Title

Alteration of RNA modification signature in human sperm correlated with sperm motility

Authors

Huanping Guo[1#], Xipeng Shen[1#], Hua Hu[3#], Peng Zhou[3], Tong He[1,4], Lin Xia[1], Dongmei Tan[4], Xi Zhang[1]* and Yunfang Zhang[1,2]*

Supplementary Figures:

Supplementary Figure S1: Abbreviations, names, transitions, and structural formula of different RNA modification marks that detected on sperm RNA samples.

Supplementary Figure S2: The levels of different RNA modification marks on the total RNAs of human sperm.

Supplementary Figure S3: Alteration of RNA modification abundance on human sperm RNA fragments >80 nt in AZS and TZS samples.

Supplementary Figure S4: Alteration of RNA modification abundance on human sperm 50-80 nt RNA fragments in AZS and TZS samples.

Supplementary Figure S5: Alteration of RNA modification abundance on human sperm 25-50 nt RNA fragments in AZS and TZS samples.

Supplementary Figure S6: Alteration of RNA modification abundance on human sperm 17-25 nt RNA fragments in AZS and TZS samples.

Supplementary Figure S7: Linear regression analyses between different RNA modification marks in all detected sperm RNA samples.

Supplementary Figure S8: Correlation analyses between human age and sperm motility scores for all participants.

Supplementary Figure S9: Original figures of microscope picture and full uncropped gels.
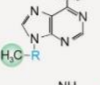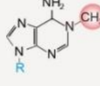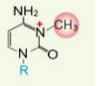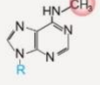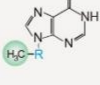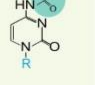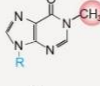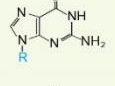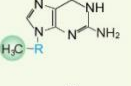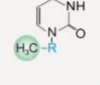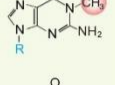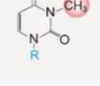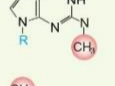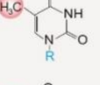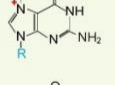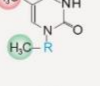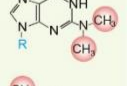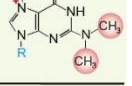
Supplementary Tables:

Supplementary Table SI: Primer sequences for RT-PCR.

Supplementary Table SII: The concentration of RNA modification standards.

Supplementary Table SIII: Clinical parameter analyses of ejaculated human semen samples.

Supplementary Table SIV: The R^2 values of Pearson correlation analyses in Figure 4A-B.

Abbreviation	Name	Transitions	Structural Formula	Abbreviation	Name	Transitions	Structural Formula
A	Adenosine	268 >136		C	Cytidine	244 >112	
Am	2'-O-Methyladenosine	282 >136		Cm	2'-O-Methylcytidine	258 >112	
m¹A	N1-Methyladenosine	282 >150		m³C	N3-Methylcytidine	258 >126	
m⁶A	N6-Methyladenosine	282 >150		m⁵C	5-Methylcytidine	258 >126	
I	Inosine	269 >137		hm⁵C	5-Hydroxymethylcytidine	274 >142	
Im	2'-O-Methylinosine	283 >137		ac⁴C	N4-Acetylcytidine	286 >154	
m¹I	N1-Methylinosine	283 >151		G	Guanosine	284 >152	
U	Uridine	245 >113		Gm	2'-O-Methylguanosine	298 >152	
Um	2'-O-Methyluridine	259 >113		m¹G	N1-Methylguanosine	298 >166	
m³U	3-Methyluridine	259 >127		m²G	N2-Methylguanosine	298 >166	
m⁵U	5-Methyluridine	259 >127		m⁷G	N7-Methylguanosine	298 >166	
m⁵Um	5,2'-O-Dimethyluridine	273 >127		m²₂G	N2,N2-Dimethylguanosine	312 >180	
Ψ	Pseudouridine	243 >153		m²₂⁷G	N2,N2,N7-Trimethylguanosine	326 >194	

Supplementary Figure S1: Abbreviations, names, transitions, and structural formula of different RNA modification marks that detected on sperm RNA samples.

