The effect of DFMO on levels of polyamines in colon mucosa

Summary

The treatment of colon cancer is an important area of current oncology research. Pharmaceutical treatment options are sought to improve outcomes. One potential therapeutic pathway is the inhibition of ornithine decarboxylase (ODC), which participates in the synthesis of a number of carcinogenic markers known as polyamines. Difluoromethyl ornithine (DFMO) has been shown to be an irreversible inhibitor of ODC in animal and human models. We conducted a phase II double-blind randomized trial consisting of 114 patients to investigate and characterize the suppression of polyamine levels by DFMO over time. Four treatment arms were included in the study: zero (placebo), 0.075, 0.2 and 0.4g/sq m/day, and each arm was followed by a 12 month treatment period and a 3 month off-treatment period. Measurements of polyamine levels were taken at randomization and at 6, 12, and 15 months. Overall, DFMO's inhibition of ODC resulted in decreased polyamine levels for all three treatment groups, as measured by the ratio of spermidine to spermine in treatment groups compared to the placebo group (mean ratio differences of -0.210, -0.172, -0.263 for dose groups 0.075, 0.2, and 0.4 respectively; all of these differences were statistically significant with p-values less than 0.05). Furthermore, the effect of DFMO did not increase significantly over time, and any of the drug's effect on polyamine levels tended to be reversed by discontinuing therapy for a period of three months. These findings suggest that DFMO may be a viable option for the treatment of colon cancers.

Comment [A1]: sex, age

Comment [A2]: presumably at 12 months

Comment [A3]: I would have given the CI and p values for each dose group

Comment [A4]: I think this was a good way to summarize these findings

Background

According to the National Center for Health Statistics, the incidence of colon cancer in the US is estimated at 61.4-66.8 cases per 100,000 in the 1980's. This accounts for approximately 14% of all cancer cases. Surviving this disease is directly related to early detection, as treatments are lacking for the fully progressed disease state. Surgery is a proven therapy for localized disease. However, due to the size of the colon and ease of development of metastases, chemotherapeutic treatments are sought to improve outcomes for more severe stages of carcinoma.

Polyamines represent a class of metabolites that are essential for the growth and proliferation of all human cells. Furthermore, it is hypothesized that alterations to polyamine metabolism may occur during carcinogenesis.[1] Activity of ODC, a metabolic enzyme, has been shown to increase in carcinogenesis with the addition of carcinogens and decrease with the addition of tumor inhibitors.[2] It has been demonstrated that ODC is the controlling enzyme in polyamine synthesis and that its effects regulate the growth of cellular tissues by modulating both tumor necrosis factor and interleukin-1.[3] It is hypothesized that inhibition of ODC by chemical means could decrease the severity of or cure carcinomas of the colon by directly down-regulating the growth of tumors.

The biochemical association between ODC and polyamine synthesis as well as the cellular activity of DFMO's irreversible inhibition of ODC has been previously studied in rat and human models of several types of cancers including lung and colon carcinoma.[4-6] The proposed mechanism of action of DFMO is the irreversible inhibition of ornithine decarboxylase. This enzyme is responsible for the direct conversion of ornithine to putrescine. In normal cells, the polyamine metabolic pathway progresses by the rapid conversion of putrescine to spermidine. It is unknown whether or not this pathway exists equally in cancerous cells. However, a fourth polyamine, spermine, tends to be related to spermidine in a ratio that decreases with the inhibition of ornithine decarboxylase. Upp and colleagues have demonstrated that the ratio of spermidine to spermine is the most accurate measure of polyamine concentration for both diagnosis and staging of colon cancer severity.[7]

Comment [A5]: Personally, I would soften the wording to "have found".

