was extracted from blood or bone marrow cells using the ArchivePure Kit (5Prime, Gaithersburg, MD) from bone marrow mononuclear cells or peripheral blood mononuclear cells isolated by Ficoll-Hypaque density centrifugation. DNA was amplified using primers 5-GTTCCTAAGGGAGAGTGTGAAGCA-3(forward) and 5-GCCTCTTCCTGTACATCTTCCTCT-3(reverse) (accession number NM_001785). Amplicons were generated with Expand High Fidelity^{PLUS} PCR System (Roche Applied Science, Indianapolis, IN) using 50ng genomic DNA. PCR conditions were: initial denaturation at 94°Cx4minutes, 30 cycles with denaturation at 94°Cx30s, annealing at 56°Cx30s, and elongation at 72°Cx40s. Amplicons were purified using the Montage PCR96 Cleanup Kit (Millipore,Billerica, MA) and sequenced using Big DyeTerminator v3·1 (Applied Biosystems, Foster City, CA), with forward primer. Sequencing reactions were purified using the Montage SEQ96 Sequencing Reaction Cleanup Kit (Millipore) and run on a 3100-Avant Genetic Analyzer (Applied Biosystems).

For QRT-PCR *(accession number NM_028176.1)*, primers were 5'-CDAAGGGTGACCTTGATTCACACACCA-3'(forward); 5'-CDATGGAATACCCGTGTCTTTGGGAGTACC-3'(reverse).

Decitabine pharmacokinetic studies in CD-1 mice: Procedures with CD-1 strain mice were approved by the IACUC of Avanza. Animals were dosed with decitabine or its vehicle via oral gavage at a dose volume of 10 mL/kg (based on the most recent body weight). Blood samples (~0.5 mL, or maximum possible) were collected via intra-cardiac puncture from non-fasted, anesthetized (70% CO2/30% O2) animals 15, 30, 60, 90, 120, and 180 minutes after administration of decitabine. Sample collection tubes were prepared prior to each collection

day by adding 10 µL/tube of a 10 mg/mL THU solution. This THU solution was prepared by adding sodium phosphate dibasic (1.5 mg/mL), sodium phosphate monobasic (0.4 mg/mL), and THU (10 mg/mL) to sterile water for injection and mixing until visually clear. Samples were collected from the first available 3 animals per time point. All samples were collected within 5 minutes of the target time. Two-way (gender vs. time) ANOVA tests (this is reasonable because each measurement was obtained from a separate mouse and is thus independent) were used to compare drug levels in female versus male mice; the R function `lm()` was used with time as a factor. In the high dose group the interaction terms were not significant, so an additive model was used. In the low dose group gender-time interaction terms were significant at 30 and 60 minutes, so interactions were kept.

Overlap probability. The two planes in Figure 5 represent the probability P of overlap between the fraction of time of adequate drug exposure D and the fraction of time of cell susceptibility S. The following R code snippet generates P: `D=seq(0,1,0.01); S=seq(0,1,0.01); P=outer(D,S,FUN="+"); P[P>1]=1.`
The R package `rgl` was then used to plot P as a surface in three dimensions. Algebraically, one derives P by letting D occupy positions 0 to D on the perimeter of a circle indexed 0 to 1, by noting that S and D miss each other if the beginning of S lies between D and 1-S, by noting that this happens with probability 1-S-D, and by noting that P is 1 minus this, i.e. P=S+D, unless S>1-D, in which case P=1.

Statistical Analysis of Clinical Data. SAS (Cary, NC) was used for all statistical analyses save those performed in R (above). Sample characteristics between patients of different gender were compared using the Wilcoxon test or Chi-square test. PROC LIFETEST to generate Kaplan-Meier product-limit survival estimates was used to model time from diagnosis to death (overall survival) and PROC PHREG (Cox proportional-hazards regression analysis) was used to assess the impact of multiple variables on time to death. All statistical significance levels were α=0.05. In the Cox model, variables assessed for influence on time from diagnosis to death were age, myeloblast% at diagnosis, metaphase karyotyping result (categorical variable: [i] Chromosome 7 or ≥3 chromosome abnormalities, [ii] Other chromosome abnormalities, [iii] Normal cytogenetics), and CDA genotype (categorical variable: [i] homozygous ancestral allele–AA, [ii] heterozygous ancestral allele–AC, [iii] homozygous variant allele–CC). Univariate variables with an $\alpha \leq 0{\cdot}05$ were retained in the multivariate model.

