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SAFE USE OF ANTI-CD154 MONOCLONAL ANTIBODY IN PIG ISLET XENOTRANSPLANTATION IN MONKEYS

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Abstract

Background—Anti-CD154mAb is a powerful costimulation blockade agent that is efficacious in preventing rejection, even in xenogeneic settings. It has been used in the majority of successful long-term pig-to-nonhuman primate islet transplantation models. However, its clinical use was halted as a result of thromboembolic complications that were also observed in pre-clinical and clinical organ transplantation models.

Methods—An anti-CD154mAb was administered to 14 streptozotocin-induced diabetic cynomolgus monkey recipients of porcine islets, some of which received the agent for many months. Monkeys were monitored for complications, and blood monitoring was carried out frequently. After euthanasia, multiple biopsies of all organs were examined for histological features of thromboembolism.

Results—Anti-CD154mAb prevented rejection of genetically-engineered pig islets in all monkeys. No significant complications were attributable specifically to anti-CD154mAb. There was no evidence of thromboembolism in multiple histological sections from all major organs, including the brain.

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David Ayares is an employee of Revivicor, Inc. The other authors have no conflicts of interest to disclose

Conclusions—Our data suggest that in diabetic monkeys with pig islet grafts, anti-CD154mAb would appear to be an effective and safe therapy, and is not associated with thromboembolic complications.

Keywords

Anti-CD154mAb; Immunosuppression; Islets of Langerhans; xenotransplantation

INTRODUCTION

Costimulation blockade was developed to target (and block) communication along the secondary pathway required for T cell activation (1), and has proven to be a powerful form of immunosuppressive therapy, even in preclinical models of xenotransplantation where the T cell response is generally considered to be stronger than after allotransplantation (2). It is usually directed to block the CD28/B7 and/or CD40/CD154 pathways. Historically, CTLA4-Ig is used to block the CD28/B7 pathway and an anti-CD154 monoclonal antibody (mAb) or anti-CD40mAb to block the CD40/CD154 pathway (1).

The combination of these agents successfully prevented kidney allograft rejection in a nonhuman primate (NHP) model (3). Anti-CD154mAb monotherapy worked well in monkeys with renal (4) or islet (5) allotransplants, whereas CTLA4-Ig monotherapy showed only modest ability to prolong similar allograft function (6). In a 2000 study on the transplantation of porcine peripheral blood progenitor cells into baboons, anti-CD154mAb successfully prevented sensitization to the graft (7). This success in a xenograft model led to anti-CD154mAb being widely used in xenotransplantation studies involving NHPs (2), including those of islet xenotransplantation (8-10).

The broadly powerful immunotherapeutic effects of anti-CD154mAb are likely derived through several mechanisms. It impacts (i) T cells by blocking the secondary signal, and (ii) B cell function through lack of T cell help. It also acts (iii) by blocking soluble CD154 released by platelets (11), and (iv) by its effect on vascular endothelial cells by modulating CD154-mediated adhesion molecules and cytokine receptors (12,13).

Following promising results in animal models, anti-CD154mAb was tested in clinical trials. Phase 1 and 2 trials were conducted (by Biogen, Cambridge, MA) from 1997-1999 in patients with immune thrombocytopenic purpura, systemic lupus erythematosus, factor VIII antibody syndrome, and in patients undergoing renal transplantation. These trials showed efficacy, but were halted when several patients suffered thromboembolic events (14). In Phase 1 and 2 trials conducted (by IDEC, San Diego, CA) from 2001-2002 in patients with systemic lupus erythematosus, the agent proved safe but was not efficacious (15). Clinical use of anti-CD154mAb was put on hold.

Thromboembolic events have also been reported in NHPs in association with use of anti-CD154mAb (16-20). The mechanisms behind these secondary effects appear to involve the formation of immune complexes between anti-CD154mAb and CD154, with subsequent platelet activation via the IgG receptor FcγRIIA, thus triggering coagulation (21). A role for

the IgG receptor FcγRIIA on adjacent platelets was also proposed for increasing the risk of thrombosis (22). Anti-CD154mAb may also affect arterial thrombi stabilization (23).

Heparin and other anticoagulants have shown efficacy in reducing anti-CD154mAb-mediated thromboembolism (16-19,24).