The levels of different RNA modification marks on human sperm **total RNAs**
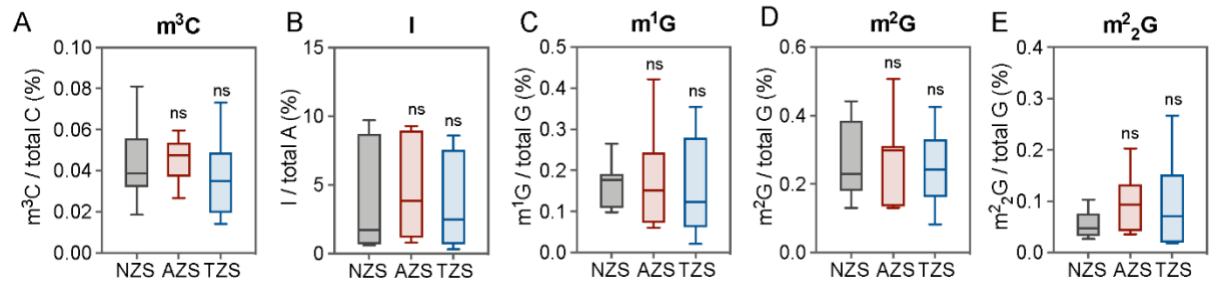
Supplementary Figure S2: The levels of different RNA modification marks on the total RNAs of human sperm. **(A-E)** comparation of RNA modification abundance on sperm total RNAs between NZS (n=6), AZS (n=6) and TZS (n=5) samples, which are shown as box plots for **(A)** m^3C, **(B)** I, **(C)** m^1G, **(D)** m^2G and **(E)** m^2_2G. NZS: Normozoozpermia; AZS: Asthenozoospermia; TZS: Teratozoospermia; ns. not significant (AZS vs NZS; TZS vs AZS). Statistical analyses were performed with one-way ANOVA and uncorrected Fisher's least square difference (LSD) for multiple comparisons of RNA modification levels by GraphPad Prism 8.
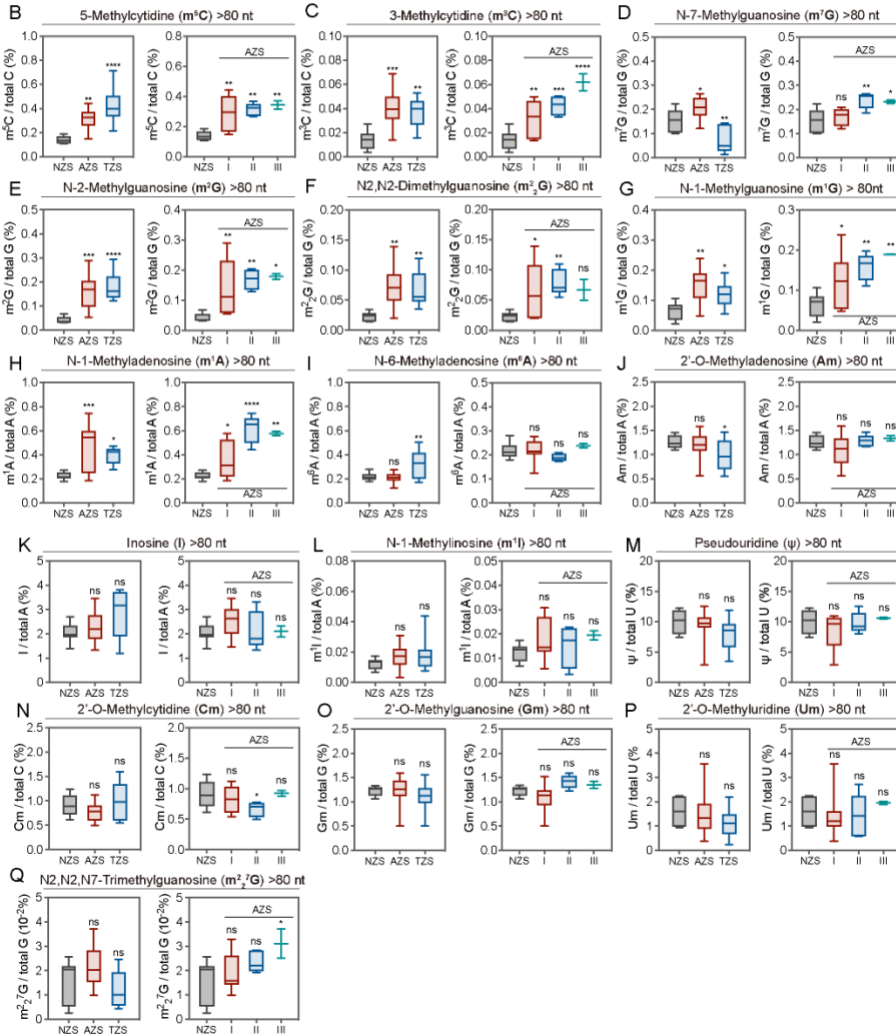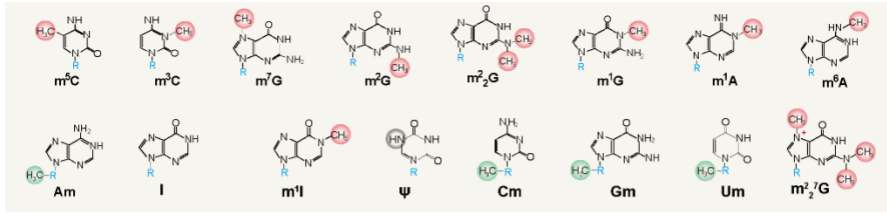
Supplementary Figure S3: Alteration of RNA modification abundance on human sperm RNA fragments >80 nt in AZS and TZS samples. **(A)** structural formula of different RNA modification marks. **(B-Q)** levels of different RNA modification marks on sperm RNA fragments >80 nt in NZS (n=7), AZS (n=15) and TZS (n=10) samples, and in AZS I (n=8), II (n=5), III (n=2) samples, which are shown as box plots for **(B)** m⁵C, **(C)** m³C, **(D)** m⁷G, **(E)** m²G, **(F)** m²₂G, **(G)** m¹G, **(H)** m¹A, **(I)** m⁶A, **(J)** Am, **(K)** I, **(L)** m¹I, **(M)** Ψ, **(N)** Cm, **(O)** Gm, **(P)** Um, **(Q)** m²₂⁷G. NZS: Normozoozpermia; AZS: Asthenozoospermia; TZS: Teratozoospermia; *P<0.05 (AZS or AZS I, II, III vs NZS; TZS vs NZS), **P<0.01(AZS or AZS I, II, III vs NZS; TZS vs NZS), ***P<0.001(AZS or AZS II vs NZS), ****P<0.0001(AZS II, III vs NZS; TZS vs NZS), ns. not significant (AZS or AZS I, II, III vs NZS; TZS vs NZS). Statistical analyses were performed with one-way ANOVA and uncorrected Fisher's least square difference (LSD) for multiple comparisons of RNA modification levels by GraphPad Prism 8.