RESULTS AND DISCUSSION

The A79C SNP and female gender are associated with decreased plasma CDA enzyme activity. DNA from normal volunteers, MDS and AML patients was sequenced to identify cases with the A79C SNP. Plasma samples from equal numbers of individuals homozygous for the ancestral allele (AA, n=32), heterozygous for the A79C SNP (AC, n=32) and homozygous for the A79C SNP (CC, n=32) were then examined for CDA enzyme activity, using an HPLC based method described previously[30]. Consistent with observations from other groups[22,23,25,31], plasma CDA activity was significantly decreased in samples homozygous for the A79C SNP (CC), compared to samples homozygous for the ancestral allele (AA) ($p<0.01$) (**figure1A**). Male gender has been associated with lower cytidine analogue drug levels[27,28], however, the mechanism underlying this association has not been elucidated. Therefore, to evaluate a potential role of CDA in gender differences in cytidine analogue metabolism, the plasma samples were further sub-classified by gender. Male gender was associated with significantly higher CDA enzyme activity ($p<0.001$) (**figure1B**). Notably, the difference in CDA activity between male and female gender (>2.5-fold, **figure1B**) was greater than the difference in activity between AA and CC genotype within each gender (<1.5-fold) (**figure1B**).

Gender difference in CDA mRNA expression in the liver and in leukemic cells. CDA expression levels are highest in the liver (**figureS1**). To evaluate the basis for higher CDA enzyme activity in males compared to females, CDA gene expression was measured in murine liver tissue by QRT-PCR. CDA expression was >3-fold higher in murine male versus female liver tissue (n=6/group, $p=0.01$, **figure2**). Because higher CDA expression within malignant myeloid cells themselves is a possible mechanism of resistance to cytidine analogues[32-39], we explored a public database of microarray gene expression in AML cells (GSE15434[40]): there was significantly higher CDA expression in myeloid leukemia cells from males compared to females (**figureS2**). Furthermore, in a gene set enrichment analysis[41] of microarray data from male versus female lymphoblastoid cell lines[41], we found CDA amongst the top 20% of genes with higher expression in males versus females (data not shown).

Decitabine pharmacokinetics in female and in male mice. Higher CDA expression and enzyme activity in males compared to females can be expected to produce lower plasma cytidine analogue levels in males. To investigate this possibility, decitabine 0.4mg/kg (**figure3A**) or 1.0mg/kg (**figure3B**) was administered to CD1 mice by oral gavage and plasma drug levels were measured by LC-MS/MS at 15, 30, 90, 120 and 180 minutes. At both dose levels, plasma drug levels were decreased in males compared to females: this decrease was significant collectively across all time-points in the higher dose group (p=0.005, gender main effect in a genderxtime two-way ANOVA analysis) (**figure3B**) and at 30 minutes (p=0.0003) and at 60 minutes (p=0.01) in the lower dose group (**figure3A,** p-values for two-way ANOVA interaction terms).

Overall survival was significantly worse in male MDS patients treated with 5-azacytidine/decitabine. Higher CDA enzyme activity and expression in males, and hence lower 5-azacytidine or decitabine drug levels, could affect treatment outcomes. Overall survival (OS) stratified by gender was evaluated in MDS and AML patients treated with 5-azacytidine or decitabine or induction cytarabine between January 2002 and December 2007 at Cleveland Clinic. These patients also had IRB approved tissue-banked bone marrow samples available for sequencing analysis for the A79C SNP, and had verifiable survival annotation. Prognostically relevant pre-treatment characteristics (karyotype, bone marrow myeloblast percentage, A79C status) were similar in male and female MDS patients treated with 5-azacytidine or decitabine, except for significantly higher age in males (**table1**). In these patients, OS was significantly worse in males (n=69, median 563 days) compared to females (n=21, median 1033 days)(Log-Rank p=0.01)(**figure4A**); this difference remained significant in a multivariate Cox model analysis that controlled for age, karyotype, myeloblast% and the A79C SNP by including them as covariates (**table2**)**.** In AML patients treated with induction cytarabine (n=76) and stratified by gender or by A79C, there was no difference in OS (**figure4B**, data not shown).