The last 10 years have brought forth several successful pre-clinical pig-to-NHP islet xenotransplantation studies, reviewed by Park CG et al (25), including our own (9,10). Successful models have been differentiated by characteristics of the pig islet donor while the studies were almost universal in using costimulation blockade immunosuppression, most frequently based on an anti-CD154mAb (25).

The aim of the present study was to determine whether relatively prolonged administration (up to one year) of an anti-CD154mAb was associated with thromboembolic events in monkeys with porcine islet transplants.

MATERIALS AND METHODS

Porcine islet donors

Islets were harvested from wild-type (WT) pigs (n=2) (Wally Whippo, Enon Valley, PA) or genetically-engineered (GE) pigs with one or more modifications (see Table 1 for information on the genetics of donor pigs); α1,3-galactosyltransferase gene-knockout (GTKO, n=2), transgenic for hCD46 (n=5), 3 GE (GTKO,hCD46,hCD39, n=1), 4 GE (GTKO,hCD46,hCD39,hTFPI, n=2), or 5 GE (GTKO,hCD46,hCD39,hTFPI,CTLA4-Ig n=2) pigs (all from Revivicor, Blacksburg, VA) (9,10).

Diabetic monkey recipients

Diabetes was induced in 14 cynomolgus monkeys (*Macaca fascicularis*; Three Springs Scientific, Perkasie, PA, and Alpha Genesis, Yemassee, SC) by the administration of streptozotocin, as previously described (10). The monkeys were 2-5yr of age and weighed 3.5±0.6kg.

All animal care was in compliance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 2011), and approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Immunosuppressive and adjunctive therapy

The immunosuppressive (and adjunctive drug) regimen was based on anti-CD154mAb and is summarized in Table 2. Anti-CD154mAbs (ABI793 [Novartis Pharma, Basel, Switzerland; n=9] or chimeric h5c8 [NIH NHP Reagent Resource, Boston, MA; n=5]) were used for induction, with the same antibody also being used for maintenance of immunosuppression. Dosage was modulated to maintain serum trough levels in the anticipated range. Trough levels were not measured in 5 monkeys who were, therefore, administered a dose proportionally higher than received by the other monkeys.

Apart from low-dose continuous heparin infusion to maintain i.v. catheter patency (Table 1), the only antiplatelet/anticoagulant/anti-inflammatory agent administered to the monkeys was aspirin (81mg/day) (Table 2).

Monitoring

The monkeys were monitored clinically for signs of infection or thromboembolism. Daily records of the following signs were kept - mobility impairment, lack of coordination, hypotonic limbs, hemiplegia, respiratory dysfunction (shortness of breath), and diarrhea, in addition to lack of appetite and lack of interest in enrichment activities. Blood was collected at least monthly for complete blood counts and tests of renal and hepatic function (Presbyterian Hospital, University of Pittsburgh Medical Center). Blood glucose was monitored at least x2 daily and insulin was administered to maintain the blood glucose <200mg/dL pre-transplant and, as necessary afterwards. The studies were designed for follow-ups of 3, 6 or 12mo, at which time monkeys were electively euthanized (9,10). Serum levels of porcine C-peptide were measured at intervals as an indicator of graft function. If undetectable, the recipient monkey was euthanized. Whole blood trough levels of anti-CD154mAb were measured by competitive inhibition ELISA (26) in 9 of the 14 monkeys. The activated clotting time (ACT) was measured using I-Stat (Abbott, Princeton, NJ) while heparin was being administered.

Necropsy and microscopic examination of major organs

After euthanasia, a full necropsy was performed in all monkeys. All major organs (brain, heart, intestine, kidney, liver, lung) were cut into blocks of approximately 5×5×5mm and macroscopically examined prior to fixation. Areas of ischemic injury, hemorrhage, or other gross pathology could therefore be excluded. Formalin-fixed tissue was embedded in paraffin. Three random biopsies, each from 3-5 areas of the organ, covering approximately 25mm^2 were stained with hematoxylin and eosin (H&E) using standard procedures and examined.

Images were captured using a Zeiss Axiovert 200 Microscope (Carl Zeiss Microscopy, Thornwood, NY) and an Olympus BX51 microscope and Nikon D300 camera (B&B Microscopes, Pittsburgh, PA).