Questions of Interest

The main question of interest is whether DFMO suppresses polyamine levels in the colon tissue. If suppression takes place, secondary questions are whether this suppression is sustained or increases over treatment periods, and whether polyamines levels return to normal levels after treatment is stopped or

Comment [A6]: Given that you are choosing the 12 month point as your primary outcome, I would have stated this question as seeing how early an effect is noticeable.

remain suppressed. To address these questions, we first analyzed whether spermidine to spermine ratios were lower in each treatment group compared to the placebo group after the 12 month treatment period. We then analyzed whether the mean difference in ratios between each treatment group and the placebo group stayed constant or increased from the first 6 months to the last 6 months. Finally, we analyzed whether mean ratios after the treatment period ended were comparable between the treatment and placebo groups. The ability of these analyses to address the major questions hinges on the presumed quality of our suppression measure and study design.

Comment [A7]: Not wrong to do, but usually we look at ratios of ratios rather than differences of ratios.

Source of Data

A phase II clinical trial was conducted with 114 volunteers with a history of colon polyps. These volunteers were randomized in a double-blind fashion to receive either placebo or one of three different doses of DFMO and were followed-up for 15 months, with a 12 month treatment period followed by a 3 month off-drug period. The doses of DFMO used in the trial were 0.075, 0.2, and 0.4 g/sq m/day. Thirty-two (32) subjects were assigned to the placebo group, 29 subjects to the 0.075 g/sq m/day, 25 subjects to the 0.2 g/sq m/day, and 28 subjects to the 0.4 g/sq m/day group. Measurements of three polyamines (putrescine, spermine, and spermidine) were obtained at the time of randomization (baseline), after 6 months of treatment, at the end of treatment period (12 months after randomization), and 3 months post-treatment. All subjects had measurements of polyamine levels at randomization. However, the number of measurements after randomization, as well as the time points at which the measurements occurred, varied for the 27 participants who did not return for some or all of the three post-randomization measurements. This lack of study adherence occurred in all dose groups but was greatest in the highest dose group (see Table 1).

Comment [A8]: This is of course an important observation, and as the loss of follow-up may represent toxicity, I would probably go into this as Results. Here I would just mention how you handle this problem.

Demographic characteristics (age and sex) were also obtained from participants, allowing for effect modification or precision analysis using these variables. Polyamine measurements, or more precisely the ratio of spermidine to spermine, were used to look at the effect of the DFMO across dose groups and over time. Two subjects (one in the 0.075 g/sq m/day group and one in the 0.4 g/sq m/day) had spermine measurements of zero, about 2 µmol/mg lower than the next lowest measurements. Given the low frequency of zero values for spermine, these ratios were excluded from the analysis.

Comment [A9]: These do in fact present a problem. Was sperimidine also zero? If so, then we are perhaps more justified in ignoring these measurements from our primary analyses, though I would not remove them from the descriptives.

Statistical methods

For our analysis, we considered the ratio of spermidine to spermine for any given subject as the best measure of polyamine suppression. To answer whether DFMO suppresses polyamine levels, we first assessed whether the ratios after the 12 month period treatment period were different between individual drug groups and the placebo group. Since we are interested only in detecting a decrease in the spermidine to spermine ratio, we used a one-sided two-sample t-test allowing for unequal variance to compare the group means of the ratios and computed two-sided 95% confidence intervals to evaluate the precision of our estimate. A negative difference between the means of the treatment group and the mean of the placebo group would indicate that DFMO would have a suppressive effect on the polyamine levels. We also considered analyzing measurements after just 6 months of treatment, but felt that if DFMO had a suppression effect on polyamine levels, it should be more easily detectable after a longer course of treatment. In addition, we looked at the per-participant change in ratios over the 12 month treatment period using a paired t-test and one-sided 95% confidence intervals; this difference should be negative if the ratio has decreased over time for patients taking DFMO.