The interaction between treatment exposure time and S-phase fraction of disease. A potential explanation for the significant association of gender with OS in MDS patients treated with 5-azacytidine or decitabine, but not in AML patients treated with cytarabine, is an interaction between treatment exposure time and S-phase fraction of disease: even a short exposure to S-phase specific therapy may treat a major portion of high S-phase fraction aggressive AML cells[42], but not more indolent low S-phase fraction disease (relatively

low S-phase fraction has been documented in MDS[43]). This question is amenable to mathematical modeling as follows: let D be the proportion of time that a cell is exposed to drug concentrations that are above the efficacy threshold (the threshold of 5-azacytidine or decitabine required to deplete DNMT1), and let S be the fraction of time spent in S-phase. Randomly placing intervals of length D and S on a unit perimeter circle, the probability P that D and S overlap as a function of their lengths is comprised of two planes as shown in **figure5**, with the plateaued upper plane representing a 100% probability of overlap. The expectation is that a progressive decrease in malignant cell burden over time will require P values above 0.5, that is, a greater than 50% possibility that a malignant cell division should overlap with drug levels above minimum thresholds required to deplete DNMT1. Thus, for larger S values as found in aggressive disease, operating points could be high enough up on the plane that gender differences in D have little to no significant effect on P (**figure5**). In contrast, for smaller S values as found in more indolent disease, lower values for D in males may be more likely to transit P below the 0.5 threshold (**figure5**).

Exposure time is a critical consideration with 5-azacytidine or decitabine treatment, since a major molecular objective of therapy, the depletion of DNMT1, is an S-phase specific effect. Accordingly, higher CDA expression and enzyme activity and consequently lower cytidine analogue plasma levels seen in males could contribute to worse outcomes in male MDS patients treated with 5-azacytidine or decitabine. Furthermore, upregulation of CDA expression in malignant cells has been implicated as a mechanism of resistance[32-39]; therefore, higher CDA expression in malignant myeloid cells from males could also contribute to poorer outcomes in this gender. In contrast to 5-azacytidine/decitabine treated MDS patients, we did not observe a gender difference in OS in AML patients treated with cytarabine. The mathematical modeling demonstrated that treatment exposure time is a more important consideration in low S-phase fraction disease, providing one potential explanation for a role of gender in MDS but not AML outcomes. Supporting this inference, in clinical trials from the same institution, relatively frequent administration of decitabine (10-20mg/m^2 for 5-10 days every 4 weeks) produced a response rate of 50% in the lowest risk MDS category and 28% in the higher risk categories (Intermediate-2 and High) whereas administration on fewer days (45mg/m^2/day for 3 days every 6 weeks) produced a response rate of 14% in the lowest risk MDS category and a response rate of 18% in the higher risk categories[11,13]. That is, the greatest detrimental impact of fewer days of treatment exposure was in

low risk disease. Another factor that may be contributing to an impact of gender in 5-azacytidine/decitabine but not cytarabine therapy could be the lower doses of 5-azacytidine or decitabine used; these lower levels of drug could be more vulnerable to falling below minimum thresholds for efficacy in patients with higher CDA. In contrast, with high dose therapy with cytarabine or gemcitabine, a concern is that pharmacogenetic factors that decrease drug metabolism and thereby increase drug levels may increase the risk for toxic death, since drug levels may already be close to maximum tolerated thresholds. Accordingly, female gender has been associated with higher drug levels and greater toxicity in studies of gemcitabine[25,27,44]. In other words, female gender could be a favorable factor with relatively low dose cytidine analogue therapy (e.g., with 5-azacytidine or decitabine), but conversely, may increase the risk of toxicity with high dose therapy, especially if these individuals concurrently carry SNPs in *CDA* (such as A79C) that additionally decrease enzyme activity[25].

Have gender differences in OS been observed in other MDS patient cohorts? Significantly worse OS in males has been reported in an analysis of 856 MDS patients, 50% of whom did not receive any therapy, and 17% of whom received therapy such as hypomethylating agents[45]. In a separate cohort of 897 untreated MDS patients, significantly poorer OS was again observed in males[46]. In most reports of clinical trials of 5-azacytidine or decitabine to treat MDS, the effects of gender on OS were not described. In studies which did examine for an association between gender and OS, statistically non-significant worse OS for males versus females (14 versus 17 months) was seen in one study of 177 mostly higher risk MDS patients treated with decitabine[47]. In another study of 358 higher risk MDS patients randomly assigned to receive either 5-azacytidine or other treatments including low dose or induction cytarabine, statistically non-significant worse OS was observed in males[48]. We suggest that response and OS stratified by gender should be more widely examined in MDS patient cohorts, especially in patients with lower risk disease, to further evaluate the role of this genetic factor in 5-azacytidine or decitabine treatment outcomes. However, the gender differences in OS documented in untreated patients, and in MDS incidence (**figureS3**), indicate that additional unknown factors beyond higher CDA expression contribute to poorer OS in males.