RESULTS

Clinical observations

Follow-up after islet transplantation ranged from 8 to 365 days with a mean of 163 days and a median of 125 days (Table 1). Nine monkeys were followed for >3months, 5 for >6months, and 3 for 12months. Of the 12 monkey recipients with graft function warranting follow-up beyond 40 days, the mean follow-up was 187 days with a median of 147 days. Anti-CD154mAb was administered weekly throughout the entire period of follow-up. Serum levels of anti-CD154mAb were between 609-1663μg/mL (mean 1025μg/mL; median 1021μg/mL). Intravascular catheters with auxiliary i.v. heparin infusion remained in situ for between 8 and 98 days in 13 monkeys (mean 39 days; median 29 days) (Table 1).

No monkey suffered a complication attributed to anti-CD154mAb therapy. There were no transient complications during or following i.v. anti-CD154mAb administration (e.g., vomiting, loss of appetite, diarrhea, fever, seizures), and no major clinical complications (e.g., infections, malignancies, or thromboembolism) occurred. In 2 monkeys (15%), we detected evidence of reactivation of cytomegalovirus, despite the fact that all monkeys received prophylaxis in the form of ganciclovir or valganciclovir, but there were no clinical features of cytomegalovirus disease.

Parameters such as white and red blood cell and platelet counts, as well as those of hepatic and renal function did not show significant changes during the post-transplant phase in the study animals (9,10). ACT levels were 127±25s (mean±SD, n=12) while heparin (and aspirin) was being administered.

There was no evidence of thromboembolic events in the monkeys regardless of whether they received a higher or lower dose of anti-CD154mAb (Table 1).

Histopathology of major organs

At necropsy no macroscopic evidence of abnormalities was seen.

Multiple H&E-stained sections from the major organs in all monkeys were examined: no microscopic features of thrombosis or embolism were detected. Figure 1 shows representative images from H&E-stained tissues of two recipients that were followed-up for one year.

DISCUSSION

Of the several groups that have reported relatively long-term successful pig islet transplantation in diabetic monkeys (25), only one recorded any severe adverse events associated with anti-CD154mAb in recipient NHPs treated with the antibody, affecting 8 of 9 monkeys tested (27). In that study, however, it was difficult to distinguish the specific side-effects of anti-CD154mAb from those of other agents that were administered, e.g., everolimus, which can also increase thrombotic events (28). Previous studies, including relevant work by Cardona et al (29) and Thompson et al (30) with WT as well as GTKO neonatal islets, observed no adverse anti-CD154mAb-derived events. Although follow-up was for <1 year, this finding is significant in view of the fact that neonatal pig islets (that express galactose-α1,3-galactose [Gal]) are more thrombogenic than adult islets (31).

In our own preclinical studies of porcine islet xenotransplantation, an anti-CD154mAb-based immunosuppressive regimen has proved effective in maintaining islet graft function for at least 1year and has been well-tolerated and safe over relatively long periods of administration (9,10).

We have employed both the ABI793 and h5c8 varieties of the monoclonal antibody in cynomolgus monkey and baboon recipients of other porcine organs/tissues. We have reported thrombosis in pig artery patch (17) and organ (18) xenotransplantation models.

Our observations in the present study on the lack of adverse events are relevant in the light of earlier reports of thromboembolism in both pre-clinical NHP studies (16,18-20) and in clinical trials (14) that were halted because of the incidence of this complication. To note, none of those earlier pre-clinical and clinical studies involved islet cells.

In our present study, 14 monkeys were administered anti-CD154mAb during follow-up, with 12 being followed for >45 days, with a median of 187 days in this sub-group. None experienced an adverse event. A WT pig-to-NHP islet transplantation study published by Shin et al (32), which reported use of anti-CD154mAb immunosuppression associated with normoglycemia in 4 of 5 monkeys for >6 months, including one for >600 days, confirmed our own observation of no evidence of thromboembolism or other adverse events (32).

Considering data from both groups reporting this level of long-term success with pig-to-NHP islet transplantation, at least 6 monkeys have now received anti-CD154mAb treatment for >300 days with demonstrated beta cell function and no ill effects associated with its use (9,10,32). Although more definitive evaluation including confirmative diagnostic tools would be beneficial, such a prolonged period of time of treatment is reassuring regarding the lack of adverse events, particularly in the absence of evidence of any histopathological features of thromboembolism in the present study.