Comment [A10]: Good. And hopefully using a p value of 0.025 as the threshold

Comment [A11]: No travelog. I would explain that the 6 month results were primarilarily of interest to describe how early effects might be noticed

Our second question is whether the suppression of polyamine levels is sustained or whether it increases over longer treatment periods. For this question we looked whether the difference between each treatment group and placebo was greater at 12 than at 6 months. A negative difference between mean difference of ratios at 12 months and mean difference of ratios at 6 months would suggest that the suppression effect of DFMO increases, while a positive difference would indicate that the effect was reversed in the second half of the treatment period. Since our major concern is detecting an increase in suppression, we again used a one-sided two-sample t-test of unequal variances with two-sided 95% confidence intervals.

For our last question, whether polyamines levels return to normal after treatment is stopped, we compared each treatment group's mean ratio at 15 months to the placebo group mean ratio. A negative difference

would mean that the polyamine levels were still suppressed after stopping the treatment. Since we only wish to detect if the suppression is still present after discontinuing treatment for 3 months, we again used a one-sided two-sample t-test with unequal variances and two-sided 95% confidence intervals.

The level of significance of all our tests was 0.05. Missing data was considered to be potentially significant and is considered in the results and discussion in conjunction with analyses performed (necessarily) excluding the missing data.

Comment [A12]: With one-sided tests, we ought to use 0.025. (To my mind, as well as to many others)

Statistical analyses were performed using STATA 10 for Macintosh (StataCorp LP, College Station, TX, USA) and R 2.4.0.

Results

Participants were randomized to the treatment groups in approximately equal numbers, with a slightly larger number assigned to the placebo group (Table 1). Age distributions were fairly similar across groups, participants being 45.4-81 years with mean group ages ranging from 61.3-65.9 ± 7.7-8.5 (mean ± SD). In keeping with the disease profile of those at risk for colon cancer, a greater proportion of study participants were male. Randomization of the small number of females resulted in no females being assigned to the 0.075 dose group and 21% of females assigned to the 0.4 dose group. An association between sex and polyamine levels or between sex and response to DFMO treatment could thus potentially confound results, but stratification by sex showed no tendencies towards different polyamine levels across groups at any time point and suggested no need for sex-adjustment.

Comment [A13]: This is still disproportionate amount of males, however. (A VA hospital was one of the recruiting centers.)

Comment [A14]: This would have been exploratory if not prespecified

Measurements for spermidine and spermine were distributed fairly similarly across dose groups at the time of randomization, confirming a successful reduction of potential confounding by initial polyamine levels. These distributions were all right-skewed around their means (which were comparable across dose groups). The main distributional difference between polyamines was that spermine measurements were more tightly clustered around the mean, but its outliers were much more extreme. Over time, spermine levels generally decreased. Trends for spermidine were less generalizable, with slight decreases in the lower three dose groups and a more marked decrease in the highest dose group. The ratio of spermidine/spermine, our measure of interest in detecting DFMO effects, seemed to follow the same pattern as spermine, except in the placebo group in which it generally increased over time of treatment (Figure 1). It is worthy to mention that the means of the ratios were fairly similar at the time of randomization, indicating that baseline measurements should not confound the difference when comparing treatment groups to placebo.

Noting the change in number of participants (N) with time interval reveals patterns in lack of study adherence, summarized by the difference across groups in mean number of measurements per patient (# mmts/person in table 1). Of the 32 subjects in the placebo group, 30 had measurements after 6 months, 28 after 12 months, and 27 after 15 months. Of the 29 subjects in the 0.075 g/sq m/day group, 28 had also measurements after 6 months, and 26 after 12 months and 15 months (note that these 26 subjects are not the same at both times). Of the 25 subjects in the 0.2 g/sq m/day group, 23 had also measurements after 6 months, and 21 after 12 months and 15 months. Finally, of the 28 subjects in the 0.4 g/sq m/day group, 25 had also measurements after 6 months, 20 after 12 months, and 18 after 15 months. These numbers illustrate that two higher dose groups had proportionally fewer people returning for later follow-ups, particularly for 12 and 15 months. This is especially true for the 0.4 dose group, which lost 36% participants by the final follow-up, a non-ignorable difference when compared to follow-up in the placebo group (a loss of 13% for the final follow-up). This must be considered when assessing the analysis results.