To prospectively address decreased treatment efficacy secondary to higher CDA enzyme activity one strategy could be to empirically administer higher doses of 5-azacytidine or decitabine to males. However, it may be

more ideal to use biomarkers of the intended pharmacodynamic effect (e.g., DNA methylation or DNMT1 levels), to guide adjustments to therapy. Biomarkers could simultaneously account for the effects of other pharmacogenetic factors, e.g., A79C. Measuring CDA enzyme activity has also been proposed as an aid to rational dose modification[31]. A complementary approach might be to dampen the influence of CDA altogether, by combination therapy with a CDA inhibitor (e.g., tetrahydrouridine)[2,15]. Combination with a CDA inhibitor may also attenuate CDA-mediated cancer resistance at the cellular level[49,50], and cancer cell sanctuary from cytidine analogue effects in tissues expressing high levels of CDA (Ebrahem *et al*., manuscript submitted).

Decitabine and 5-azacytidine, cytidine analogues with a powerful S-phase specific molecular epigenetic effect, have an important and evolving role in oncotherapy. However, an important determinant of efficacy is treatment exposure time, and as the present study shows, this can be significantly impacted by gender differences in CDA expression and activity. The possibility that higher CDA expression and activity in males is a cause of inferior outcomes in male MDS patients treated with 5-azacytidine or decitabine warrants further evaluation in additional MDS patient cohorts. If confirmed, the effects of this genetic factor on outcomes should be amenable to rational intervention.

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COMPETING FINANCIAL INTERESTS

The authors have no competing financial interests in relation to this work. In the interests of full disclosure of information, a patent application has been filed for the combination of oral tetrahydrourine and decitabine.

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Table 1: Pre-treatment characteristics of female and male MDS patients treated with 5-azacytidine or decitabine.

	Female patients (n=21)	Male patients (n=69)	p-value
Age (median ± IQR)	63 ± 13	67 ± 12	0.04*
Bone marrow myeloblast% (median ± IQR)	6 ± 9	6.5 ± 11	0.41*
≥3 chr. abnormalities or chr. 7 deletion	5/18 (27%)	23/63 (37%)	0.65#
Other abnormal cytogenetics	4/18 (22%)	9/63 (14%)	
Normal cytogenetics	9/18 (50%)	31/63 (49%)	
CDA genotype AA	8/17 (47%)	21/60 (35%)	0.40#
CDA genotype AC	8/17 (47%)	28/60 (47%)	
CDA genotype CC	1/17 (6%)	11/60 (18%)	
Number of treatment cycles (median ± IQR)	7 ± 14	7 ± 8	0.42*

*Wilcoxon test; #Chi-Square test;

Table 2: Multivariate analysis (Cox Proportional Hazards model) of overall survival in MDS patients treated with 5-azacytidine or decitabine (n=90).

	Univariate p-value	Hazard Ratio (95% confidence limits)	Multivariate p-value
Gender (m v fm)	0.0129	0.356 (0.165-0.766)	0.0083
CDA genotype (AA, AC, CC)	0.7336		
Age (continuous)	0.0004	1.038 (1.002-1.075)	0.0382
BM blasts (continuous)	0.2044		
Cytogenetics (deletion 7 or complex vs other)	<0.0001	1.992 (1.435-2.764)	<0.0001

FIGURE LEGENDS

Figure 1: A) Plasma CDA enzyme activity by *CDA* genotype. DNA sequencing was used to identify individuals homozygous for the A79C SNP in *CDA* (CC genotype), heterozygous for A79C (AC genotype) or homozygous for the ancestral allele (AA genotype). Plasma samples from equal numbers of individuals with these genotypes were assessed for CDA enzyme activity using an HPLC-based assay. Box-plot boundaries = inter-quartile range, horizontal line = median, whiskers = range of values. p-value from Wilcoxon test. **B) Samples were further sub-classified by gender**. p-value from Wilcoxon test.