What accounts for the difference seen in the incidence of thromboembolism after pig islet xenotransplantation compared with pig solid organ or artery patch xenotransplantation? The answer to this particular question lies beyond the scope of our present study, but we may point to several intriguing possibilities. There are peculiarities in the islet xenotransplantation setting that may prove relevant when compared to whole organ or tissue transplantation. For example, (i) the much smaller size of the islet graft might play a significant role in reducing the antigen load (thus stimulating a much weaker antibody response); (ii) the relative absence of vascular endothelium in the islet graft may be important from the perspective of coagulation dysfunction (almost certainly initiated by anti-pig antibodies); (iii) the invariable administration of a large dose of heparin or dextran sulfate immediately before the islet graft is exposed to the recipient's blood may prevent thrombosis just as a single dose of ketorolac prior to anti-CD154mAb can prevent thrombosis in a pig artery patch graft model (17) or two doses in a NHP kidney allotransplantation model, despite anti-CD154mAb being administered for weeks or months post-transplantation (19). It may be interesting to note that the dose of anti-CD154mAb used in monkeys is greater proportionally to body weight than doses administered to humans: it would be expected, therefore, that potential ill effects would be more readily observed in the monkeys (14).

In the current study, the use of intravascular catheters with continuous infusion of fluids supplemented with very low doses of heparin (approximately 10-20U/h) would have provided some (though minimal) anticoagulant coverage at least during the period the catheters were in place (Table 1). Furthermore, a daily low-dose of aspirin was given to all recipients, which may have imparted sufficient anti-platelet/anti-inflammatory activity to minimize the risk of clotting. Schuler et al (20) observed that in the absence of aspirin, platelet counts fell in cynomolgus monkeys administered anti-CD154mAb, while the

addition of aspirin normalized the platelet count. The drop in numbers may be indicative of platelet consumption, which can be associated with thromboembolic events. Monkeys in our study all received a daily dose of aspirin and their platelet counts showed no significant variability during the course of immunosuppressive therapy (Table 3).

All of the groups that have reported long-term success in the pig-to-NHP islet xenotransplantation model using anti-CD154mAb-based immunosuppressive therapy administered an anti-coagulant (heparin or dextran sulfate) to the recipients at the time of transplantation (9,10,27,29,30,32), but only the groups with the longest follow-ups (>1yr) continued some form of anti-coagulation therapy beyond the time of islet infusion (9,10,32). Our group administered daily aspirin while Shin et al administered Plavix for one month after transplantation. Also of note, of three groups with the most sustained anti-CD154mAb treatment (9,10,27), the only group to report severe events associated with its use was also the only one not to extend anticoagulant coverage beyond the transplant (27). These observations suggest that extended administration of anticoagulants, even in relatively small doses, may be beneficial.

Is it possible that the genetic-engineering of the porcine islets may have provided protection against thromboembolic events? The expression of hCD39 and/or human tissue factor pathway inhibitor (hTFPI) in some of the pig donor islet grafts may have protected against thromboembolism (10). The addition of CTLA4-Ig or hCD46, or the deletion of Gal in pig aortic endothelial cells would not be predicted to have had a major impact on thrombosis (33). This is supported by the absence of thromboembolic complications in recipients of WT donor pig islets in our study and, after much longer follow-up, in the study by Shin et al (32). Importantly, in combinations of 5 different genetically-engineered donor islets along with 2 types of anti-CD154mAbs studied (Table 1), the lack of thromboembolic events was general. Based on our data, the specific genetic manipulation does not appear to influence the occurrence of thromboembolic events either negatively or positively. We suggest that the absence of thromboembolism irrespective of the genetic nature of the islet-source pig or of the immunosuppressive regimen strengthens our conclusion that anti-CD154mAb was not thrombogenic under the circumstances of these experiments.

Although alternative costimulation blockade agents have been – or are being – developed, our experience and that of others (32) suggests that any anti-CD154mAb, when given as monotherapy (or combined with other less potent agents), is more potent than others, e.g., CTLA4-Ig (abatacept and belatacept) and anti-CD40mAb, even though the results using this latter agent have been very encouraging following the xenotransplantation of solid pig organs (34,35). Because of their undoubted efficacy, attempts are being made to develop an anti-CD154mAb that successfully blocks the CD40/CD154 pathway, but does not activate platelets (36).