Supplementary Figure S4: Alteration of RNA modification abundance on human sperm 50-80 nt RNA fragments in AZS and TZS samples. The box plots show the comparations of RNA modification abundance between NZS (n=7), AZS (n=15) and TZS (n=10) for **(A)** Am, **(B)** m^{1}A, **(C)** m^{6}A, **(D)** I, **(E)** m^{1}I, **(F)** Cm, **(G)** m^{3}C, **(H)** m^{5}C, **(I)** Um, **(J)** Ψ, **(K)** Gm, **(l)** m^{1}G, **(M)** m^{2}G, **(N)** m$^2_2$G, **(O)** m^{7}G and **(P)** m$^2_2$^{7}G. NZS: Normozoozpermia; AZS: Asthenozoospermia; TZS: Teratozoospermia; *P<0.05 (TZS vs NZS), **P<0.01(AZS or AZS II, III vs NZS;), ns. not significant (AZS or AZS I, II, III vs NZS; TZS vs NZS). Statistical analyses were performed with one-way ANOVA and uncorrected Fisher's least square difference (LSD) for multiple comparisons of RNA modification levels by GraphPad Prism 8.

Quantitative analysis of RNA modifications in human sperm **25-50 nt** RNAs
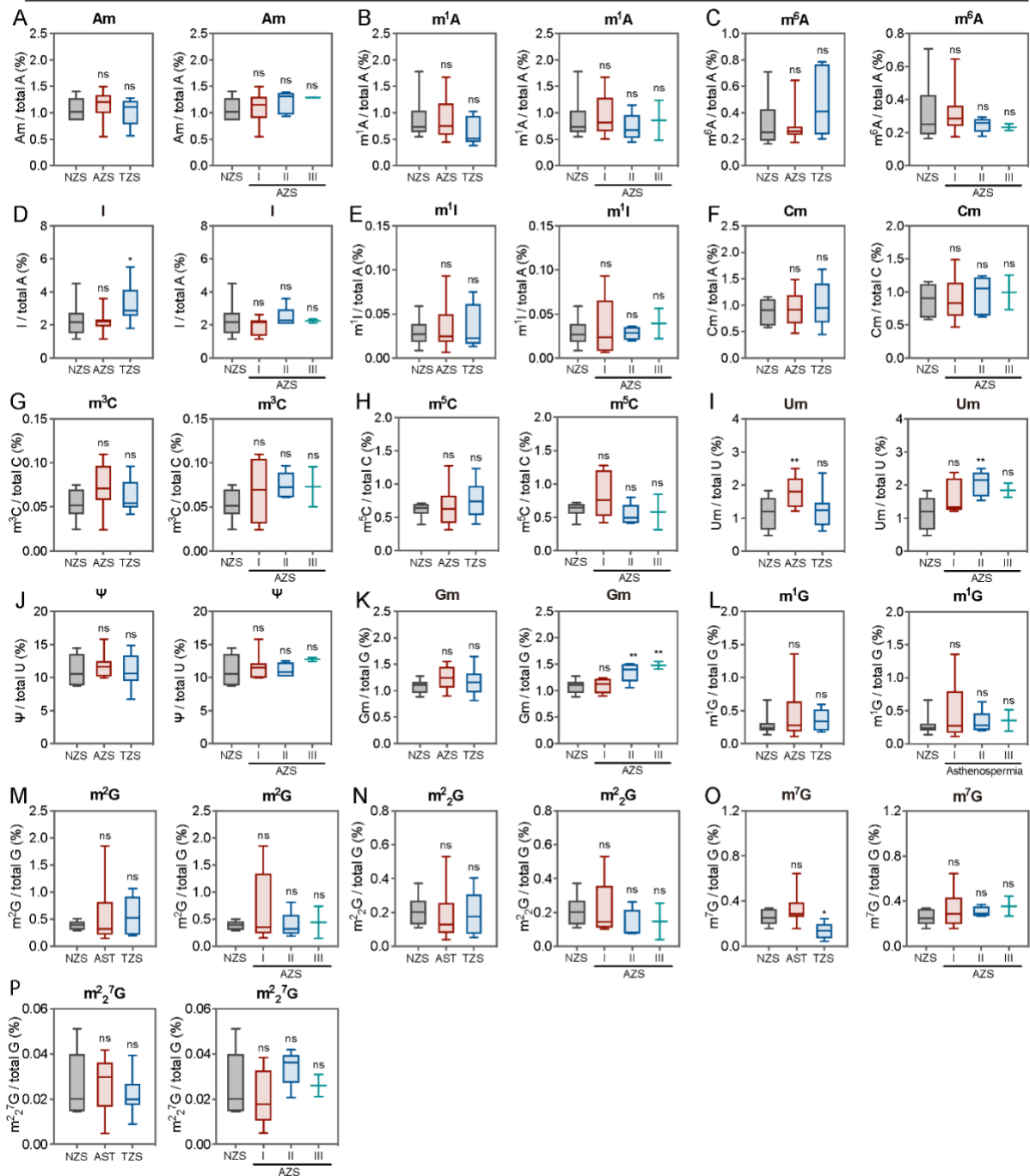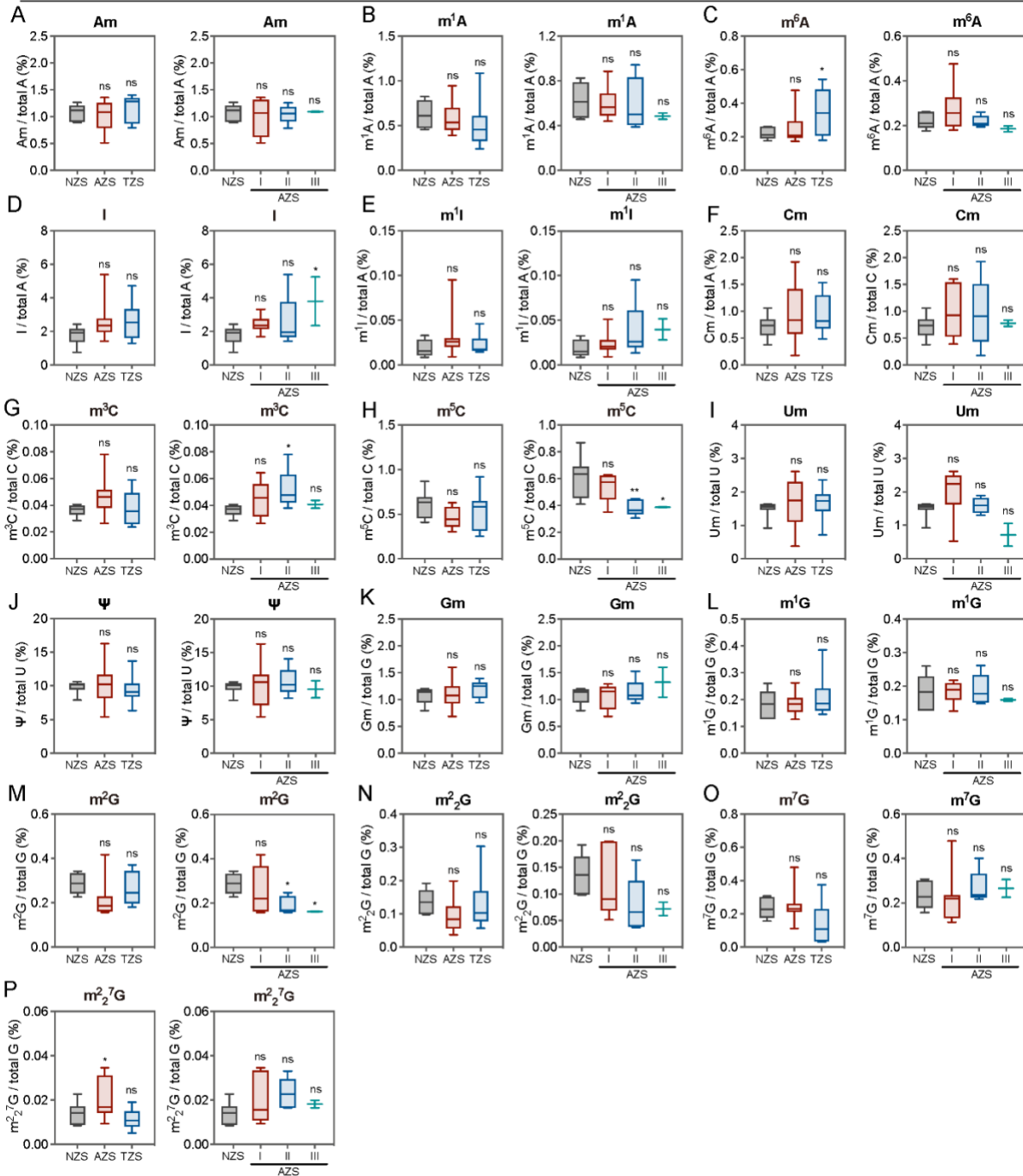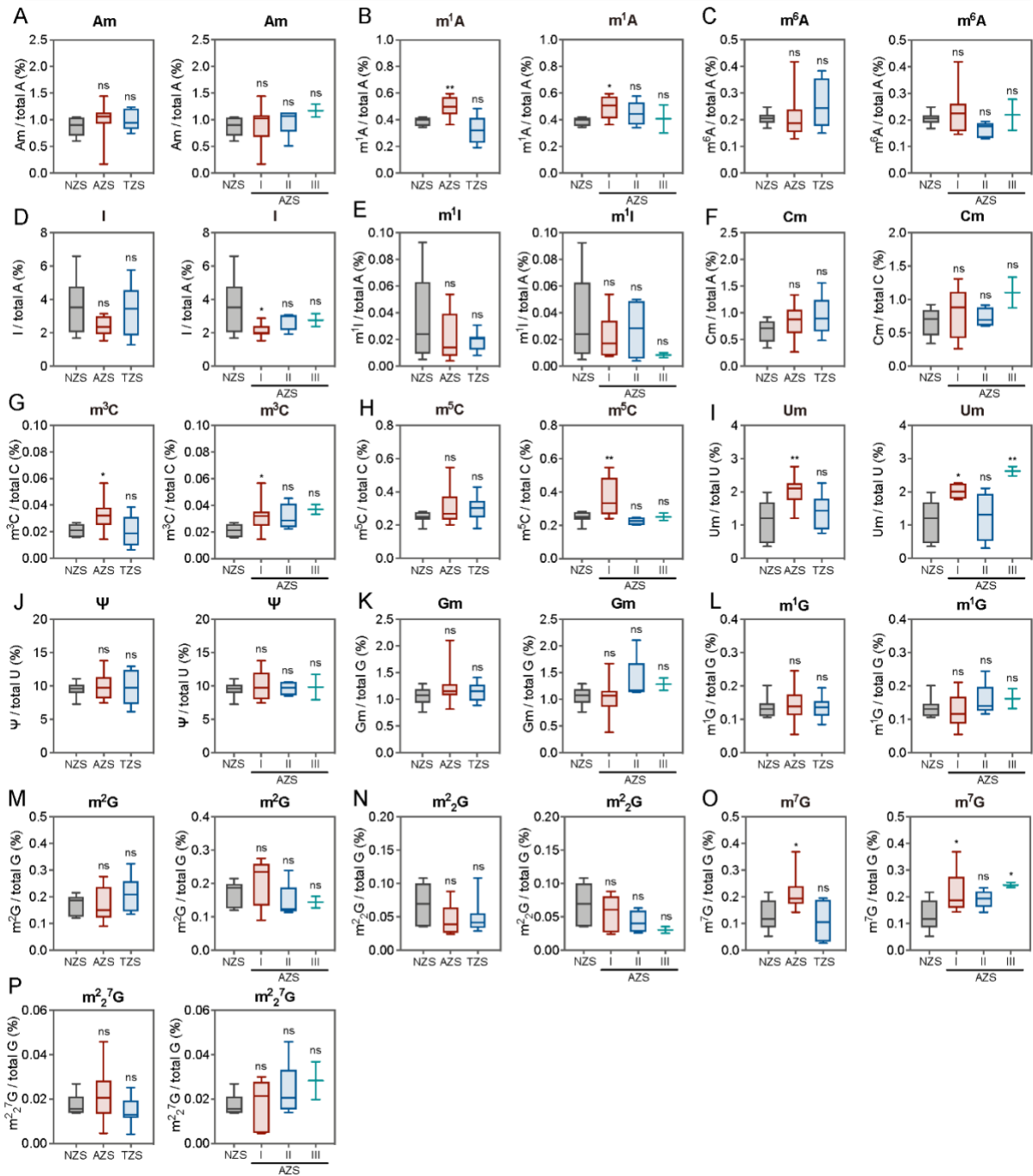
Supplementary Figure S5: Alteration of RNA modification abundance on human sperm 25-50 nt RNA fragments in AZS and TZS samples. The box plots show the comparations of RNA modification abundance between NZS (n=7), AZS (n=15) and TZS (n=10) for **(A)** Am, **(B)** m^1A, **(C)** m^6A, **(D)** I, **(E)** m^1I, **(F)** Cm, **(G)** m^3C, **(H)** m^5C, **(I)** Um, **(J)** Ψ, **(K)** Gm, **(l)** m^1G, **(M)** m^2G, **(N)** m^2_2G, **(O)** m^7G and **(P)** $m^2_2{}^7G$. NZS: Normozoospermia; AZS: Asthenozoospermia; TZS: Teratozoospermia; *P<0.05 (AZS or AZS II, III vs NZS; TZS vs NZS), **P<0.01(AZS II vs NZS), ns. not significant (AZS or AZS I, II, III vs NZS; TZS vs NZS). Statistical analyses were performed with one-way ANOVA and uncorrected Fisher's least square difference (LSD) for multiple comparisons of RNA modification levels by GraphPad Prism 8.