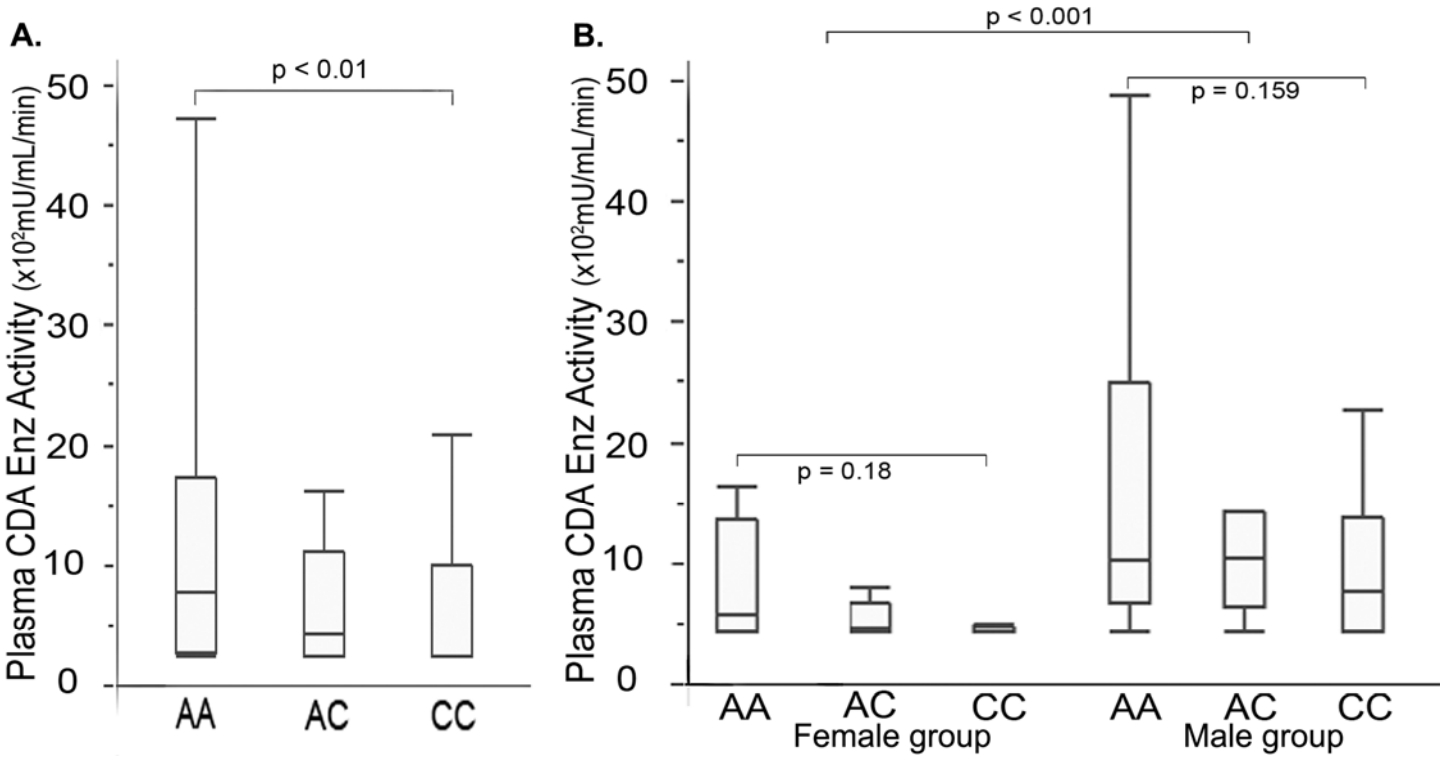
Figure 2: CDA expression was increased in liver tissue from male mice compared to female mice. CDA expression was measured by QRT-PCR.

Figure 3: Decitabine plasma drug levels were decreased in male compared to female mice at multiple time-points after administration of decitabine by oral gavage. A) Mice administered decitabine 0.4 mg/kg. Drug levels measured by LC-MS/MS. Levels from three mice per gender for each time-point. **B) Mice administered decitabine 1.0 mg/kg.**

Figure 4: Overall survival (OS) was significantly worse in male MDS patients treated with 5-azacytidine/decitabine. OS was evaluated in MDS patients initiated on treatment between January 2002 and December 2007 at Cleveland Clinic, with IRB approved tissue-banked bone marrow samples available for analysis for the A79C SNP by sequencing, and with verifiable follow-up and survival annotation. **A) OS in MDS patients treated with 5-azacytidine or decitabine. B) OS in AML patients treated with cytarabine.** All these patients received induction cytarabine in combination with daunorubicin, some received additional cycles of consolidation which included cytarabine.

Figure 5. The interaction between exposure time to S-phase specific therapy and the S-phase fraction of disease. D is the fraction of the time that drug levels are above the efficacy threshold (e.g., minimum levels

required for DNMT1 depletion in the case of 5-azacytidine or decitabine). S is the fraction of time over which malignant cells are susceptible to therapy because they are in S-phase. P is the probability of overlap between D and S. The plateau in P represents a 100% probability of overlap. The expectation is that values of P (the intersection of the red and yellow lines in the figure) should exceed 0.5 (50%) to produce a progressive decrease in malignant clone burden over time. The red lines represent relatively low versus higher S-phase fraction disease. The yellow lines represent differences in treatment exposure time that might result from gender differences in cytidine analogue metabolism, inferred from the present data and published gender differences in gemcitabine pharmacokinetics.