In conclusion, anti-CD154mAb remains one of the most potent anti-rejection drugs with, except for thromboembolism, a good safety profile. Despite earlier reports of its use in clinical trials and NHP organ transplantation being associated with serious adverse events, in our model of pig-to-NHP islet transplantation, it proved an effective and safe agent to

administer under a daily regimen of aspirin. Whether this experience is sufficient to consider its use in clinical trials of islet allotransplantation remains uncertain.

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Abbreviations

Gal	galactose-α1,3-galactose
GE	genetically-engineered
GTKO	α1,3-galactosyltransferase gene-knockout
mAb	monoclonal antibody
NHP	nonhuman primate
WT	wild-type

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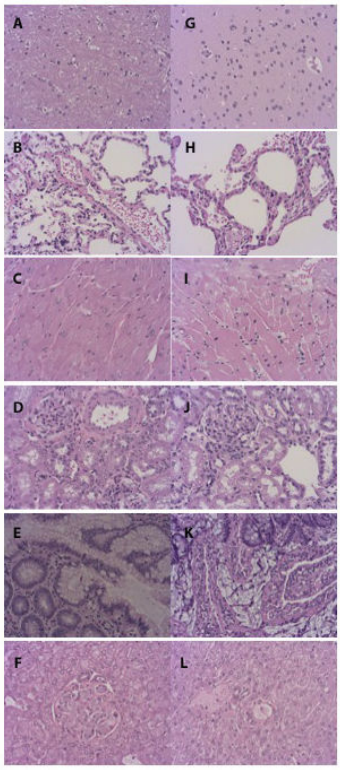

Figure 1.

H&E-stained sections of brain **(A, G),** lung **(B, H),** heart **(C, I),** kidney **(D, J)** intestine **(E, K),** liver **(F, L)** from two cynomolgus monkey recipients of pig islets treated with anti-CD154mAb for one year. No histopathological features of thromboembolism were seen in any of the multiple sections examined.

Table 1

Post-transplant follow-up of recipient monkeys

Monkey	Pig donor genetics	Post-Tx Follow-up (days)	Presence of intravascular catheters post-Tx (days)	2nd dose of ATG	Anti-CD154mAb type	Anti-CD154mAb maintenance dose (mg/kg/week)	Anti-CD154mab serum trough levels (μg/mL) mean±SD
1	GTKO	8	8	y	ABI793	5-15	641
2	WT	270	21	y	ABI793	5-15	1138±440
3	GTKO	70	42	y	ABI793	5-15	1021±489
4	WT	41	41	y	ABI793	5-15	1388±578
5	hCD46	98	98	y	ABI793	5-15	1075±514
6	hCD46	131	75	y/n*	ABI793	5-15	609±165
7	hCD46	120	0	n	ABI793	5-15	944±46
8	hCD46	183	40	n/y*	ABI793	5-15	1663±184
9	hCD46	365	78	y	ABI793	5-15	744±415
10	4-GE	364	29	y	h5c8	25	n/a
11	4-GE	365	16	y	h5c8	25	n/a
12	5-GE	162	14	y	h5c8	25	n/a
13	3-GE	58	21	n	h5c8	25	n/a
14	5-GE	52	21	y	h5c8	25	n/a

All monkeys received; Mycophertolate mofetil, Dextran sulfate, Prostacyclicn, Methylprednisolone, Aspirin, Ganciclovir or Valganciclovir, Famotidine, and Heparin as detailed in Table 2.

*Monkeys 6,8 received two transplants and two courses of ATG.

n/a = not measured

GE (genetically-engineered)

GTKO (α1,3-galactosyltransferase gene-knockout)

hTFPI (human tissue factor pathway inhibitor)

WT (wild-type)

3-GE (GTKO/hCD46/hCD39)

4-GE (GTKO/hCD46/hTFPI/CTLA4-Ig)

5-GE(GTKO/hCD46/hCD39/hTFPI/CTLA4-Ig)