Supplementary Figure S6: Alteration of RNA modification abundance on human sperm 17-25 nt RNA fragments in AZS and TZS samples. The box plots show the comparations of RNA modification abundance between NZS (n=7), AZS (n=15) and TZS (n=10) for **(A)** Am, **(B)** m^1A, **(C)** m^6A, **(D)** I, **(E)** m^1I, **(F)** Cm, **(G)** m^3C, **(H)** m^5C, **(I)** Um, **(J)** Ψ, **(K)** Gm, **(l)** m^1G, **(M)** m^2G, **(N)** m^2_2G, **(O)** m^7G and **(P)** $m^2_2{}^7G$. NZS: Normozoozpermia; AZS: Asthenozoospermia; TZS: Teratozoospermia; *P<0.05 (AZS or AZS I, III vs NZS), **P<0.01(AZS or AZS I, III vs NZS), ns. not significant (AZS or AZS I, III vs NZS; TZS vs NZS). Statistical analyses were performed with one-way ANOVA and uncorrected Fisher's least square difference (LSD) for multiple comparisons of RNA modification levels by GraphPad Prism 8.
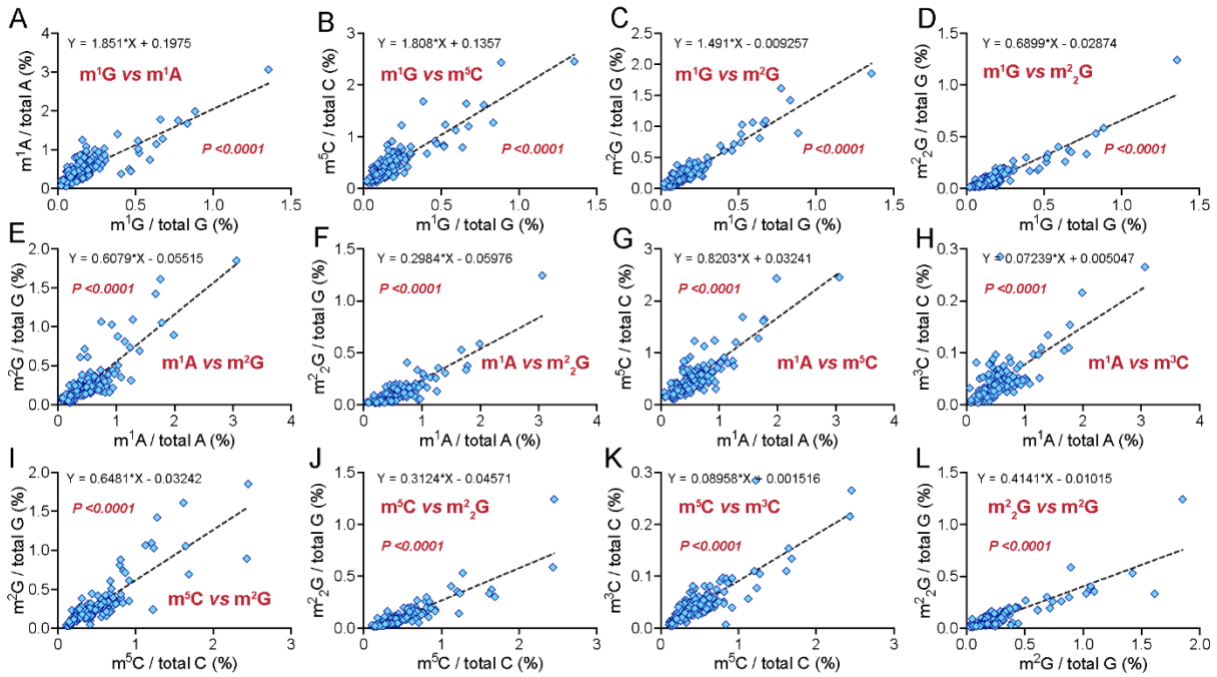
Supplementary Figure S7: Linear regression analyses between different RNA modification marks in all detected sperm RNA samples. (**A**) m^{1}G *vs* m^{1}A, (**B**) m^{1}G *vs* m^{5}C, (**C**) m^{1}G *vs* m^{2}G, (**D**) m^{1}G *vs* m$^2_2$G, (**E** m^{1}A *vs* m^{2}G, (**F**) m^{1}A *vs* m$^2_2$G, (**G**) m^{1}A *vs* m^{5}C, (**H**) m1A *vs* m^{3}C, (**I**) m^{5}C *vs* m^{2}G, (**J**) m^{5}C *vs* m$^2_2$G, (**K**) m^{5}C *vs* m^{3}C and (**L**) m$^2_2$G *vs* m^{2}G. The linear regression analyses were performed by GraphPad Prism 8, n=145. The equations and *p* values were shown on each panel.
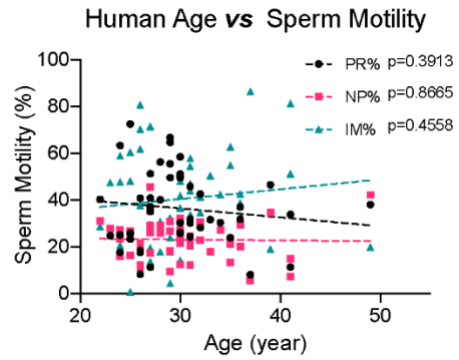
Supplementary Figure S8: Correlation analyses between human age and sperm motility scores for all participants. IM%: percentage of immotile sperm; PR%: percentage of progressive motility sperm; NP%: percentage of non-progressive sperm. The linear regression analysis was performed by GraphPad Prism 8, n=49.
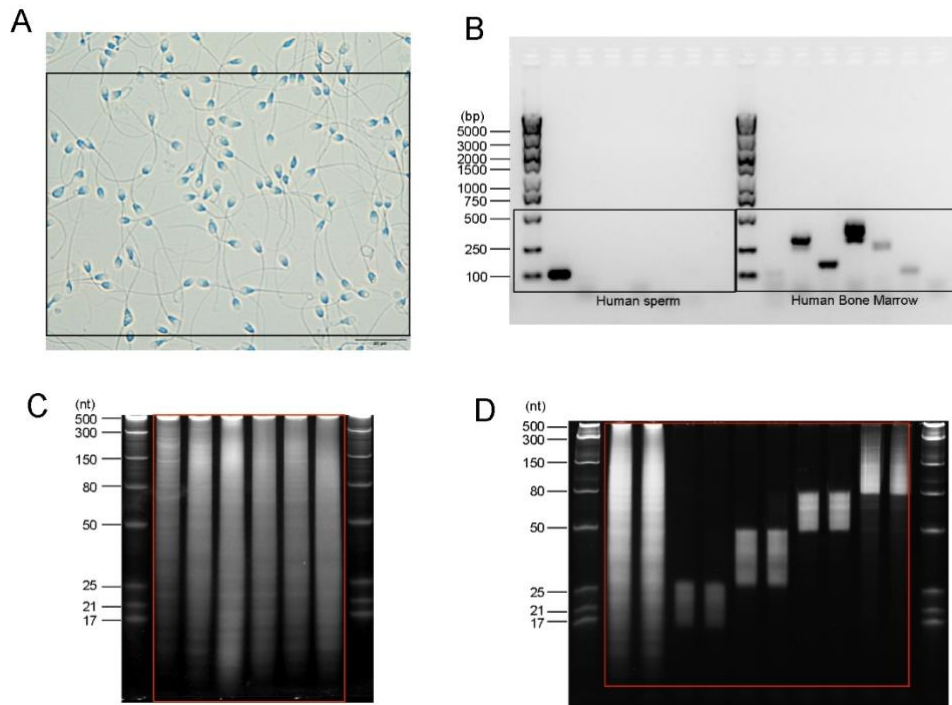
Supplementary Figure S9: Original figures of microscope picture and full uncropped gels. **(A)** Full microscopic picture of Figure 1A; **(B)** Full uncropped agarose gel of Figure 1B; **(C)** Full uncropped gel of Figure 1C. **(D)** Full uncropped gel of Figure 2A.