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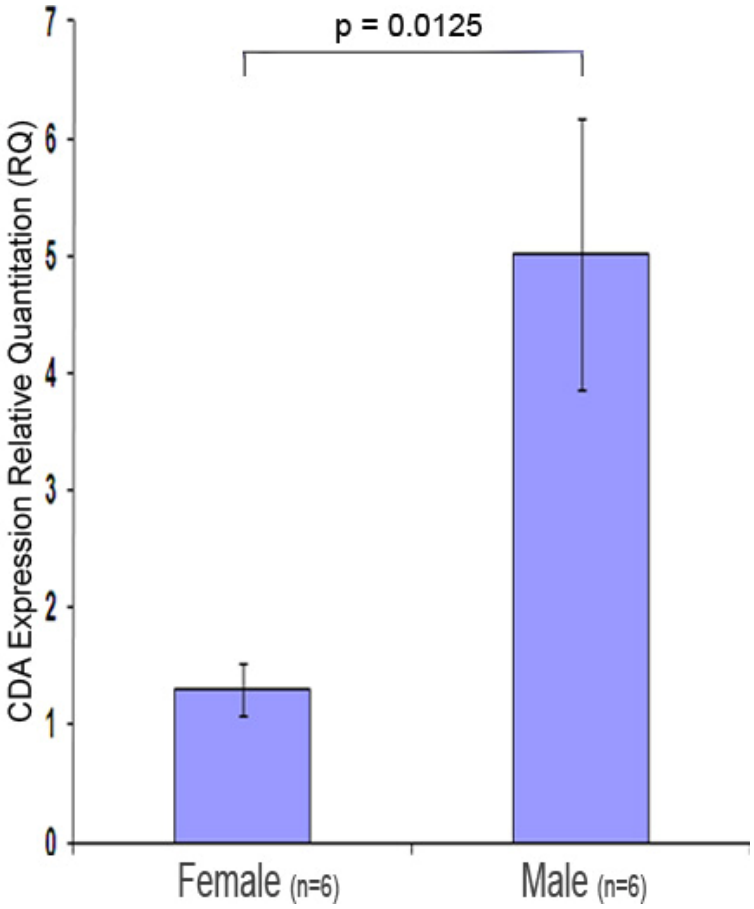