Comment [A15]: Very good to note. And you might speculate on toxicity.

After 12 months of therapy, the difference between each dose group and the placebo group showed significant decreases in polyamine levels (Table 2 (A)). The 0.075 dose group had a 0.210 decrease in its ratio (p-value=0.01), the 0.2 dose group had a 0.172 ratio decrease (p-value=0.049), and the 0.4 dose group had a 0.263 ratio decrease (p-value=0.003). However, even though these decreases were significant, the levels of polyamines were fairly constant across measurements for each individual dose group, as it can be seen in Figure 1. It is clear from the figure that the placebo group had a slight increase in their ratio across measurements.

Comment [A16]: A difference in ratios is a little hard to interpret without knowing what the baseline ratio was. As noted above, ratios of ratios is a bit more common to examine. Also, greater statistical precision is generally achieved when analyzing on log transformed data. But this approach is not wrong.

To better understand the relative trends within each group, we also compared the effect of particular doses on the polyamine levels at month 12 with respect to baseline (Table 2 (B)), using paired t-tests. It is worthy to mention that we only obtained a significant decrease in the ratio for the highest (0.4) dose group of 0.129 (p-value=0.043). Hence, the significance of the differences between the two other treatment groups and placebo must be caused by the increase of the mean ratio in the placebo group.

Comment [A17]: Quite descriptive, as we did not randomize to time, only to dose. We had the placebo group to remove confounding by aging, seasonal trends in diet, laboratory drift, etc.

The difference in mean ratio between the treatment groups and placebo at 12 months was not significantly greater than the difference at 6 months for any dose level (Table 2 (C)). Hence, for all treatment groups, we cannot reject the null hypothesis that the effect of DFMO on polyamines is constant or increases in the second half of the treatment period.

Differences in the mean ratios of spermidine to spermine between the treatment and placebo groups at 15 months were not statistically significant greater than zero in any of the groups (Table 2 (D)). Mean differences between the three dosage groups and the placebo group were positive (0.077, 0.225 and 0.004, with p values=0.889, 0.97, and 0.532, respectively), and we thus do not reject the null hypothesis that post-treatment levels resemble those in the placebo group.

Discussion

These results partially confirm previous studies of the effect of DFMO on polyamine levels[4-6]. With DFMO therapy, polyamine levels are suppressed when compared to the placebo (as measured by the ratio of spermidine to spermine) via inhibition of ODC. This suppression occurred with the greatest effect in the highest dose group (0.4g/sq m/day), indicating that higher doses lead to greater suppression. Due to the size limitation of our study, a true dose-response curve does not appear estimable.

To determine if treatment for longer periods of time resulted in variable therapeutic effects, we analyzed differences in suppression in two sequential six month periods. Our analysis shows that suppression does not increase over time (p values > 0.1), indicating that once the ODC enzyme has been inhibited, polyamine levels do not get suppressed further. This is consistent with the proposed irreversible inhibition of ODC by DFMO.

In addition, our investigation of whether or not the suppression would continue after discontinuing DFMO treatment showed that the ratio of spermidine to spermine post-treatment compared between on-drug groups and placebo group had a non-significant difference. Even for the highest dose of DFMO, stopping treatment leads to a return to initial polyamine levels (p values > 0.5), indicating that high doses must be maintained to see suppression effects. Furthermore, these results indicate that long term toxic effects of DFMO on other cellular tissues should not exist.

As mentioned previously, Figure 1 depicts that the ratio of spermidine to spermine in the dose groups and the placebo group, and while all the dose groups tended to increase their polyamine levels after the end of the treatment, the polyamine levels in the placebo group decreased back to the initial level. This effect in the placebo seems unusual. The natural assumption would be that the polyamine levels (represented by the spermidine to spermine ratio) are increasing over time for patients with history of colon polyps. Under this assumption, however, the mean ratio would continue to increase after the end of the treatment for the placebo group, but instead it drops back to the initial level. It can be seen in Figure 1 that at month 12, the placebo group has what it seems to be 3 influential points, that might over-influence the mean. Post-hoc analysis would suggest that other measures (like the geometric mean) would have been more accurate to asses the importance of these points and whether there was a true difference across groups. Also, given that the data is positively skewed, the geometric mean would have given more precise estimates than the mean in this situation.