Supplementary Tables

Supplementary Table SI. Primer sequences for RT-PCR.

Target gene	Oligononucleotide sequences (5'- 3')	
PRM2	F: GGATCCACAGGCGGCAGCATCGCT;	R: GCATGTTCTCTTCCTGGTTCTGCA
CDH1	F: CACCTTCCATGACAGACCC;	R: AACGCATTGCCACATACAC
CDH2	F: CTGAAGTGACTCGTAACGACG;	R: CATGTCAGCCAGCTTCTTGAAG
CD4	F1: TTCAACTGTAAAGGCGAGTG;	R1: CGGATTGACTGCCAACTCT
	F2: GTGAACCTGGTGGTGATGAGAGC;	R2: GGCTACATGTCTTCTGAAACCGGTG
C-KIT	F: TACAACGATGTGGGCAAGA;	R: TACGAAACCAATCAGCAAAG

Supplementary Table SII. The concentration of RNA modification standards.

Abbreviation	Original (ng/ml)	Diluted times							Abbreviation	Original (ng/ml)	Diluted times						
		2X	5X	25X	50X	200X	1000X	2000X			2X	5X	25X	50X	200X	1000X	2000X
A	250	125	50	10	5	1.25	0.25	0.125	C	2500	1250	500	100	50	12.5	2.5	1.25
m^1A	50	25	10	2	1	0.25	0.05	0.025	Cm	500	250	100	20	10	2.5	0.5	0.25
I	2500	1250	500	100	50	12.5	2.5	1.25	ac^4C	100	50	20	4	2	0.5	0.1	0.05
Im	50	25	10	2	1	0.25	0.05	0.025	m^5C	200	100	40	8	4	1	0.2	0.1
m^6A	20	10	4	0.8	0.4	0.1	0.02	0.01	m^3C	25	12.5	5	1	0.5	0.125	0.025	0.0125
Am	10	5	2	0.4	0.2	0.05	0.01	0.005	hm^5C	100	50	20	4	2	0.5	0.1	0.05
m^1I	50	25	10	2	1	0.25	0.05	0.025	G	1000	500	200	40	20	5	1	0.5
U	2500	1250	500	100	50	12.5	2.5	1.25	m^2_2G	5	2.5	1	0.2	0.1	0.025	0.005	0.0025
m^3U	200	100	40	8	4	1	0.2	0.1	m^2G	100	50	20	4	2	0.5	0.1	0.05
m^5U	100	50	20	4	2	0.5	0.1	0.05	Gm	50	25	10	2	1	0.25	0.05	0.025
Um	1000	500	200	40	20	5	1	0.5	m^1G	10	5	2	0.4	0.2	0.05	0.01	0.005
m^5Um	100	50	20	4	2	0.5	0.1	0.05	m^7G	100	50	20	4	2	0.5	0.1	0.05
Ψ	500	250	100	20	10	2.5	0.5	0.25	$m^2_2{}^7G$	5	2.5	1	0.2	0.1	0.025	0.005	0.0025

Supplementary Table SIII. Clinical parameter analysis of human ejaculated semen samples.

Characteristic	Normozoospermia (SEM.)	Asthenozoospermia (SEM.)	Teratozoospermia (SEM.)	Reference range (WHO 2010)
Age (year)	29.6 (0.48)	29.7 (0.82)	33.3 (1.18)	—
BMI	24.5 (1.00)	24.2 (0.83)	23.2 (0.95)	—
Liquefaction time (min)	30.3 (2.06)	33.1 (2.40)	31.3 (2. 68)	< 60
Semen volume (ml)	3.6 (0.22)	4.3 (0.23)	3.8 (0.24)	≥ 1.5
Sperm concentration (10^6/ml)	63.3 (7.88)	56.5 (3.90)	59.4 (4.98)	≥ 15.0
Total sperm number (10^6/ml)	211.6 (24.25)	248.1 (23.61)	222.5 (20.69)	≥ 39.0
Sperm motility (%)				
Immotility (IM) %	22.5 (2.68)	57.4 (2.36)****	32.4 (2.69)####	< 60.0
Progressive motility (PR) %	52.9 (2.64)	22.7 (1.32)****	41.3 (1.91)####	≥ 32.0
Non-progressive (NP) %	24.6 (1.66)	20.0 (1.30)	26.2 (1.68)#	—
Total motility (PR+NP) %	77.5 (2.68)	42.6 (2.36)****	67.6 (2.69)####	≥ 40.0
Sperm morphology (%)				
Sperm with normal morphology	8.0 (0.68)	6.0 (0.27) ####	2.0 (0.15)****	≥ 4.0
Sperm abnormality	92.0 (0.68)	94.0 (0.27) ####	98.0 (0.15)****	—
(Sperm **Head**)	81.2 (1.39)	84.5 (0.98) ####	95.7 (0.29)****	—
(Sperm **Neck**)	48.7 (2.41)	52.7 (1.44)	50.3 (1.20)	—
(Sperm **Tail**)	4.7 (0.47)	4.6 (0.69)	3.9 (1.17)	—
Cytoplasmic droplet (CD)	10.7 (0.44)	10.9 (0.79)	13.8 (0.98)*	—
Teratozoospermia index (TZI)	1.5 (0.02)	1.5 (0.01)	1.5 (0.02)	—
Sperm deformity index (SDI)	1.4 (0.03)	1.4 (0.01)#	1.5 (0.02)**	—

* Compared with NZS   # Comparison between AZS and TZS   */# $P<0.05$;   **/## $P<0.01$;   ****/### $P<0.0001$

NormoZooSpermia (NZS, n=13)   AsthenoZooSpermia (AZS, n=21)   TeratoZooSpermia (TZS, n=15)

All the participants do not perform the basic genetic analysis.

Supplementary Table SIV: The R^2 values of Pearson correlation analyses in Figure 4A-B.