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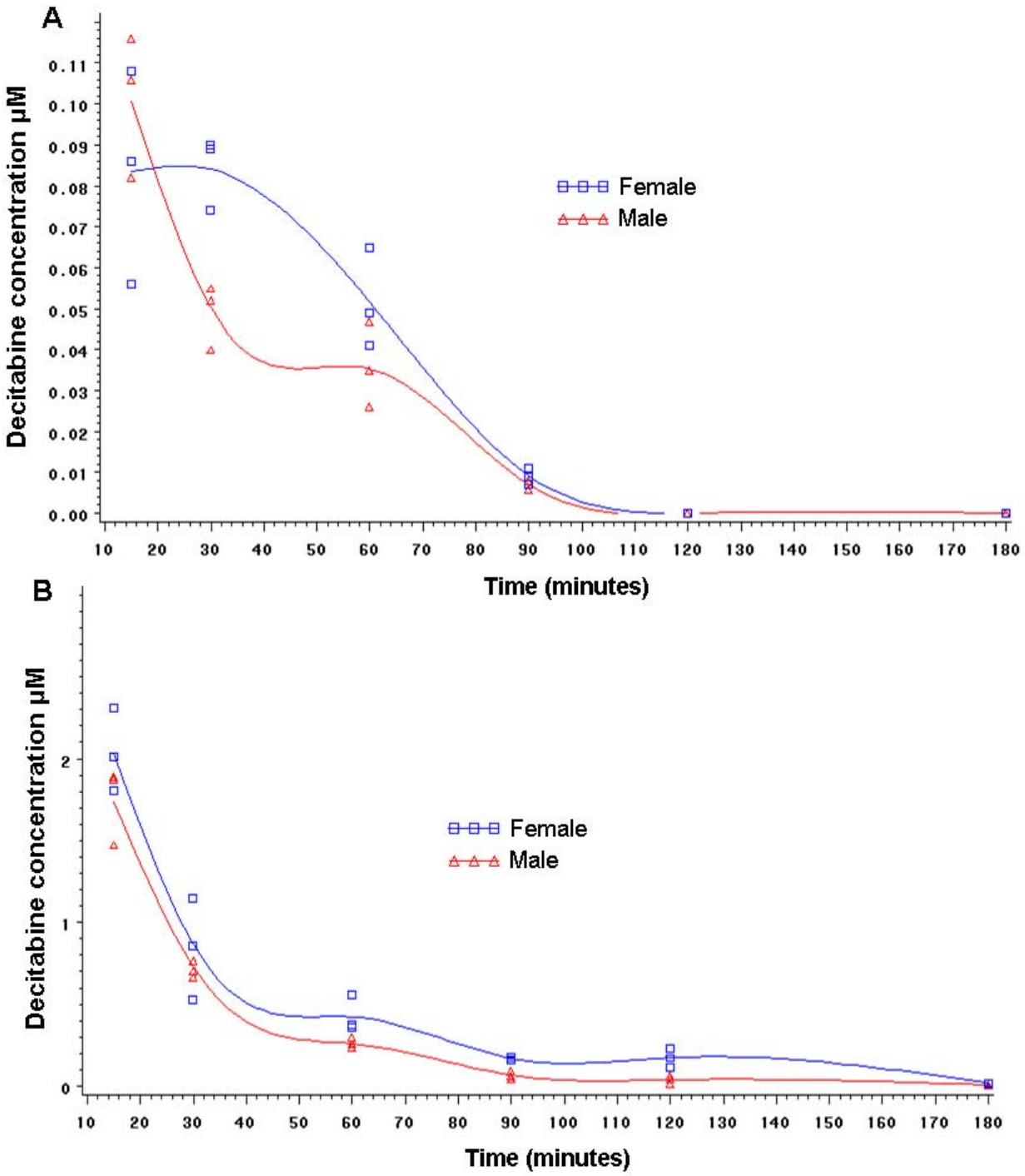

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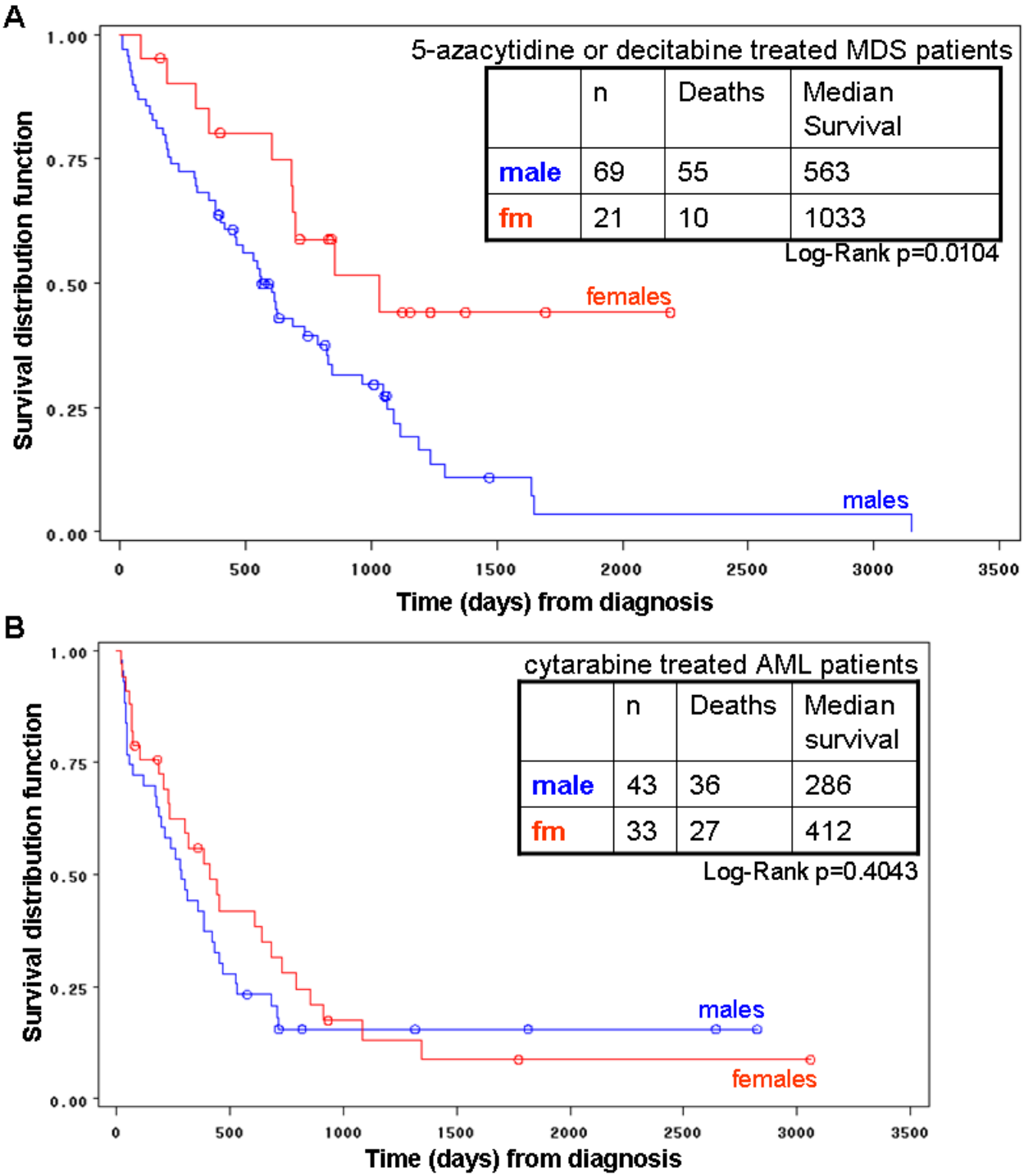