Table 2

immunosuppressive and supportive therapy

Induction
Antithymocyte globulin, i.v. 25mg day −3 (ATG, Thymoglobulin, Genzyme, Cambridge, MA). A second variable dose was administered on day −1 if the CD3+ T cell count remained >500cells/mL.
Anti-CD154mAb, i.v. 25mg/kg on days −1, 0, 3, 7, 11 and 15 (ABI793 [n=9], Novartis Pharma, Basel, Switzerland, or chimeric h5c8 [n=5], NIH NHP Reagent Resource, Boston, MA)
Maintenance
Anti-CD154mAb, i.v. 5–15mg/kg weekly from day 22 to maintain serum trough levels of >500μg/mL. In the absence of trough levels, a dosage of 25mg/kg was administered weekly.
Mycophenolate mofetil, p.o**.** 50-100mg/kg/day to maintain whole blood trough levels of 3-5μg/mL (Cellcept; Roche, Nutley, NJ).
Supportive therapy on the day of transplantation
Dextran sulfate before islet infusion, i.v. 5mg/kg **(**Sigma-Aldrich, St. Louis, MO)
Prostacyclin, i.v. 20ng/kg/min (Flolan; GlaxoSmithKline, Philadelphia, PA) starting before islet infusion and for additional 3h.
Methylprednisolone, i.v. 10mg/kg before islet infusion (SoluMedrol; Pfizer, New York, NY**).**
Supportive therapy during follow-up
Aspirin, p.o. 81mg daily starting 1 week before islet transplantation
Ganciclovir, i.v. 5mg/kg/day (Cytovene; Roche, Welwyn Garden City, UK) **or**
Valganciclovir, p.o. 15mg/kg x2 daily (Valcyte; Genentech, San Francisco, CA) (to prevent cytomegalovirus reactivation).
Famotidine, i.v. 0.25mg/kg/day or p.o. 1mg/kg/day while i.v. catethers were in place (APP Pharmaceuticals, Schaumburg, IL, and Baxter Healthcare, Deerfield, IL) (to prevent peptic ulceration).
Heparin, i.v. 10-20U/h while i.v. cathethers were in place (Hospira, Lake Forest, IL).

Table 3

Complete blood counts (means and ranges) in monkey recipients followed for at least 3 months after pig islet transplantation

Monkey		WBC X10E+09/L	RBC X10E+12/L	Hematocrit %	Platelet x10E+09/L	Platelet volume fL
				mean±SD (range)		
2	n=9	7.4±2.3 (4.5-10.9)	3.9±0.6 (2.6-4.4)	29.3±4.6 (20.2-35.1)	400±98 (219-537)	9.1±0.8 (8.3-10.9)
5	n=9	5.0±3.3 (1.2-10.3)	3.5±0.5 (2.8-4.5)	28.5±4.2 (19.3-33.4)	267±81 (156-380)	9.2±1.1 (7.1-10.3)
6	n=9	4.4±1.7 (1.9-8.1)	4.0±0.5 (3.2-5.2)	31.7±4.1 (23.4-39.5)	250±68 (142-436)	10.6±0.9 (8.9-12.4)
7	n=12	8.7±3.3 (5.0-15.6)	4.3±0.3 (3.3-4.7)	33.8±3.2 (26.0-39.0)	422±104 (249-580)	9.0±0.7 (8.3-10.5)
8	n=6	4.5±1.2 (2.8-7.1)	4.6±0.3 (4.3-4.8)	33.3±1.5 (30.5-34-7)	323±71 (230-533)	10.7±0.7 (9.2-11.6)
9	n=13	6.7±1.5 (1.9-10.7)	4.5±0.4 (4.1-5.1)	36.3±3.5 (31.2-41.0)	321±57 (207-440)	10.0±0.6 (8.9-11.4)
10	n=3	6.6±1.6 (5.5-8.4)	5.6±0.1 (5.5-5.6)	37.0±1.2 (36.7±38.9)	447±50 (390-476)	9.0±0.5 (8.6-9.6)
11	n=7	7.0±2.7 (3.0-11.7)	5.2±0.3 (4.7-5.4)	37.0±1.8 (34.3±39.1)	460±95 (292-501)	8.3±0.4 (7.9-9.0)
12	n=7	8.3±4.1 (9.5-13.8)	4.4±0.5 (3.6-5.1)	32.3±4.0 (30.0±38.5)	439±140 (311-710)	84-0.4 (8.0-9.2)