It is important to note that if the missing post-randomization measurements were indeed non-ignorable and biased towards patients experiencing greater polyamine suppression, these results do not properly represent the effects of higher doses of DFMO. Reasons for patient non-adherence could range from chance to uncomfortable side effects resulting in self-removal from the study, and in the latter case this study would not be capturing those participants most affected by DFMO.

Comment [A18]: Because toxicity is always a concern, and especially so in early studies, and because this missing data may be biasing, I would probably have started the discussion commenting on the missing data. (Others might have followed your organization)

Further investigation into the true dose-response relationship of DFMO and the psychological effects of placebo treatment on polyamine levels is necessary to properly characterize the therapeutic dosing guidelines for DFMO. Investigation into reasons for study adherence or non-adherence will also clarify whether studies with DFMO may be suffering from a lack of representative data and whether effective doses are in fact somewhat toxic.

References

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	PLACEBO								DOSE = 0.075								DOSE = 0.200								DOSE = 0.400							
	N	*mean*	*SD*	*min*	*25th %ile*	*med*	*75th %ile*	*max*	*N*	*mean*	*SD*	*min*	*25th %ile*	*med*	*75th %ile*	*max*	*N*	*mean*	*SD*	*min*	*25th %ile*	*med*	*75th %ile*	*max*	*N*	*mean*	*SD*	*min*	*25th %ile*	*med*	*75th %ile*	*max*
age	32	65.9	8.5	45.5	61.1	66.4	73.5	77.2	28	61.3	7.7	47.8	56.5	61.4	65.8	76.9	25	62.8	8.3	45.4	59.2	63.7	68.3	77.6	28	63.9	7.8	48.5	60.1	65.0	69.4	81.0
sex*	32	0.19	0.40	-	-	-	-	-	29	0.17	0.38	-	-	-	-	-	25	0.00	0.00	-	-	-	-	-	28	0.21	0.42	-	-	-	-	-
# mmts/person	-	3.65	-	-	-	-	-	-	-	3.76	-	-	-	-	-	-	-	3.60	-	-	-	-	-	-	-	3.25	-	-	-	-	-	-
spermidine																																