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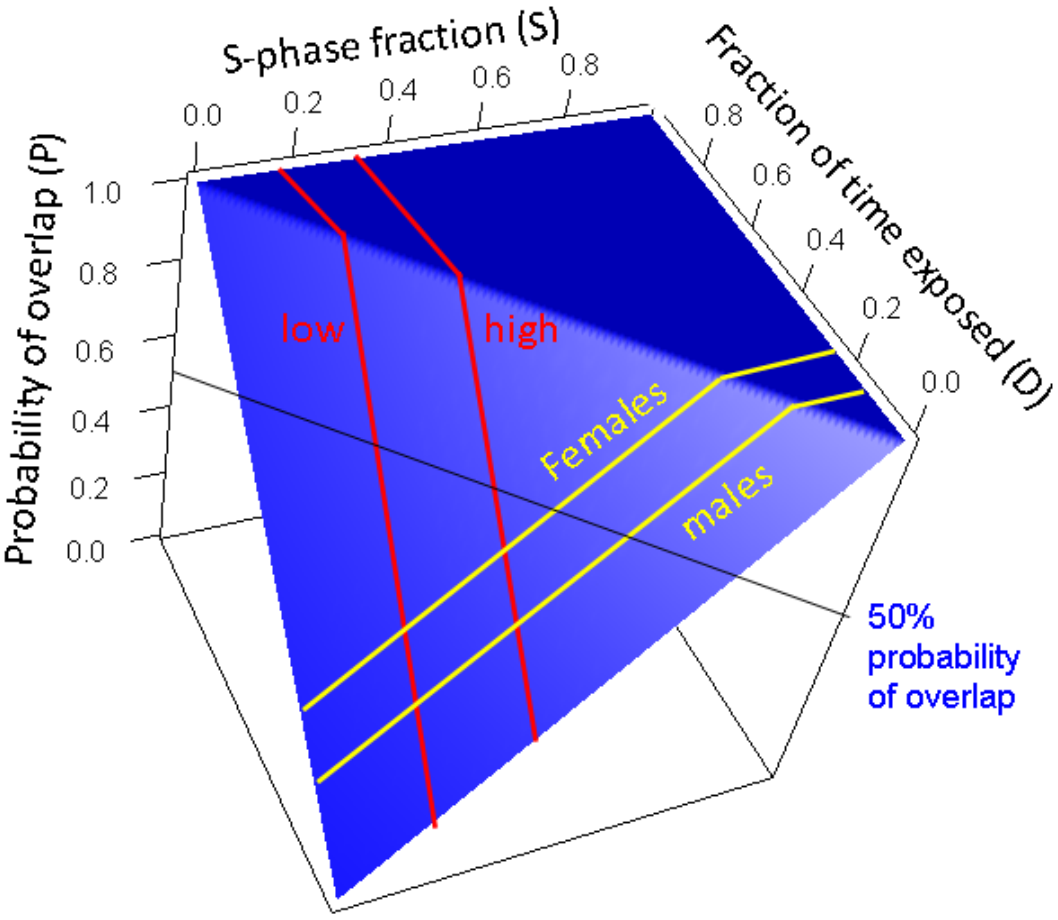

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