The R^2 values of Pearson correlation analyses in Figure 4A

Type	RNA fragments > 80 nt				RNA fragments 50 - 80 nt				RNA fragments 25 - 50 nt				RNA fragments 17 - 25 nt			
	Age	PR%	NP%	IM%	Age	PR%	NP%	IM%	Age	PR%	NP%	IM%	Age	PR%	NP%	IM%
A	0.01998	0.03611	0.1042	0.07314	0.00204	0.0004431	0.0167	0.0006849	0.0006243	0.1827	0.04913	0.1884	0.03491	0.01112	0.0198	0.001556
m^{1}A	0.002555	0.5975	0.1414	0.5814	0.002381	0.01042	0.03171	0.02131	0.002899	0.0001034	0.02231	0.003558	0.01045	0.003553	0.07381	0.001884
I	0.01068	0.002186	0.0802	0.01873	0.0006441	0.0007707	0.0136	0.0002631	3.298E-06	0.1995	0.1115	0.2385	0.03222	0.008446	0.01546	0.001138
Am	0.007632	0.000227	0.0004904	0.0003894	0.01383	0.03522	0.0009677	0.02797	0.007101	9.399E-05	0.03877	0.003497	2.176E-05	0.04089	0.008915	0.04062
m^{1}I	0.007683	0.1053	0.02385	0.1059	0.003753	0.02705	3.611E-06	0.01902	0.01116	0.08587	0.129	0.1358	0.05784	0.008445	0.05614	2.804E-05
m^{6}A	0.0728	0.002841	0.0006032	0.00126	0.06262	0.01138	0.03842	0.02445	0.08777	0.00339	0.08002	0.002294	0.01207	0.01523	0.05962	0.03514
U	0.02236	0.0001739	0.1123	0.01588	0.03008	0.02164	0.0007119	0.01316	0.03324	0.005078	0.005938	0.001123	0.0318	0.01338	0.1317	0.04943
Um	9.482E-05	0.0003626	0.02687	0.001596	5.536E-06	0.07872	0.00874	0.0412	0.002045	0.0171	0.1428	0.05701	0.0119	0.04489	0.006595	0.04238
ψ	0.02644	0.0004204	0.08433	0.01367	0.03997	0.01087	7.784E-05	0.007235	0.04944	0.01625	0.00143	0.01438	0.02592	0.0284	0.1555	0.07706
C	0.004093	0.00139	0.00742	0.003702	0.008214	0.0112	0.02046	0.01889	0.002193	0.001143	0.1159	0.02093	0.05522	0.04926	0.0154	0.02043
m^{5}C	0.04766	0.5738	0.07853	0.607	0.02668	0.0342	0.04521	0.05169	0.0004464	0.0346	0.06136	0.0578	0.01042	0.01367	0.07758	5.16E-06
Cm	0.000798	0.06091	0.001929	0.0493	0.006386	0.009705	0.007049	0.01231	0.008744	0.01684	0.07721	0.000186	0.06642	0.05398	0.002005	0.03196
m^{3}C	0.03153	0.6252	0.3194	0.6909	0.002453	6.085E-05	0.005683	0.0003495	0.006781	6.479E-05	0.06152	0.006106	0.0439	0.05876	0.008026	0.02959
G	0.02884	0.3588	0.1973	0.4352	0.0284	0.004872	8.406E-05	0.003897	0.002762	0.01946	0.01306	0.02421	0.02331	0.0173	0.05215	0.001004
Gm	0.02974	0.02606	0.08943	0.05645	0.03003	0.0445	0.01377	0.04737	0.02787	0.04667	0.009731	0.04634	0.04866	0.0417	0.03751	0.01077
m^{7}G	0.05085	0.4212	0.5134	0.6377	0.01734	0.09164	0.05092	0.1103	0.054	0.004463	0.02127	0.01145	0.01604	0.1262	0.1552	0.1881
m^{2}G	0.0673	0.5875	0.04657	0.6689	0.0512	0.007338	0.00788	0.01032	0.0002093	0.1734	0.07258	0.1947	0.0002394	0.0002885	0.05933	0.00952
m$^2_2$G	0.07101	0.4905	0.03477	0.4666	0.01127	0.008358	0.01715	0.01462	0.006444	0.04542	0.02443	0.05384	0.008723	0.006131	0.1374	0.03726
m^{1}G	0.01218	0.5896	0.1785	0.6853	0.02972	0.000983	0.001047	0.0002529	0.001117	0.02769	0.02437	0.03712	0.00183	0.0007539	0.02807	0.001175
m$^2_2$^{7}G	0.03308	0.3253	0.1661	0.3891	1.279E-05	0.01447	0.03739	0.02784	0.005703	0.1023	0.002625	0.06276	0.01174	0.0631	0.05214	0.08381

The R^2 values of Pearson correlation analyses in Figure 4B

Type	A	m^{1}A	I	Am	m^{6}A	U	Um	ψ	C	m^{5}C	Cm	m^{3}C	G	Gm	m^{7}G	m^{2}G	m$^2_2$G	m^{1}G	m$^2_2$^{7}G
Age	0.02161	0.1275	0.1098	0.393	0.08512	0.2864	0.4113	0.2382	0.2864	0.08523	0.4086	0.08623	0.3633	0.3771	0.03773	0.01648	0.03359	0.08379	0.01716
PR%	0.0274	0.2039	0.2892	0.6179	0.6712	0.7035	0.3447	0.7203	0.2612	0.02365	0.5483	0.08162	0.4638	0.4177	0.1942	0.0001	0.01458	0.01907	0.4057
NP%	0.3135	0.1089	0.00949	0.2362	0.4908	0.3753	0.1112	0.4055	0.3146	0.1465	0.262	0.02428	0.5345	0.2457	0.5792	0.01375	0.2718	0.04702	0.03585
IM%	0.1078	0.2337	0.1667	0.6615	0.8352	0.81	0.3568	0.8399	0.3747	0.06611	0.6151	0.03299	0.6566	0.4923	0.3885	0.00227	0.07715	0.03493	0.346