baseline	32	3.26	1.45	1.4	2.08	2.93	4.17	7.1	29	3.47	1.55	1.51	2.29	2.91	4.62	7.02	25	3.35	1.33	1.7	2.42	2.92	4.01	6.22	28	3.57	1.89	0.66	2.15	3.08	4.7	7.6
6 mos.	30	3.37	1.53	1.51	2.2	3.03	4.28	6.9	28	2.64	0.89	1.39	1.94	2.46	3.05	5.12	23	2.58	1.64	1.07	1.49	1.85	3.9	7.84	25	2.68	1.43	1.06	1.67	2.07	2.9	6.34
12 mos.	28	3.26	1.31	1.01	2.26	2.82	4.27	5.9	26	2.92	0.99	1.35	2.13	2.86	3.64	4.92	21	2.71	1.40	0.29	1.76	2.51	3.78	6.45	20	1.95	0.80	0	1.48	1.93	2.46	3.42
15 mos.	27	2.69	0.93	1.25	2.04	2.45	3.37	4.6	26	2.95	0.99	0	2.4	2.98	3.63	4.83	21	2.98	0.90	1.81	2.2	2.81	3.71	4.81	18	2.70	0.87	1.29	2.36	2.69	3.02	4.47
spermine																																
baseline	32	8.22	5.54	1.46	6.04	7.52	8.77	36	29	8.43	5.86	4.13	6.29	7.32	8.75	37.7	25	9.03	7.04	2.54	6.7	7.53	8.89	41.7	28	8.08	5.50	2.28	5.57	6.81	8.78	34
6 mos.	30	7.34	2.71	3.31	5.58	6.51	8.69	14	28	8.08	2.49	4.6	6.2	8.01	9.21	15.7	23	7.18	2.49	2.35	5.46	7.37	8.74	12.1	25	8.04	3.95	2.76	5.43	8.02	9.81	17.2
12 mos.	28	6.55	3.59	2.32	3.65	5.24	9.06	15	26	7.75	3.12	3.15	4.78	8.35	10.1	14.1	21	7.16	3.15	2.96	4.48	6.63	8.78	13.8	20	5.93	2.58	0	4.45	5.97	7.5	10.7
15 mos.	27	6.40	2.45	2.83	4.5	5.79	8.67	12	26	6.69	3.32	0	4.39	6.3	9.36	12.4	21	6.08	3.56	1.93	3.14	4.61	9.64	12.4	18	6.43	2.53	2.53	4.45	6	8.07	11.5
spermidine/spermine																																
baseline	32	0.46	0.23	0.12	0.3	0.37	0.64	1.2	29	0.46	0.22	0.13	0.29	0.43	0.56	1.11	25	0.46	0.31	0.12	0.3	0.34	0.48	1.73	28	0.48	0.24	0.2	0.31	0.41	0.59	1.16
6 mos.	30	0.51	0.27	0.14	0.32	0.41	0.59	1.1	28	0.34	0.12	0.2	0.25	0.31	0.43	0.64	23	0.40	0.33	0.17	0.21	0.28	0.43	1.72	25	0.45	0.44	0.16	0.19	0.32	0.53	2.2
12 mos.	28	0.62	0.43	0.21	0.4	0.49	0.64	2.1	26	0.41	0.14	0.19	0.3	0.39	0.49	0.73	21	0.45	0.28	0.03	0.27	0.37	0.59	1.03	19	0.36	0.14	0.18	0.23	0.35	0.41	0.76
15 mos.	27	0.45	0.16	0.24	0.32	0.46	0.55	1	25	0.53	0.27	0.27	0.31	0.38	0.76	1.21	21	0.68	0.50	0.25	0.34	0.54	0.68	2.18	18	0.46	0.15	0.22	0.36	0.41	0.59	0.75

* mean represents proportion female

Table 1. Descriptive statistics for demographic indicators, number of measurements at each time point, and polyamine levels and level ratios

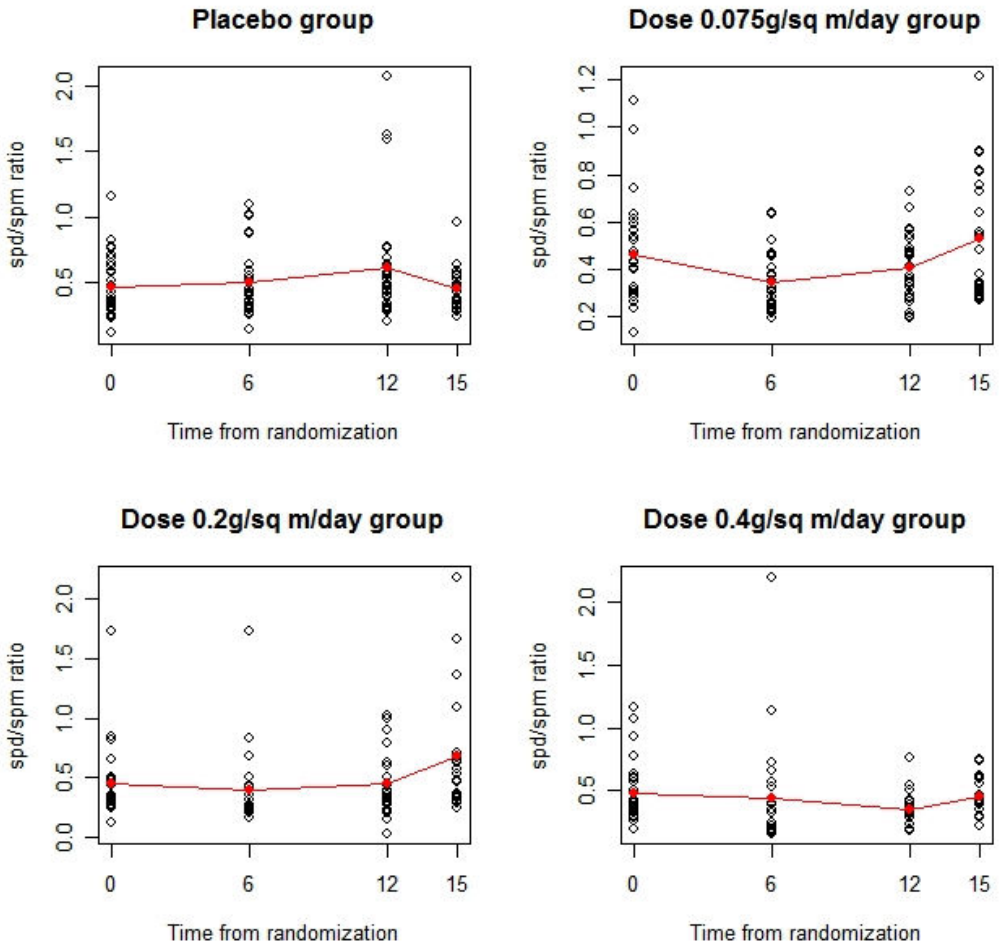

Figure 1. Spermidine/spermine ratio distributions at each time point for each dose group. Mean values shown in red.

	(A) *Comparison of spermidine/spermine at 12 months between each treatment group and the placebo group*			**(B)** *Comparison of the change in spermidine/spermine from 0-12 mos. within each treatment group*			**(C)** *Comparison of differences in spermidine/spermine between each treatment group and the placebo group, between 12 mos. and 6 mos.*			**(D)** *Comparison of spermidine/spermine at 15 mos. between each treatment group and the placebo group*		
Dose group	**Estimate**	**95% confidence interval**	**1-sided p value**	**Estimate**	**95% confidence interval**	**1-sided p value**	**Estimate**	**95% confidence interval**	**1-sided p value**	**Estimate**	**95% confidence interval**	**1-sided p value**
0	-	-	-	0.180	(-0.006, 0.366)	0.972	-	-	-	-	-	-
0.075	-0.210	(-0.385, 0.034)	0.010	-0.032	(-0.141, 0.077)	0.278	-0.013	(-0.205, 0.178)	0.444	0.077	(-0.048, 0.202)	0.889
0.2	-0.172	(-0.377, 0.032)	0.049	-0.005	(-0.243, 0.233)	0.482	-0.008	(-0.201, 0.186)	0.469	0.225	(-0.010, 0.460)	0.970
0.4	-0.263	(-0.441, -0.085)	0.003	-0.129	(-0.279, 0.021)	0.043	-0.175	(-0.465, 0.115)	0.114	0.004	(-0.093, 0.101)	0.532

Table 2. Inferential statistics for four analysis: (Comparisons are for means of spermidine/spermine ratios for the indicated groups and times)
(A) Null: (treatment at 12 mos. – placebo 12 mos.) ≥ 0; Alternative: < 0. Two-sample t-test, unequal variances.
(B) Null: (treatment at 12 mos.– treatment at 0 mos.) ≥ 0; Alternative: < 0. Paired t-test.
(C) Null (treatment – placebo) at 12 mos. ≥ (treatment– placebo) at 6 mos; Alternative: < . Two-sample t-test, unequal variances.
(D) Null: (treatment at 15 mos. – placebo 15 mos.) ≥ 0; Alternative: < 0. Two-sample t-test, unequal variances.